Tomasz Maciej Grzywa

Zbadanie roli komórek erytroidalnych wykazujących ekspresję cząsteczki CD71 (CECs) w regulacji odpowiedzi układu odpornościowego

Rozprawa na stopień doktora nauk medycznych i nauk o zdrowiu

w dyscyplinie nauki medyczne

Promotor: prof. dr hab. n. med. Jakub Gołąb

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Spis treści

Wykaz stosowanych skrótów

ARG – arginaza

CEC – komórki erytroidalne wykazujące ekspresję cząsteczki CD71, ang. *$CD71^+$ erythroid cells*

HSC – komórka macierzysta hematopoezy, ang. *hematopoietic stem cell*

IFN-γ – interferon γ

OVA – owalbumina, ang. *ovalbumin*

PBMCs – komórki jednojądrzaste krwi obwodowej, ang. *peripheral blood mononuclear cells*

PHZ – fenylohydrazyna, ang. *phenylhydrazine*

RFT – reaktywne formy tlenu

TGF-β – transformujący czynnik β, ang. *transforming growth factor β*

TNF-α – czynnik martwicy nowotworu α, ang. *tumor necrosis factor α*

Streszczenie w języku polskim

Komórki erytroidalne wykazujące ekspresję cząsteczki CD71 (CEC, ang. *CD71$^+$ erythroid cells*) to progenitory i prekursory erytrocytów o niedawno zidentyfikowanej roli w regulacji odpowiedzi układu odpornościowego. Głównym czynnikiem indukującym ekspansję komórek CEC jest niedokrwistość. Jak dotąd jednak nie wykazano immunoregulatorowej roli komórek CEC w warunkach niedokrwistości. Celem niniejszej rozprawy doktorskiej składającej się z cyklu trzech publikacji było opisanie roli komórek CEC w regulacji odpowiedzi układu odpornościowego oraz zbadanie wpływu tych komórek na odpowiedź immunologiczną w niedokrwistości.

Pierwszą publikacją wchodzącą w skład cyklu jest praca przeglądowa pt. "The role of CD71$^+$ erythroid cells in the regulation of the immune response" opublikowana w czasopiśmie *Pharmacology & Therapeutics*. W artykule omówiono mechanizmy regulacji erytropoezy oraz opisano rolę komórek CEC w regulacji odpowiedzi układu odpornościowego w fizjologicznych i patologicznych procesach zachodzących u myszy oraz ludzi. Opisano między innymi rolę komórek CEC u noworodków, u kobiet w ciąży, w czasie wykształcania tolerancji matczyno-płodowej, w chorobie nowotworowej, chorobach zakaźnych, chorobach zapalnych, oraz w niedokrwistości. Następnie opisano poznane mechanizmy regulacji odpowiedzi odpornościowej przez komórki CEC, w tym hydrolizę L-argininy przez arginazę (ARG), wytwarzanie transformującego czynnika wzrostu β (TGF-β, ang. *transforming growth factor β*), regulację przez cząsteczki tworzące tak zwane punkty kontroli układu odpornościowego, wydzielanie immunomodulujących cytokin oraz wytwarzanie reaktywnych form tlenu (RFT). W pracy opisano potencjalne strategie terapeutyczne mające na celu regulację procesu powstawania oraz dojrzewania komórek CEC, jak również sposoby modulowania ich właściwości immunoregulatorowych. Dodatkowo, w pracy przedyskutowano braki w wiedzy dotyczącej komórek CEC, które wymagają dalszych badań oraz omówiono kierunki i perspektywy dalszych badań nad rolą tych komórek.

W pracy pt. „Tumor Immune Evasion Induced by Dysregulation of Erythroid Progenitor Cells Development" opublikowanej w czasopiśmie *Cancers* opisano zmiany w procesie erytropoezy, które indukowane są przez chorobę nowotworową. Na podstawie dostępnej

literatury przedstawiono zaburzenia w procesie powstawania oraz różnicowania komórek CEC w różnych typach nowotworów. Następnie opisano rolę komórek CEC w procesach immunoregulacji oraz porównano ich funkcje z komórkami układu odpornościowego o dobrze opisanej roli w regulacji odpowiedzi przeciwnowotworowej, między innymi z komórkami supresorowymi pochodzenia szpikowego (MDSCs, ang. *myeloid-derived suppressor cells*) i limfocytami T regulatorowymi. Następnie przedstawiono rolę komórek CEC w regulacji odpowiedzi przeciwnowotworowej oraz systemowej odpowiedzi odpornościowej u chorych z nowotworem. Dodatkowo opisano wpływ komórek CEC na proliferację i inwazyjność komórek nowotworowych oraz progresję choroby, w zależności od etapu różnicowania komórek CEC. Ponadto opisano mechanizmy prowadzące do ekspansji komórek CEC, które są zaangażowane w zwiększenie ryzyka progresji choroby nowotworowej oraz potencjalne strategie terapeutyczne promujące dojrzewanie komórek CEC i zmniejszające ich ekspansję. Ostatecznie przedstawiono kliniczne znaczenie komórek CEC jako regulatorów progresji choroby oraz markerów prognostycznych.

Trzecią publikacją wchodzącą w skład rozprawy doktorskiej jest artykuł oryginalny pt. „Potent but transient immunosuppression of T-cells is a general feature of $CD71^+$ erythroid cells" opublikowany w czasopiśmie *Communications Biology*. W pracy opisano ekspansję komórek CEC we wczesnych stadiach ich dojrzewania w mysich modelach ostrej niedokrwistości. Wykazano, że ekspansja komórek CEC nie wpływa na wytwarzanie przeciwciał klasy IgG przeciwko owalbuminie (OVA, ang. *ovalbumin*) w odpowiedzi na immunizację u myszy z niedokrwistością ani na produkcję czynnika martwicy nowotworu α (TNF-α, ang. *tumor necrosis factor α*) przez komórki wykazujące ekspresję cząsteczki CD11b w śledzionie. Wykazano natomiast, że niedokrwistość i towarzysząca jej ekspansja komórek CEC prowadzą do upośledzenia proliferacji limfocytów T cytotoksycznych rozpoznających peptyd pochodzący z OVA. Następnie w doświadczeniach *ex vivo* wykazano, że komórki CEC indukowane niedokrwistością są odpowiedzialne za upośledzenie proliferacji limfocytów T. Opisano wysoką ekspresję mitochondrialnej izoformy ARG – ARG2, oraz wydzielanie dużych ilości RFT w mysich komórkach CEC. Następnie wykazano, że kluczowymi mechanizmami regulacji limfocytów T przez komórki CEC w niedokrwistości są ARG2 i RFT. Ponadto potwierdzono, że komórki CEC znajdujące się we wczesnych stadiach dojrzewania, mianowicie przed enukleacją, są

odpowiedzialne za hamowanie proliferacji limfocytów T. W dalszej części pracy opisano ekspansję komórek CEC w krwi osób z niedokrwistością oraz ich wpływ na zahamowanie wytwarzania interferonu γ (IFN-γ) przez limfocyty T. Zaobserwowano, że w komórkach CEC wyizolowanych ze szpiku zdrowych dawców dochodzi do wytwarzania obu izoform ARG oraz zbadano rolę tych enzymów w regulacji proliferacji limfocytów T. Wykorzystując komórki CEC różnicowane z komórek jednojądrzastych krwi obwodowej (PBMCs, ang. *peripheral blood mononuclear cells*) zdrowych dawców krwi oraz modelowe ludzkie linie komórek erytroidalnych opisano rolę ARG i RFT w regulacji odpowiedzi T-komórkowej przez ludzkie komórki CEC. Wykazano, że komórki CEC znajdujące się we wczesnych stadiach dojrzewania mają najsilniejsze właściwości immunoregulatorowe, które zanikają wraz z dalszym różnicowaniem komórek CEC.

Podsumowując, przedstawione w niniejszej pracy doktorskiej wyniki wskazują, iż komórki CEC pełnią istotne funkcje immunoregulatorowe w wielu fizjologicznych i patofizjologicznych warunkach. Opisano rolę komórek CEC w regulacji odpowiedzi immunologicznej w niedokrwistości i zależność tej regulacji od ARG i RFT. Wykazano, że immunoregulatorowe właściwości komórek CEC ulegają znacznym zmianom w trakcie ich różnicowania i są najsilniejsze we wczesnych stadiach dojrzewania. Modulacja dojrzewania komórek CEC oraz wykorzystywanych przez nie mechanizmów regulacji odpowiedzi immunologicznej są obiecującymi strategiami terapeutycznymi w wielu jednostkach chorobowych.

Streszczenie w języku angielskim

Elucidating the role of CD71$^+$ erythroid cells (CECs) in the regulation of immune response

CD71$^+$ erythroid cells (CECs) are progenitors and precursors of erythrocytes that have been recently identified as the regulators of the immune response. Anemia is the main factor that induces their expansion. However, the immunoregulatory role of anemia-induced CECs remained unknown. The aim of this dissertation that includes three publications was to describe the role of CECs in the regulation of immune response and to elucidate the immunoregulatory properties of anemia-induced CECs.

The first article included in the publication series is a review article “The role of CD71$^+$ erythroid cells in the regulation of the immune response” published in *Pharmacology & Therapeutics*. This article discusses the mechanisms of erythropoiesis regulation and describes the role of CECs in the regulation of immune response in physiological and pathological processes in mice and humans. The role of CECs in neonates, in pregnant women, during the development of maternal-fetal tolerance, in cancer, in patients with infectious diseases, inflammatory diseases, and anemia is described. Next, we described the mechanisms of immune response regulation by CECs, including hydrolysis of L-arginine by arginase (ARG), production of transforming growth factor β (TGF-β), expression of immune checkpoints, production of immunomodulatory cytokines, and production of reactive oxygen species (ROS). This article presents potential therapeutic strategies to regulate the formation and maturation of CECs as well as methods to modulate their immunoregulatory properties. Additionally, it indicates what elements in the knowledge about CECs require further investigation and what are the prospects for further research on the role of these cells.

In the article "Tumor Immune Evasion Induced by Dysregulation of Erythroid Progenitor Cells Development", published in the *Cancers* journal, changes in erythropoiesis induced by cancer were described. Based on literature data, the dysregulation in the process of formation and differentiation of CECs in different types of cancer is presented. Next, the role of CECs as immunoregulatory cells is described and their functions are compared with well-described immune cells that play a role in the regulation of anti-tumor response,

including myeloid-derived suppressor cells (MDSCs) and regulatory T cells. Next, the role of CECs in regulating antitumor and systemic immune responses in patients with cancer is presented. In addition, the effects of CECs on tumor cell proliferation and invasiveness and disease progression, depending on the differentiation stage of CECs, are described. Furthermore, the mechanisms leading to the expansion of CECs that are involved in increasing the risk of cancer progression and potential therapeutic strategies to promote the maturation of CECs and reduce their expansion are discussed. Finally, the clinical significance of CECs as regulators of disease progression and prognostic markers is presented.

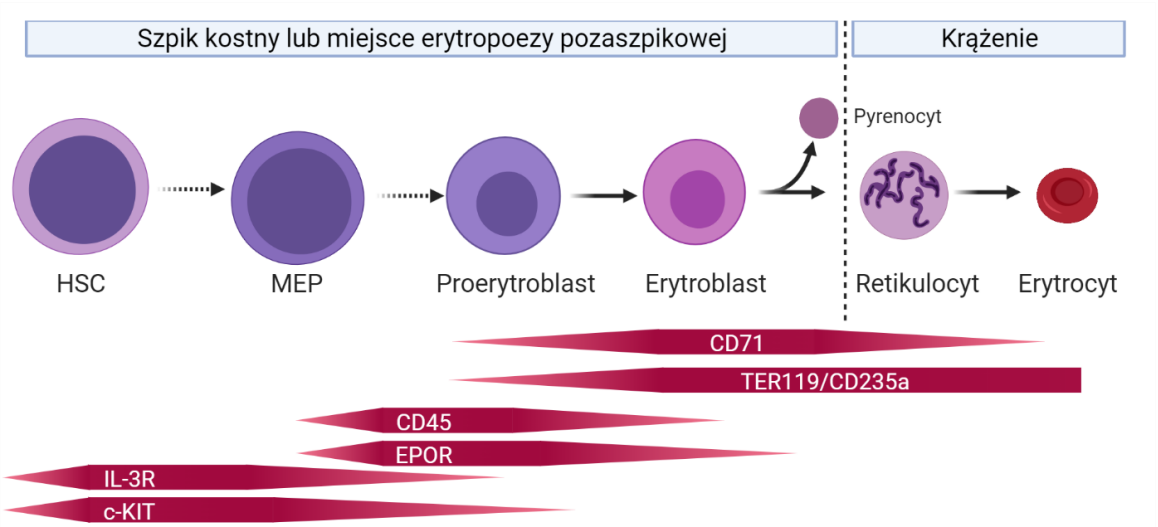
The third publication is the original article "Potent but transient immunosuppression of T-cells is a general feature of $CD71^+$ erythroid cells" published in the journal *Communications Biology*. The article describes the expansion of CECs at early stages of maturation in murine models of acute anemia. In this work, it was shown that expansion of CECs does not affect the production of IgG class anti-ovalbumin (OVA) antibodies in response to immunization in anemic mice or the production of tumor necrosis factor α (TNF-α) by cells expressing the CD11b molecule in the spleen, while it leads to the impairment of the proliferation of cytotoxic T lymphocytes that recognize the OVA-derived peptide. Subsequently, anemia-induced CECs were shown to be responsible for impaired T-cell proliferation in *ex vivo* experiments. High expression of the mitochondrial ARG isoform, ARG2, and high levels of ROS in mouse CECs were described. Subsequently, ARG2 and ROS were shown to be crucial mechanisms of T-cell regulation by CECs in anemia. Furthermore, it was confirmed that CECs at the early stages of maturation, before enucleation, are responsible for the inhibition of T-cell proliferation. We further described the expansion of CECs in the peripheral blood of anemic patients and their effect on the inhibition of interferon γ (IFN-γ) production by T-cells. We observed the expression of both ARG isoforms and their role in regulating T-cell proliferation by CECs in the bone marrow of healthy donors. Using CECs differentiated from peripheral blood mononuclear cells (PBMCs) of healthy blood donors and model human erythroid cell lines, the role of ARG and ROS in the regulation of T-cell responses by human CECs was confirmed. It was demonstrated that CECs at the early stages of maturation possess the strongest immunoregulatory properties, which disappear with further differentiation of CECs.

In summary, the results presented in this dissertation indicate that CECs have important immunoregulatory functions in a variety of physiological and pathophysiological conditions. The role of CECs in the regulation of the immune response in anemia and the dependence of this regulation on ARGs and ROS were demonstrated. The immunoregulatory functions of CECs have been shown to be significantly altered during their differentiation and found to be the strongest at the earliest stages of erythroid cell maturation. Modulation of the differentiation of CECs and the mechanisms they use to regulate the immune response are promising therapeutic targets in many diseases.

1. Wstęp

Komórki erytroidalne wykazujące ekspresję cząsteczki CD71 (CEC, ang. *CD71$^+$ erythroid cells*) to progenitory (EPCs, ang. *erythroid progenitor cells*) oraz prekursory erytrocytów. Ich główną rolą w organizmie człowieka jest generacja ponad 2 milionów transportujących tlen erytrocytów w ciągu jednej sekundy [1]. Dodatkowo, ostatnie lata badań ujawniły kluczową rolę komórek CEC w regulacji odpowiedzi układu odpornościowego u noworodków [2], ciężarnych [3, 4], osób z zaawansowaną chorobą nowotworową [5] oraz przechodzących infekcję ludzkim wirusem niedoboru odporności (HIV) [6] lub drugim koronawirusem ciężkiego ostrego zespołu oddechowego (SARS-CoV-2) [7].

Erytropoeza to proces namnażania i różnicowania erytrocytów z komórek macierzystych hematopoezy (HSCs, ang. *hematopoietic stem cells*) (Ryc. 1) [8]. W warunkach fizjologicznych proces erytropoezy zapewniającej podstawową produkcję erytrocytów zachodzi u dorosłych ludzi w szpiku kostnym, a u myszy w szpiku kostnym i w niewielkim stopniu w lokalizacjach pozaszpikowych, głównie w śledzionie [8-10]. W procesie erytropoezy HSCs różnicują we wspólną komórkę progenitorową krwi linii mieloidalnej i granulocytarnej (CMP, ang. *common myeloid progenitor*), następnie w komórkę progenitorową megakariocytów i erytrocytów (MEP, ang. *megakaryocyte–erythroid progenitor cell*), która różnicuje w linię erytroblastów. Dalsze stadia dojrzewania obejmują proerytroblast, erytroblast zasadochłonny, erytroblast polichromatyczny oraz erytroblast kwasochłonny, który traci jądro komórkowe przekształcając się w retikulocyt. Retikulocyty opuszczają szpik i po około tygodniu stają się dojrzałymi erytrocytami [8]. W warunkach, w których erytropoeza podstawowa nie zapewnia odpowiedniej liczby erytrocytów, aktywowana jest erytropoeza stresowa. Jest ona regulowana przez dodatkowe czynniki, takie jak białko morfogenetyczne kości 4 (BMP4, ang. *bone morphogenetic protein 4*) oraz szlak sygnałowy sonic hedgehog [11]. U ludzi zachodzi głównie w szpiku, a u myszy w miejscach pozaszpikowych, głównie w śledzionie, i jest związana ze znaczącą ekspansją komórek CEC w stosunku do warunków fizjologicznych [12, 13].



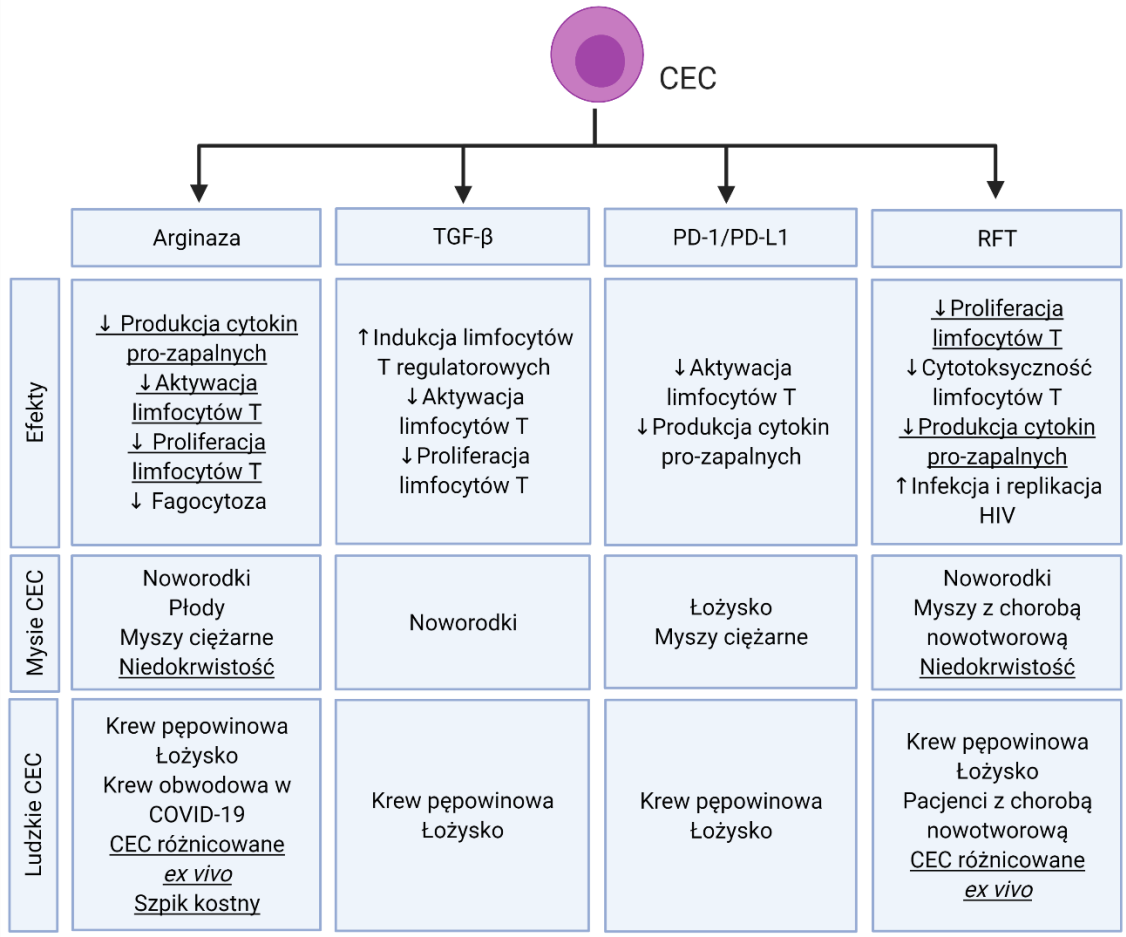
Rycina 1. Ekspresja wybranych markerów na różnych stadiach rozwojowych komórek erytroidalnych. Na podstawie Grzywa i wsp. 2021 [13], z modyfikacjami. Przygotowano przy użyciu programu Biorender.com.

Komórki CEC charakteryzują się ekspresją dwóch markerów – cząsteczki CD71 (receptor transferryny I) oraz TER119 (białko związane z glikoforyną A) u myszy i CD235a (glikoforyna A) u ludzi [13]. W trakcie różnicowania z HSCs komórki erytroidalne nabywają zdolność do wytwarzania cząsteczki CD71, która jest najbardziej nasilona w stadium erytroblastu, a następnie cząsteczka ta jest tracona z powierzchni dojrzewającego retikulocytu (Ryc. 1). Ekspresja TER119 i CD235a stopniowo rośnie w trakcie różnicowania komórek erytroidalnych, osiągając najwyższy poziom w stadium dojrzałego erytroblastu, retikulocytu oraz erytrocytu [13, 14].

Immunoregulatorowa rola komórek CEC została opisana w roku 2013 [2]. Fizjologiczna ekspansja tych komórek w śledzionie mysich noworodków prowadzi do zahamowania syntezy czynnika martwicy nowotworu alfa (TNF-α) przez komórki pochodzenia szpikowego w odpowiedzi na zabite termicznie bakterie *Listeria monocytogenes* oraz zahamowania aktywacji limfocytów T po stymulacji przeciwciałami przeciwko CD3 [2]. Podobne efekty zostały opisane dla ludzkich komórek CEC pochodzących z krwi pępowinowej [2]. W przytoczonej pracy autorzy zasugerowali, iż immunoregulacyjne właściwości komórek CEC są ograniczone do okresu noworodkowego [2]. Kolejne badania wykazały jednak, iż komórki CEC wykazują istotną rolę w regulacji odpowiedzi układu

odpornościowego w licznych warunkach, zarówno w okresie okołoporodowym, jak i na dalszych etapach rozwoju [13].

Komórki CEC wykorzystują wiele mechanizmów regulacji odpowiedzi immunologicznej, opisanych między innymi dla limfocytów T regulatorowych lub komórek supresorowych pochodzenia szpikowego (MDSCs, ang. *myeloid-derived suppressor cells*) [15] (Ryc. 2). Jednym z nich jest aktywność arginazy (ARG), wytwarzanej zarówno przez mysie jak i ludzkie komórki CEC [2, 4]. ARG hydrolizuję L-argininę do L-ornityny i mocznika, uczestnicząc w cyklu mocznikowym odpowiedzialnym za metabolizm amoniaku [16]. U ssaków obecne są dwie izoformy ARG katalizujące tę samą reakcję, ale ulegające ekspresji w różnych komórkach, w odmiennej lokalizacji komórkowej. ARG1 jest białkiem cytoplazmatycznym wytwarzanym głównie w wątrobie, podczas gdy ARG2 jest izoformą mitochondrialną ulegającą ekspresji w większości komórek. Poza rolą w metabolizmie, ARG jest kluczowym enzymem regulującym odpowiedź układu odpornościowego [17]. ARG, poprzez hydrolizę L-argininy w mikrośrodowisku, prowadzi do zmniejszenia ilości podjednostki zeta (ζ) kompleksu CD3 (CD247) odpowiedzialnej za przekazywanie sygnału aktywacji limfocytu T do wnętrza komórki [18]. Brak L-argininy w mikrośrodowisku skutkuje zahamowaniem progresji cyklu komórkowego [19], zaburzeniami w formowaniu synapsy immunologicznej [20], jak również zahamowaniem wydzielania cytokin oraz proliferacji limfocytów [21]. Kolejnym mechanizmem zaangażowanym w regulację odpowiedzi immunologicznej przez komórki CEC jest wytwarzanie reaktywnych form tlenu (RFT), które w nadmiarze prowadzą do upośledzenia odpowiedzi limfocytów T [5, 22]. Komórki CEC mogą modulować odpowiedź immunologiczną również poprzez wydzielanie transformującego czynnika wzrostu β (TGF-β, ang. *transforming growth factor β*), hamując aktywację i proliferację limfocytów T, a jednocześnie promując ich różnicowanie w limfocyty T regulatorowe [23]. W pracy Delyea i wsp. (2018) opisano również rolę komórek CEC w regulacji odpowiedzi limfocytów T w wyniku interakcji receptora programowanej śmierci 1 (PD-1, ang. *programmed cell death protein 1*) i jego liganda (PD-L1, ang. *programmed death-ligand 1*) [3].



Rycina 2. Mechanizmy regulacji odpowiedzi układu odpornościowego przez komórki CEC izolowane z różnych źródeł. Podkreślono mechanizmy opisane w ramach pracy doktorskiej. Na podstawie Grzywa i wsp. 2021 [13], z modyfikacjami. Przygotowano przy użyciu programu Biorender.com.

Komórki CEC, rozróżniane jako komórki wykazujące ekspresję cząsteczki CD71 oraz TER119/CD235a, są heterogenną populacją komórek na różnych etapach dojrzewania. W trakcie różnicowania komórek CEC ich transkryptom, proteom, jak również funkcje immunoregulatorowe ulegają znaczącym zmianom [24, 25]. Komórki CEC w najwcześniejszych stadiach różnicowania charakteryzują się ekspresją cząsteczki CD45, która jest tracona w trakcie dojrzewania [5, 26, 27]. Biorąc pod uwagę jej obecności lub brak na powierzchni komórki wyróżnia się dwie populacje komórek CEC, różniące się profilem ekspresji genów oraz rolą w regulacji odpowiedzi immunologicznej [13, 15].

Dotychczas sugerowano, iż komórki CEC wykazujące ekspresję cząsteczki CD45 są odpowiedzialne za właściwości immunoregulatorowe heterogennej populacji komórek CEC. Sugerowano, że bardziej dojrzałe komórki CEC niewykazujące ekspresji CD45, nazywane również komórkami TER (ang. *TER-Cells*, $CD45^-$ $CD71^+$ $TER119^+$), nie mają tej funkcji [28]. CD45-negatywne ($CD45^-$) komórki CEC wydzielają arteminę, neurotroficzny czynnik wzrostu, promując proliferację oraz inwazyjność komórek nowotworowych [28, 29]. Stwierdzono, że wysoka liczba $CD45^-$ komórek CEC w śledzionie osób chorych na gruczolakoraka przewodowego trzustki jest predyktorem gorszej prognozy oraz zaawansowania choroby nowotworowej [29]. Niemniej rola cząsteczki CD45 jako głównego markera komórek CEC mających właściwości immunoregulatorowe jest niejasna, ponieważ wykazano, że również komórki CEC niemające CD45 są w stanie modulować odpowiedź immunologiczną [30, 31]. Dodatkowo, dokładne zmiany w immunoregulatorowej roli komórek CEC w trakcie ich dojrzewania pozostają nieznane.

Przedstawiona rozprawa doktorska stanowi monotematyczny cykl 3 publikacji opisujących rolę komórek CEC w regulacji odpowiedzi immunologicznej. Doktorant jest pierwszym, wiodącym autorem we wszystkich pracach. Łączna wartość współczynnika oddziaływania (*impact factor*) czasopism, w których artykuły zostały opublikowane, wynosi 25.217, a sumaryczna liczba punktów MEiN wynosi 360.

Pierwszym celem pracy doktorskiej było opisanie dotychczasowej wiedzy na temat roli komórek CEC w regulacji odpowiedzi układu odpornościowego. W tym celu przygotowano dwie publikacje przeglądowe. Pierwszą publikację cyklu stanowi artykuł pt. „The role of $CD71^+$ erythroid cells in the regulation of the immune response" opublikowany w czasopiśmie *Pharmacology & Therapeutics* [13]. Jest to pierwszy obszerny przegląd literatury prezentujący obecną wiedzę na temat komórek CEC i ich roli w regulacji odpowiedzi immunologicznej. W pracy opisano mechanizmy regulacji procesu erytropoezy oraz sytuacje fizjologiczne oraz patologiczne, w których zaobserwowano zmiany w liczebności komórek CEC. Następnie omówiono rolę komórek CEC u noworodków, ciężarnych, osób z chorobą nowotworową, osób z chorobami zapalnymi oraz infekcjami wirusowymi. Przedyskutowano rolę ARG, TGF-β, cząsteczek immunomodulujących i cytokin oraz RFT w regulacji odpowiedzi immunologicznej przez

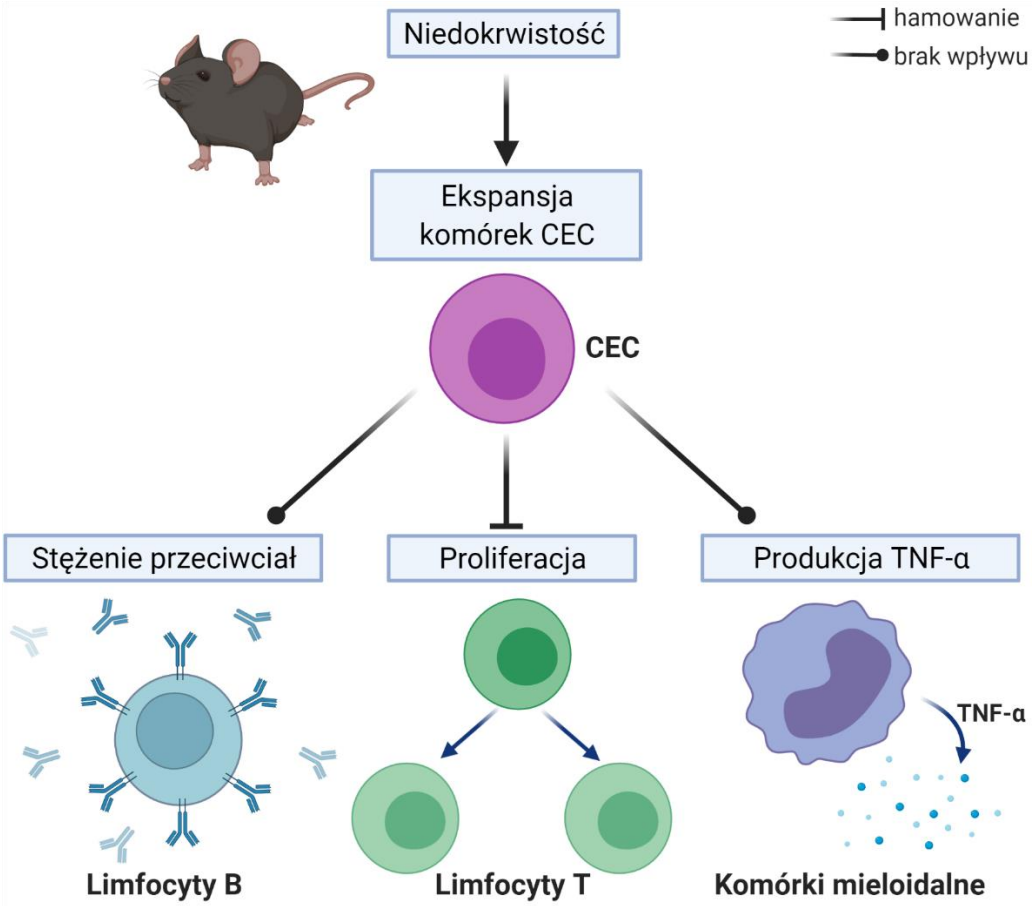
komórki CEC. Wskazano również potencjalne strategie terapeutyczne modulacji ekspansji i dojrzewania komórek CEC oraz ich mechanizmów immunoregulatorowych. Dodatkowo, analiza danych literaturowych wykazała brak jednoznacznych informacji dotyczących właściwości immunoregulatorowych komórek CEC powstających w wyniku erytropoezy podstawowej (ang. *steady-state erythropoiesis*) lub w wyniku erytropoezy stresowej (ang. *stress erythropoiesis*) indukowanych niedokrwistością bez współwystępującego zapalenia.

Drugą publikacją cyklu jest artykuł przeglądowy pt. „Tumor Immune Evasion Induced by Dysregulation of Erythroid Progenitor Cells Development" opublikowany w czasopiśmie *Cancers* [15]. W pracy przedyskutowano zmiany w erytropoezie indukowane przez rozwój choroby nowotworowej, powodujące upośledzenie dojrzewania komórek erytroidalnych. Wykazano, że rola komórek CEC jest największa w warunkach, w których dochodzi do zwiększenia ich liczby we wczesnych stadiach różnicowania, między innymi w wyniku selektywnego niszczenia bardziej dojrzałych komórek erytroidalnych, w tym erytrocytów, lub w wyniku zatrzymania dojrzewania komórek CEC na etapie wczesnych progenitorów.

Wyniki badań w ramach projektu doktorskiego zostały przedstawione w artykule oryginalnym „Potent but transient immunosuppression of T-cells is a general feature of $CD71^+$ erythroid cells" opublikowanym w czasopiśmie *Communications Biology* [32]. W pracy przedstawiono trzy mysie modele ostrej niedokrwistości – niedokrwistość niehemolityczną indukowaną dwukrotnym pobraniem krwi (NHA, ang. *non-hemolytic anemia*), niedokrwistość hemolityczną indukowana fenylohydrazyną (HA-PHZ, ang. *hemolytic anemia – phenylhydrazine*) lub przeciwciałem monoklonalnym przeciwko TER119 (HA-TER119, ang. *hemolytic anemia – TER119*). Opisano ekspansję komórek CEC w śledzionie myszy z niedokrwistością ze zwiększeniem udziału frakcji komórek CEC znajdujących się w najwcześniejszych stadiach różnicowania.

Głównym celem pracy było zbadanie wpływu indukcji ostrej niedokrwistości i ekspansji komórek CEC na odpowiedź układu immunologicznego – odpowiedź humoralną, odpowiedź T-komórkową, oraz odpowiedź nieswoistą komórek pochodzenia szpikowego (Ryc. 3). Wykazano brak istotnego wpływu ostrej niedokrwistości na wytwarzanie przeciwciał klasy IgG przeciwko białku jaja kurzego, owalbuminie (OVA, ang. *ovalbumin*) po immunizacji tym białkiem oraz na wydzielanie TNF-α w odpowiedzi na stymulację

zabitymi termicznie bakteriami pałeczki okrężnicy (*Escherichia coli*). Następnie zbadano odpowiedź T-komórkową w modelu adoptywnego transferu limfocytów cytotoksycznych izolowanych od myszy transgenicznych OT-I, selektywnie rozpoznających peptyd SIINFEKL z białka OVA. Zaobserwowano istotnie upośledzoną proliferację limfocytów OT-I w śledzionie myszy NHA w porównaniu do myszy kontrolnych.



Rycina 3. Wpływ ekspansji komórek CEC na odpowiedź komórek układu odpornościowego. W pracy wykazano, iż ekspansja komórek CEC w śledzionie myszy z niedokrwistością prowadzi do upośledzenia proliferacji limfocytów T, bez wpływu na wytwarzanie przeciwciał przez limfocyty B oraz wydzielanie TNF-α przez komórki pochodzenia szpikowego. Przygotowano przy użyciu programu Biorender.com.

Następnie zbadano, czy to komórki CEC indukowane przez niedokrwistość są odpowiedzialne za supresję proliferacji limfocytów OT-I. W tym celu wyizolowano komórki CEC ze śledzion myszy z niedokrwistością przy pomocy selekcji immunomagnetycznej, a następnie przeprowadzono testy proliferacji limfocytów T *ex vivo* w obecności komórek CEC. Doświadczenia te wykazały, że mysie komórki CEC

indukowane niedokrwistością hamują proliferację limfocytów T, podobnie do komórek CEC indukowanych przez inne czynniki lub warunki patofizjologiczne.

Kolejnym celem pracy było opisanie mechanizmu regulacji odpowiedzi limfocytów T przez mysie komórki CEC. W tym celu sprawdzano ekspresję obu izoform ARG oraz wytwarzanie RFT. Wykazano, że mysie komórki CEC indukowane niedokrwistością wykazują ekspresję ARG2 oraz charakteryzują się nasilonym wytwarzaniem RFT, podczas gdy ilość ARG1 jest bardzo mała lub niewykrywalna. Wytwarzanie obu izoform ARG oraz RFT było największe w komórkach CEC znajdujących się w najwcześniejszych stadiach dojrzewania, które ulegają istotnej ekspansji w śledzionie w odpowiedzi na niedokrwistość, i malało wraz z dalszym różnicowaniem komórek CEC osiągając najniższe wartości w dojrzałych erytrocytach.

Ilość ARG1 w komórkach CEC była podwyższona w jednym z modeli niedokrwistości, HA-PHZ, jednak sama aktywność enzymatyczna ARG była obniżona. Dodatkowo, komórki CEC izolowane ze śledzion myszy HA-PHZ wykazywały najsłabsze właściwości immunosupresyjne *ex vivo*, jak również nie zaobserwowano wpływu indukcji niedokrwistości PHZ na proliferację limfocytów OT-I *in vivo.* Wykazano, iż PHZ zmniejsza aktywność enzymatyczną ARG, prowadząc do zamaskowania właściwości immunoregulatorowych komórek CEC. Wyniki te tłumaczą brak obserwowanego efektu immunoregulatorowego komórek CEC indukowanych przez PHZ w poprzednich badaniach [5].

Następnie zbadano, jakie są mechanizmy supresji limfocytów T przez mysie komórki CEC indukowane przez niedokrwistość. Wykazano, że komórki CEC hamują proliferację mysich limfocytów T w wyniku aktywności ARG i RFT. Zahamowanie tych mechanizmów przez zastosowanie inhibitora obu izoform ARG, OAT-1746 [33], lub N-acetylocysteiny, związku o działaniu antyoksydacyjnym doprowadziło do zniesienia supresyjnego działania komórek CEC na proliferację limfocytów T. Dodatkowo, wykazano kluczową rolę ARG2 w immunoregulatorowej roli komórek CEC. Zaobserwowano bowiem, że komórki CEC wyizolowane z myszy pozbawionych ARG2 o aktywności katalitycznej charakteryzują się upośledzonymi właściwościami immunosupresyjnymi wobec limfocytów T. Zaobserwowano, że ekspansja komórek CEC w śledzionach myszy

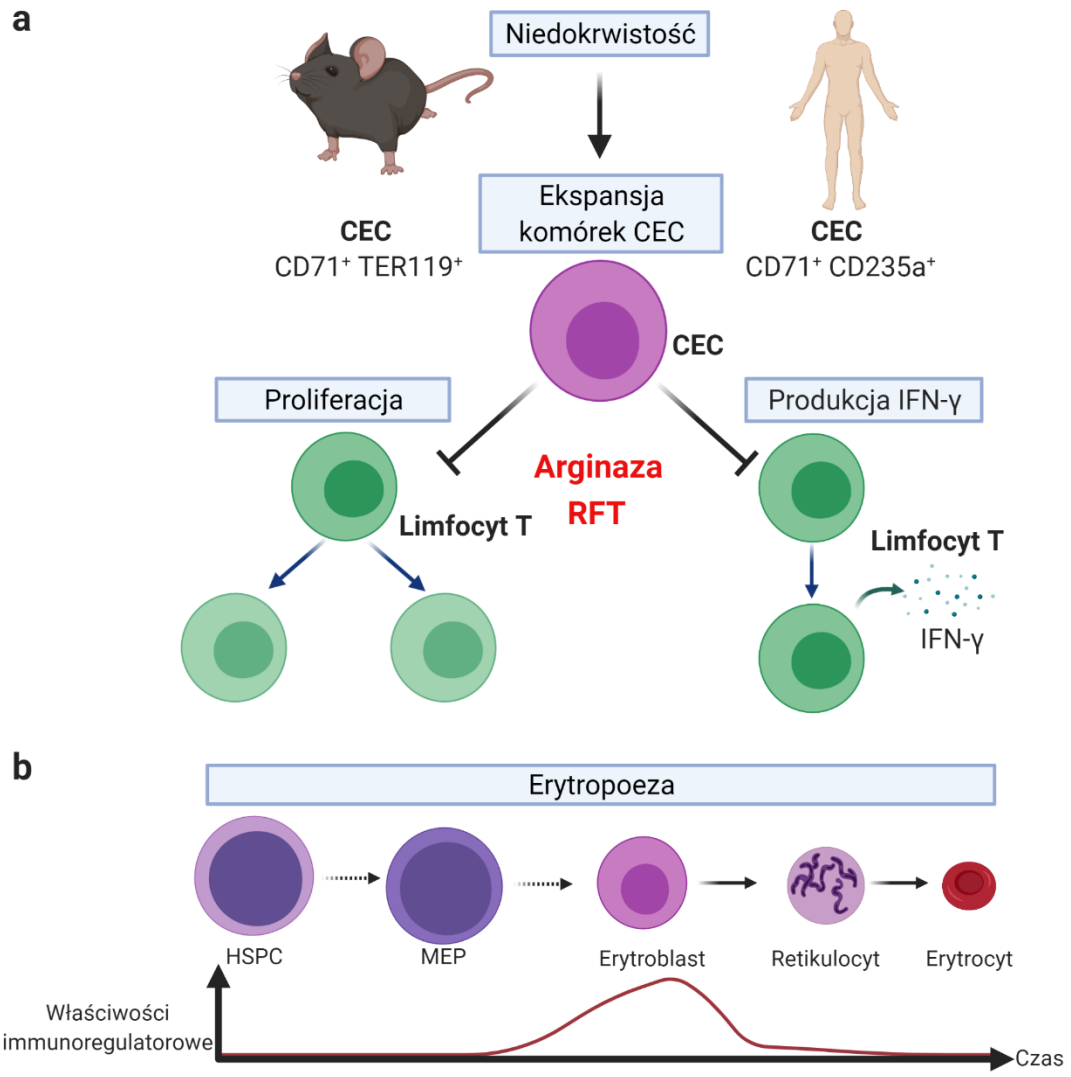
z niedokrwistością prowadzi do zwiększenia wytwarzania ARG2 w mikrośrodowisku śledziony, a także do wzrostu aktywności ARG, prowadząc do obniżenia ilości podjednostki ζ kompleksu CD3. Tym samym, ekspansja komórek CEC w śledzionie może prowadzić do supresji limfocytów T w mechanizmach podobnych do tych obserwowanych u osób z chorobą nowotworową, chorych we wstrząsie septycznym lub u ciężarnych [34-36].

W dalszym etapie badań sprawdzono, czy obserwacje uzyskane dla mysich komórek CEC są prawdziwe również dla ich ludzkich odpowiedników. Wykazano ekspansję komórek CEC we krwi osób z niedokrwistością. Liczba komórek CEC korelowała negatywnie ze stężeniem hemoglobiny. Następnie wykazano, że komórki CEC wyizolowane z aspiratów szpiku zdrowych dawców wykazują ekspresję zarówno ARG1 i ARG2 oraz hamują proliferację limfocytów T w mechanizmie zależnym od aktywności ARG.

Następnie przy wykorzystaniu komórek CEC różnicowanych z PBMCs zdrowych dawców krwi, jak również modelowych linii komórek erytroidalnych wyprowadzonych z ludzkich komórek erytroleukemii wykazano rolę ARG i RFT w regulacji odpowiedzi T-komórkowej przez ludzkie komórki CEC.

Ostatnim celem pracy było zbadanie zmian w ekspresji ARG1 i ARG2 oraz potencjału immunoregulatorowego ludzkich komórek CEC znajdujących się w różnych etapach różnicowania. Wykazano, że wytwarzanie ARG1 i ARG2 jest większe w mniej zróżnicowanych $CD45^+$ komórkach CEC pochodzących z aspiratów szpiku zdrowych dawców w porównaniu do $CD45^-$ komórek CEC. Pomimo ekspresji ARG1 i ARG2 w ludzkich komórkach macierzystych i progenitorowych hematopoezy (HSPCs, ang. *hematopoietic stem and progenitor cells*) nie zaobserwowano ich wpływu na proliferację limfocytów T. Przy wykorzystaniu systemu ciągłej hodowli i różnicowania komórek CEC z PBMCs wykazano, że silna supresja aktywacji, wytwarzania IFN-γ oraz proliferacji limfocytów T jest charakterystyczna dla komórek CEC znajdujących się we wczesnych stadiach różnicowania, na etapie komórek charakteryzujących się wysoką ekspresją cząsteczki CD71 i niską ekspresją cząsteczki CD235a. Potencjał immunoregulatorowy zanika wraz z dalszym dojrzewaniem i jest nieobecny w dojrzałych erytrocytach pobranych od zdrowych dawców.

Podsumowując, w pracy po raz pierwszy opisany został wpływ ostrej niedokrwistości na odpowiedź immunologiczną w mysim modelu, jak również rola i mechanizmy komórek CEC indukowanych niedokrwistością w regulacji odpowiedzi immunologicznej. W pracy opisano również zmiany potencjału immunoregulatorowego komórek CEC w trakcie ich różnicowania (Ryc. 4). Dodatkowo wykazano, że supresja limfocytów T jest cechą wspólną mysich i ludzkich komórek CEC, niezależnie od czynnika indukującego ich ekspansję.



Ryc. 4. Wpływ mysich i ludzkich komórek CEC na funkcje limfocytów T oraz zmiany w potencjale immunoregulatorowym komórek CEC w trakcie ich dojrzewania. a, Niedokrwistość u myszy oraz ludzi prowadzi do ekspansji komórek CEC, które hamują poprzez aktywność arginazy oraz RFT proliferację limfocytów T oraz wytwarzanie przez limfocyty IFN-γ. **b,** W trakcie procesu erytropoezy komórki erytroidalne nabywają właściwości immunoregulatorowe, które są najsilniejsze we wczesnych stadiach

dojrzewania komórek CEC. Właściwości te ulegają osłabieniu wraz z dalszym różnicowaniem i są nieobecne w dojrzałych erytrocytach. Przygotowano przy użyciu programu Biorender.com.

W pracach wchodzących w skład rozprawy doktorskiej opisano rolę komórek CEC w regulacji odpowiedzi układu odpornościowego, skupiając się na komórkach CEC indukowanych niedokrwistością i ich wpływem na odpowiedź T-komórkową. Wykazana ekspansja komórek CEC i ich działanie supresorowe w stosunku do limfocytów T może tłumaczyć mechanizm obserwowanego zwiększenia ekspresji ARG1 i obniżoną sygnaturę aktywacji limfocytów T $CD4^+$ [37], jak również upośledzoną odpowiedź T-komórkową i odpowiedź przeciwbakteryjną leukocytów u dzieci z niedokrwistością [38, 39]. Dodatkowo, ekspansja immunoregulatorowych komórek CEC może być jedną z przyczyn zwiększonego ryzyka infekcji u chorych poddawanych operacjom chirurgicznym, u których przed operacją lub bezpośrednio po niej była obecna niedokrwistość [40]. Rola immunoregulatorowych funkcji komórek CEC pozostaje niejasna. Dane sugerują, że ekspansja komórek CEC może zapobiegać progresji odpowiedzi immunologicznej w przewlekłe zapalenie oraz ograniczać syntezę cytokin zapalnych, w tym IFN-γ, wpływających hamująco na proces erytropoezy [13, 41]. Wyniki przedstawione w rozprawie doktorskiej wskazują na istotną rolę, którą pełnią komórki CEC w regulacji odpowiedzi układu odpornościowego zależnej od limfocytów T.

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3. Założenia i cel pracy

Istotne właściwości immunoregulatorowe komórek CEC opisano w wielu modelach mysich oraz u ludzi na różnych etapach życia w fizjologicznych oraz patologicznych warunkach. Dotychczas dane literaturowe sugerowały, iż komórki CEC tylko w niektórych warunkach regulują odpowiedź immunologiczną. Dlatego, głównym celem niniejszej pracy doktorskiej było zbadanie roli komórek CEC w regulacji odpowiedzi immunologicznej oraz określenie czy immunoregulatorowe właściwości są cechą wspólną komórek CEC indukowanych różnymi czynnikami. Kluczowe było również poznanie czy w trakcie dojrzewania komórek erytroidalnych dochodzi do zmian immunoregulatorowych właściwości tych komórek. Szczegółowe cele pracy obejmowały:

- opisanie właściwości immunoregulatorowych komórek CEC oraz mechanizmów regulacji odpowiedzi układu odpornościowego;
- zbadanie wpływu ostrej niedokrwistości niehemolitycznej na odpowiedź układu odpornościowego;
- zbadanie poziomu ARG1, ARG2 oraz RFT w mysich komórkach CEC indukowanych niedokrwistością;
- określenie zmian w ekspresji ARG1, ARG2 oraz RFT w mysich komórkach CEC znajdujących się w różnych stadiach dojrzewania;
- zbadanie właściwości immunoregulatorowych mysich komórek CEC indukowanych niedokrwistością;
- zbadanie roli ARG oraz RFT w regulacji odpowiedzi immunologicznej przez mysie komórki CEC;
- zbadanie właściwości immunoregulatorowych ludzkich komórek CEC indukowanych niedokrwistością oraz ludzkich komórek CEC ze szpiku kostnego;
- zbadanie roli ARG oraz RFT w regulacji odpowiedzi immunologicznej przez ludzkie komórki CEC;
- zbadanie zmian w ekspresji ARG1 i ARG2 oraz właściwości immunoregulatorowych ludzkich komórek CEC znajdujących się w różnych stadiach dojrzewania.

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The role of CD71$^+$ erythroid cells in the regulation of the immune response

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ABSTRACT

Complex regulation of the immune response is necessary to support effective defense of an organism against hostile invaders and to maintain tolerance to harmless microorganisms and autoantigens. Recent studies revealed previously unappreciated roles of CD71$^+$ erythroid cells (CECs) in regulation of the immune response. CECs physiologically reside in the bone marrow where erythropoiesis takes place. Under stress conditions, CECs are enriched in some organs outside of the bone marrow as a result of extramedullary erythropoiesis. However, the role of CECs goes well beyond the production of erythrocytes. In neonates, increased numbers of CECs contribute to their vulnerability to infectious diseases. On the other side, neonatal CECs suppress activation of immune cells in response to abrupt colonization with commensal microorganisms after delivery. CECs are also enriched in the peripheral blood of pregnant women as well as in the placenta and are responsible for the regulation of feto-maternal tolerance. In patients with cancer, anemia leads to increased frequency of CECs in the peripheral blood contributing to diminished antiviral and antibacterial immunity, as well as to accelerated cancer progression. Moreover, recent studies revealed the role of CECs in HIV and SARS-CoV-2 infections. CECs use a full arsenal of mechanisms to regulate immune response. These cells suppress proinflammatory responses of myeloid cells and T-cell proliferation by the depletion of L-arginine by arginase. Moreover, CECs produce reactive oxygen species to decrease T-cell proliferation. CECs also secrete cytokines, including transforming growth factor β (TGF-β), which promotes T-cell differentiation into regulatory T-cells. Here, we comprehensively describe the role of CECs in orchestrating immune response and indicate some therapeutic approaches that might be used to regulate their effector functions in the treatment of human conditions.

Contents

Abbreviations: AI, anemia of inflammation; ARG, arginase; CECs, CD71$^+$ erythroid cells; COVID-19, Corona Virus Disease 2019; DCs, dendritic cells; EBV, Epstein–Barr virus; EPO, erythropoietin; HCC, hepatocellular carcinoma; HIV, human immunodeficiency virus; HSCs, hematopoietic stem cells; IBD, inflammatory bowel diseases; IDA, iron-deficiency anemia; IFN-γ, interferon γ; IL, interleukin; MDSCs, myeloid-derived suppressor cells; NK, natural killer; PBMC, peripheral blood mononuclear cells; PD-1, Programmed cell death protein 1; PD-L1, programmed death-ligand 1; ROS, reactive oxygen species; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; TLR, Toll-like receptor; TNF-α, tumor necrosis factor-alpha; Tregs, regulatory T cells; TGF-β, transforming growth factor β; VISTA, V-type immunoglobulin domain-containing suppressor of T cell activation..

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1. Introduction

Erythroid cells are the most abundant host cells in the human body reaching over 25×10^{12} cells and constituting over 80% of the total human cell count (Sender, Fuchs, & Milo, 2016). Due to the enormous amounts of hemoglobin, that constitutes over 90% of the erythrocyte cytosol proteome, erythroid cells are mainly considered as oxygen transporters. However, recent studies have revealed a huge diversity of erythroid cell functions. Due to a huge number of erythroid cells, any gene expressed even at a low level in these cells achieves a high proportion in the organism and potentially may exert substantial biological effects.

Among many less known functions, erythroid cells were found to regulate the immune response. The first links between erythropoiesis and immune response were reported over 40 years ago (Conway de Macario & Macario, 1979; Pavia & Stites, 1979). An increase in the erythropoiesis rate induced by erythropoietin (EPO) or resulting from the induction of anemia in mice led to almost complete inhibition of antibody secretion against *E. coli* antigen, β-D-galactosidase (Conway de Macario & Macario, 1979). At the same time, impaired cell-mediated immune response and bactericidal activity of leukocytes were described in anemic children (Srikantia, Prasad, Bhaskaram, & Krishnamachari, 1976). However, only recent studies have changed our view on erythroid cells from cells mainly transporting oxygen to important regulators of both innate and adaptive immune responses (Adkins, Levy, & Betz, 2014; Anderson, Brodsky, & Mangalmurti, 2018; Bordon, 2014; Elahi, 2014, 2019; Elahi & Mashhouri, 2020). This review aims to provide a comprehensive overview of the regulation of the immune response by erythroid lineage cells.

2. Regulation of erythropoiesis

Erythropoiesis is a highly regulated process that generates from hematopoietic stem cells (HSCs) over 2×10^{11} erythrocytes per day (Hattangadi, Wong, Zhang, Flygare, & Lodish, 2011; Hom, Dulmovits, Mohandas, & Blanc, 2015; Jafari, Ghadami, Dadkhah, & Akhavan-Niaki, 2019; Valent et al., 2018). HSCs reside in the bone marrow niches, where local environment maintains and regulates their self-renewal and differentiation (Morrison & Scadden, 2014). This microenvironment is composed of multiple cell types, including leptin receptor$^+$ (LepR) stromal cells, CXC chemokine ligand (CXCL)12-abundant reticular (CAR) cells, and CD169$^+$ macrophages that collectively enable effective erythropoiesis (A. Chow et al., 2013; Andrew Chow et al., 2011; Comazzetto et al., 2019; Omatsu et al., 2010). In response to hematopoietic stress, this hematopoietic niche expands to the extramedullary sites (Inra et al., 2015; Morrison & Scadden, 2014), mainly to the spleen (Fig. 1) (Oda et al., 2018). During erythropoiesis, HSCs differentiate into megakaryocyte-erythroid progenitors (MEP), followed by the burst-forming unit-erythroid (BFU-E) progenitors, and finally the colony-forming unit-erythroid (CFU-E) progenitors (Dzierzak & Philipsen, 2013). Molecular markers used for the identification and analysis of each of the erythroid cell populations are presented in Table 1. CFU-Es differentiate in a process called terminal erythropoiesis within erythroblastic islands, which are specialized microenvironments formed by macrophages surrounded by developing erythroblasts (Fig. 1) (A. Chow et al., 2013; de Back, Kostova, van Kraaij, van den Berg, & Van Bruggen, 2014; Wei Li, Guo, Song, & Jiang, 2021). During terminal erythropoiesis, proerythroblasts undergo major changes, including cell size reduction as well as nuclear condensation, and begin to produce intensively erythroid lineage-specific proteins including hemoglobin. Proerythroblasts differentiate into basophilic, polychromatophilic, and orthochromatophilic erythroblasts, successively (Fig. 1) (Moras, Lefevre, & Ostuni, 2017). Finally, orthochromatophilic erythroblasts lose their nucleus to form pyrenocytes, i.e., nuclei surrounded by the cytoplasm and plasma membrane, and reticulocytes that enter the circulation (Moras et al., 2017). This process is accompanied by the loss of many organelles, including endoplasmic reticulum and mitochondria. Reticulocytes represent 0.5-3% of peripheral blood cells (Paterakis et al., 1993). In humans, it takes about a week for a reticulocyte to mature into an erythrocyte (Dzierzak & Philipsen, 2013).

Erythropoiesis is strictly regulated by multiple factors including hematopoietic cytokines, glucocorticoids, and activation of the peroxisome proliferator-activated receptor α (PPAR-α) (Bauer et al., 1999; H. Y. Lee et al., 2015; Tusi et al., 2018). Moreover, erythropoiesis is regulated by the availability of selected nutrients, including glucose, L-glutamine, and iron (Oburoglu et al., 2014; Papanikolaou & Pantopoulos, 2017). At the earliest stages, erythroid progenitors expansion mainly depends on stem cell factor (SCF) and c-Kit and is additionally supported by thrombopoietin (TPO), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 3 (IL-3), and IL-11 (Dzierzak & Philipsen, 2013; von Lindern et al., 1999). At later stages, EPO together with the SCF are the main regulators of erythroid cell differentiation (Kuhrt & Wojchowski, 2015; Wu, Klingmüller, Besmer, & Lodish, 1995).

At the molecular level, erythropoiesis is orchestrated by the GATA-1 transcription factor, which regulates expression of all known erythroid genes (Gutiérrez, Caballero, Fernández-Calleja, Karkoulia, & Strouboulis, 2020). Moreover, GATA1 is responsible for the repression of GATA2, which regulates proliferation and maintenance of HSCs and inhibits erythroid differentiation, as well as PU.1, which promotes myeloid cell differentiation suppressing erythroid commitment (Nerlov, Querfurth, Kulessa, & Graf, 2000). Other transcription factors crucial for regulating erythroid cell fate include GFI1b, Klf1, Tal1, Lmo2, and c-Myc (Capellera-Garcia et al., 2016; Love, Warzecha, & Li, 2014; Osawa et al., 2002). Erythropoiesis is negatively regulated by death receptors, including Fas (CD95). Their activation triggers caspase-mediated degradation of GATA-1 leading to the maturation arrest and apoptosis (De Maria et al., 1999). EPO is known to downregulate the expression of Fas and FasL on erythroblasts to counterbalance the negative signals and prevent erythroblasts apoptosis (Y. Liu et al., 2006). Recent high-throughput methods uncovered the complexity of mechanisms regulating erythropoiesis on transcriptional (An et al., 2014; Gillespie et al., 2020; Huang et al., 2020; J. Li et al., 2014; X. Liu et al., 2017; Shi et al., 2014; Yan et al., 2018; Y. Yang et al., 2013), proteomic (Amon et al., 2019; Brand et al., 2004; Gautier et al., 2016; Jassinskaja et al., 2017; X. Liu et al., 2017), and epigenomic levels (Alvarez-Dominguez et al., 2014; Ding et al., 2016; Ludwig et al., 2019; Schulz et al., 2019) in both murine and human erythroid cells. Moreover, single-cell RNA-seq analysis revealed previously underappreciated heterogeneity of erythroid cells adding more complexity to the already complicated picture (Huang et al., 2020; Pellin et al., 2019; Tusi et al., 2018; Xie et al., 2020).

During human embryogenesis, erythropoiesis takes place in the yolk sac (Crawford, Foley, & Elmore, 2010), followed by liver (Fanni et al., 2018; Popescu et al., 2019), and spleen (Sharma, Pati, Ahuja, Takkar, & Kochupillai, 1997; Wilkins, Green, Wild, & Jones, 1994). In adult humans, bone marrow is almost exclusively responsible for steady-state erythropoiesis (Vácha, Holá, Dungel, & Znojil, 1982). In contrast, in mice about

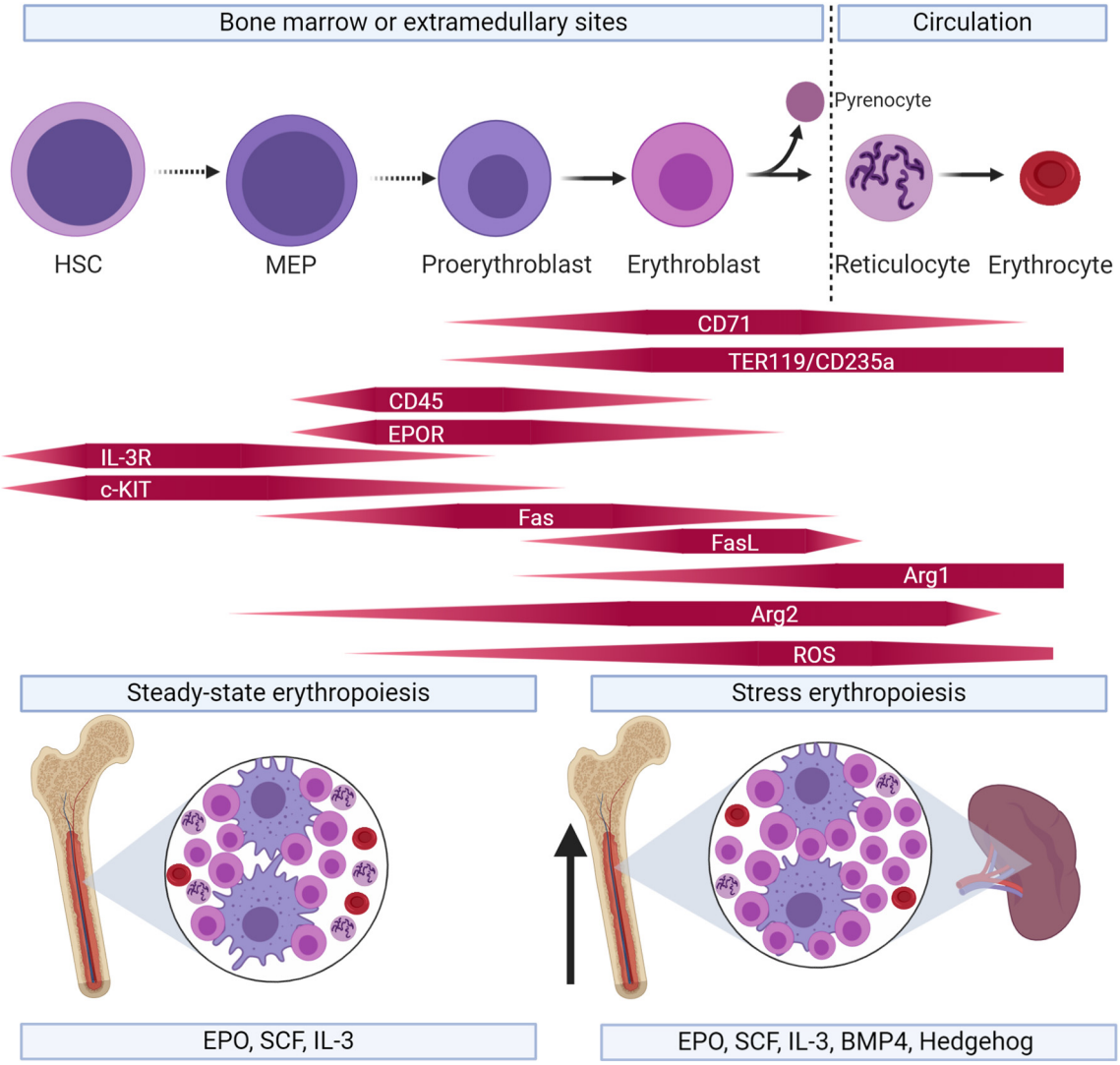

Fig. 1. Development of erythroid cells leading to the production of erythrocytes. Early stages of erythropoiesis include hematopoietic stem cell (HSC), megakaryocyte–erythroid progenitor cell (MEP), and burst-forming unit erythroid cells (BFU-E, not shown) and colony-forming unit erythroid cell (CFU-E, not shown) and are regulated by mainly IL-3/IL-3R and stem cell factor (SCF)/c-KIT. Further stages include proerythroblast (or pronormoblast, $CD71^{mid/hi}CD235a^{low}$), erythroblasts – basophilic (or early normoblast, $CD71^{hi}CD235a^{mid/hi}$), polychromatophilic (or intermediate normoblast, $CD71^{mid/hi}CD235a^{hi}$), and orthochromatic (or late normoblast, $CD71^{mid}CD235a^{hi}$) erythroblast, and are regulated mainly by erythropoietin (EPO). Orthochromatic erythroblasts lose nucleus giving pyrenocyte and reticulocyte ($CD71^{mid}CD235a^{hi}$) that enters the circulation and differentiates into erythrocyte ($CD71^{-}CD235a^{hi}$). Arginase 1 (ARG) expression starts to increase in proerythroblast reaching the highest level in the mature erythrocyte. In contrast, arginase 2 (ARG2) is expressed at the earliest stages of erythroid cell development, increases until the erythroblast stage, but starts to decrease in early reticulocyte and is undetectable in the erythrocyte. Reactive oxygen species (ROS) level increases during differentiation and reaches the highest level in late erythroblast. Steady-state erythropoiesis takes place in the bone marrow and is regulated by EPO, SCF, and IL-3. Stress erythropoiesis in humans occurs primarily in the bone marrow, but also the spleen. In mice, stress erythropoiesis occurs mainly in the spleen. Stress erythropoiesis is regulated by additional factors compared to steady-state erythropoiesis, including bone morphogenetic protein 4 (BMP4) and Hedgehog pathway.

10% of erythrocytes are produced in the spleen (Vácha et al., 1982). Blood loss or excessive damage of erythrocytes disrupt homeostasis between the production of erythrocytes by steady-state erythropoiesis and clearance of senescent or damaged cells by phagocytes. In these conditions, bone marrow erythropoiesis is supported by so-called stress erythropoiesis. It is regulated by multiple factors, including bone morphogenetic protein 4 (BMP4) and the Hedgehog pathway (Paulson, Ruan, Hao, & Chen, 2020). Moreover, extramedullary erythropoiesis requires involvement of other cell types, including natural killer (NK) cells, that protect extramedullary niches and facilitate extramedullary hematopoiesis (Jordan et al., 2013). Stress erythroid cells derive from short-term HSCs (Table 1), that migrate into the spleen (Paulson et al., 2020) and extensively proliferate, but do not differentiate until serum EPO reaches sufficient concentrations (Chen et al., 2020). Moreover, stress erythropoiesis depends on the pool of resident erythroid progenitors in the spleen (Lenox, Perry, & Paulson, 2005). Extramedullary erythropoiesis in the spleen may become a key source of erythrocytes (Kam, Ou, Thron, Smith, & Leiter, 1999), especially when EPO concentration remains high (Nijhof, Goris, Dontje, Dresz, & Loeffler, 1993). Spleen is a highly complex organ that is responsible for the filtration and destruction of pathogens and the induction of adaptive immune response as well as the regulation of erythrocytes turnover (Mebius & Kraal, 2005). It is organized into white pulp and red pulp regions, which are separated by the marginal zone (Bronte & Pittet, 2013). Importantly, extramedullary erythropoiesis disrupts this architecture leading to a loss of clear borders between these two regions (Gomes et al., 2019), which may affect initiation of the immune response.

3. $CD71^{+}$ erythroid cells

$CD71^{+}$ erythroid cells (CECs) are immature red blood cells, including erythroblasts and reticulocytes (Elahi et al., 2013). CECs are characterized by the expression of transferrin receptor I (CD71) and erythroid lineage markers, glycophorin A (CD235a) in humans and glycophorin

Table 1
Current gating strategies to identify erythroid cells

Species	Cell surface markers	Ref.
Hematopoietic stem cell (HSC)		
Mouse – long term HSC	$CD150^{hi}$ $CD48^{-}$ $CD41^{-}$ $Rho123^{lo}$ $CD34^{-/lo}$ $Flt3R^{-}$ $CD49^{lo}$ $CD201^{+}$ (EPCR) $CD244^{-}$ $CD229^{-}$	(Balazs, Fabian, Esmon, & Mulligan, 2006; Iwasaki, Arai, Kubota, Dahl, & Suda, 2010; Ng & Alexander, 2017; Oguro, Ding, & Morrison, 2013)
Mouse intermediate term HSC	$Rho123^{lo}$ $CD34^{-/lo}$ $Flt3R^{-}$ $CD150^{lo}$ $CD49^{hi}$	(Ng & Alexander, 2017)
Mouse short term HSC	Lin^{-} $CD34^{hi}$ $Flt3R^{-/+}$ $Rho123^{hi}$ $CD150^{lo}$ c-kit^{+} $Sca1^{+}$	(Harandi, Hedge, Wu, McKeone, & Paulson, 2010; Ng & Alexander, 2017)
Human HSC	Lin^{-} $CD34^{+}$ $CD38^{-}$ $CD48^{-}$ $CD84^{+}$ $CD90^{+}$ $CD45RA^{-}$ $CD49f^{+}$ $CD150^{-}$ $CD201^{+}$ (EPCR) $CD229^{-}$ $CD244^{+}$	(Fares et al., 2014; Fares et al., 2017; Majeti, Park, & Weissman, 2007; Ng & Alexander, 2017; Notta et al., 2011; Sintes, Romero, Marin, Terhorst, & Engel, 2008; Subramaniam, Talkhoncheh, Magnusson, & Larsson, 2019)
Megakaryocyte-erythroid progenitor (MEP)		
Mouse	Lin^{-} $flk2^{-}$ $IL7R\alpha^{-}$ c-kit^{+} $Fc\gamma RII/III^{-/lo}$ $CD34^{-}$ $CD105^{+/-}$ $CD150^{+/-}$ $Sca1^{-}$	(Doulatov, Notta, Laurenti, & Dick, 2012; Dulmovits, Hom, Narla, Mohandas, & Blanc, 2017; Pronk et al., 2007)
Human	Lin^{-} $CD34^{+}$ $CD38^{mid/+}$ IL-$3R^{-}$ $CD7^{+}$ $CD10^{-}$ $FLT3^{-}$ $CD45RA^{-}$ $MPL^{+/-}$ (BAH1 clone) $CD71^{+/-}$ $CD105^{+/-}$ $CD36^{-}$ $CD41^{-}$ $CD135^{-}$	(Doulatov et al., 2012; Dulmovits et al., 2017; Mori, Chen, Pluvinage, Seita, & Weissman, 2015; Notta et al., 2016; Psaila et al., 2016; Sanada et al., 2016)
Burst forming unit-erythroid (BFU-E)		
Mouse	c-kit^{+} $CD45^{+}$ $Ter119^{-}$ $CD71^{low}$	(Dulmovits et al., 2017; Flygare et al., 2011)
Mouse (stress)	$CD34^{-}$ $CD133^{-}$ c-kit^{+} $Sca\text{-}1^{+}$	(Dulmovits et al., 2017; Xiang, Wu, Chen, & Paulson, 2015)
Human	IL-$3R^{-}$ GPA^{-} $CD34^{+}$ $CD36^{-}$ $CD123^{-}$ $CD71^{low}$ $T\beta RIII^{low/hi}$	(Dulmovits et al., 2017; Gao et al., 2016; J. Li et al., 2014)
Human (stress)	$CD34^{-}$ $CD133^{-}$ c-kit^{+}	(Dulmovits et al., 2017; Xiang et al., 2015)
Colony-forming unit-erythroid (CFU-E)		
Mouse	c-kit^{+} Lin^{-} $CD45^{-/dim}$ $Ter119^{-}$ $CD71^{hi}$ $Sca\text{-}1^{-}$ $IL\text{-}7R\alpha^{-}$ $IL3R\alpha^{-}$ $CD41^{-}$	(Dulmovits et al., 2017; Flygare et al., 2011; Terszowski et al., 2005)
Human	IL-$3R^{-}$ GPA^{-} $CD34^{-}$ $CD36^{+}$	(J. Li et al., 2014; L. Zhao et al., 2018)
Erythroid progenitor cells (CECs)		
Mouse	$CD71^{hi}$ $TER119^{+}$ $CD45^{-/+}$ $CD36^{+}$	(Cui et al., 2016; Elahi et al., 2013; L. Zhao et al., 2018)
Human	$CD71^{hi}$ $CD235a^{+}$ $CD45^{-/+}$ $CD36^{+}$	(Cui et al., 2016; Elahi et al., 2013; L. Zhao et al., 2018)
Reticulocytes		
Mouse	$CD71^{+}$ $TER119^{+}$ RNA^{+} DNA^{-}	(Rhodes et al., 2016)
Human	$CD71^{+}$ $CD235a^{+}$ RNA^{+} DNA^{-}	(Griffiths et al., 2012; Malleret et al., 2013)
Erythrocytes		
Mouse	$CD71^{-}$ $TER119^{+}$ RNA^{-} DNA^{-}	(Samsel & McCoy Jr., 2015)
Human	$CD71^{-}$ $CD235a^{+}$ RNA^{-} DNA^{-}	(Samsel & McCoy Jr., 2015)

A-associated protein (TER119) in mice (Fig. 1). Expression of CD71 increases from the proerythroblast stage and reaches the highest levels in basophilic and polychromatophilic erythroblasts ($CD71^{hi}$), but decreases in orthochromatophilic ($CD71^{mid}$) cells and is absent in mature erythrocytes (K. Chen et al., 2009). Expression of TER119 and CD235a constantly increases from the earliest stages of erythroblasts up to mature erythrocytes (K. Chen et al., 2009). Moreover, by evaluation of the relative cell size together with determination of the cell surface levels of CD44 in mice or α4 integrin and band 3 in humans it is feasible to identify CECs at distinct stages of their differentiation (K. Chen et al., 2009; J. Hu et al., 2013; J. Liu et al., 2013).

In healthy adult individuals, CECs are found almost exclusively in the bone marrow. Nonetheless, they are physiologically enriched in murine (Elahi et al., 2013) and human neonatal spleens (Gomez-Lopez et al., 2016). In adult mice and humans, CECs form a rather rare population, but their number increases in certain stress conditions (Table 2) (Elahi et al., 2013; L. Zhao et al., 2018). Many pathological states, including anemia, induce extramedullary erythropoiesis in the spleen of both mice and humans (Dzierzak & Philipsen, 2013; Naing et al., 2018; Socolovsky, 2007). In humans, extramedullary erythropoiesis is observed in patients suffering from hematological diseases including hereditary spherocytosis, β-thalassemia, Hodgkin's lymphoma (Meytes et al., 1983), chronic myeloproliferative disorders (Wilkins et al., 1994), myelofibrosis (X. Wang et al., 2015), as well as osteopetrosis (Freedman & Saunders, 1981).

3.1. CECs in neonates

Leaving a safe and protected intra-uterine environment and entering the external world is a genuine challenge for neonates. Many pathogens, including *Listeria monocytogenes* (Elahi et al., 2013), *Escherichia coli* (Elahi et al., 2013), and *Bordetella pertussis* (G. Dunsmore et al., 2017) pose a significant threat to the infants (Tobias R. Kollmann, Kampmann, Mazmanian, Marchant, & Levy, 2017). Sepsis, meningitis, and pneumonia are the leading causes of death of term-born neonates, together accounting for ~600,000 deaths per year (L. Liu et al., 2016). Moreover, infections are the main cause of death in children before the age of 5 (L. Liu et al., 2015).

In the past, it was believed that this vulnerability to infections in infants results from the immaturity of the immune system. Preliminary studies in the 1950s revealed that fetal exposure to antigens is associated with the development of immune tolerance (Billingham, Brent, & Medawar, 1953). Further studies in neonates demonstrated diminished T-cells functions such as reduced IL-2 and interferon-gamma (IFN-γ) secretion upon activation (Rudd, 2020). Similarly, the inflammatory response of innate immune cells to bacteria was found to be weakened in neonates (T. R. Kollmann, Levy, Montgomery, & Goriely, 2012). However, recent studies demonstrated that the decreased immune reactivity of newborns is rather an active immunosuppression, which is necessary for the induction of immune tolerance to harmless antigens in the challenging unsterile environment than a result of the immune cells immaturity (Elahi et al., 2013; Y.-M. He et al., 2018; Tobias R. Kollmann et al., 2017; T. R. Kollmann et al., 2012; Rudd, 2020; Yu, Khodadadi, Malik, Davidson, & Salles, É. d. S. L., Bhatia, J., Hale, V. L., & Baban, B., 2018).

Decreased reactivity of the neonatal immune system makes it highly plastic and tolerant to new pathogens and antigens (Yu et al., 2018). The transient suppression of the immune response is caused by the expansion of immunomodulating cells, including myeloid-derived suppressor cells (MDSCs) (Y.-M. He et al., 2018), regulatory T cells (Tregs) (Hayakawa, Ohno, Okada, & Kobayashi, 2017), and regulatory B cells (W. E. Walker & Goldstein, 2007), as well as polarization of T-cells response towards the T_H2 phenotype (Rudd, 2020). Moreover, neonatal blood has a high concentration of immunosuppressive adenosine (Pettengill et al., 2013) and S100 alarmin that regulate the immune response to prevent hyperinflammation (Ulas et al., 2017). Other studies indicate that also CECs, a population of non-immune cells, are involved in the regulation of neonatal immunity (Elahi et al., 2013). The susceptibility of human neonates to bacterial infections is recapitulated in neonatal mice. While infection with *L. monocytogenes* did not affect the survival of adult mice, it was lethal to over half of the 6-day-old newborns (Elahi et al., 2013). Adoptive transfer of adult immune cells into neonatal mice did not alter their susceptibility to infection and failed to induce tumor necrosis factor-alpha (TNF-α) production in response to infection (Elahi et al., 2013), indicating that immune response is actively suppressed in neonates. Suppressed activation of a wide variety of immune cells by murine neonatal splenocytes was observed for the first time over 40 years ago (Pavia & Stites, 1979). Newborn splenocytes

Table 2
Frequency of CECs in different models

Model	Frequency	Ref.
CECs ($CD71^+TER119^+$) in mice		
Fetus	70% of liver cells	(Delyea et al., 2018)
Newborn mice (3-day-old)	40% of splenocytes	(G. G. Dunsmore et al., 2017)
	50% of hepatic mononuclear cells	(L. Yang et al., 2020)
Newborn rotavirus-infected mice	65% of hepatic mononuclear cells	(L. Yang et al., 2020)
Neonatal mice (6-9-day-old)	60% of splenocytes	(G. Dunsmore et al., 2017; Elahi et al., 2013; Grzywa, Sosnowska, et al., 2021; Wynn et al., 2015)
	30-40% of bone marrow cells	(G. Dunsmore et al., 2017; Wynn et al., 2015)
	40% of liver cells	(G. Dunsmore et al., 2017)
	5-10% of lung cells	(G. Dunsmore et al., 2017; A. Namdar et al., 2017)
	0.5% of thymus cells	(G. Dunsmore et al., 2017)
	5% of cells in lymph node	(Elahi et al., 2013)
Mice (12-day-old)	40% of splenocytes	(G. Dunsmore et al., 2017)
Mice (21-day-old)	30% of splenocytes	(G. Dunsmore et al., 2017)
Mice (28-day-old)	10% of splenocytes	(G. Dunsmore et al., 2017)
Healthy adult mice	5% of splenocytes	(Elahi et al., 2013; Grzywa, Sosnowska, et al., 2021; Wynn et al., 2015; L. Zhao et al., 2018)
	10-30 % of bone marrow cells	(Grzywa, Sosnowska, et al., 2021; Liu et al., 2015; Wynn et al., 2015; L. Zhao et al., 2018)
	10% of liver cells	(G. Dunsmore et al., 2017; L. Zhao et al., 2018)
	5% of blood cells	(Grzywa, Sosnowska, et al., 2021; L. Zhao et al., 2018)
	1% of lymph node cells	(L. Zhao et al., 2018)
Adult tumor-bearing mice	20-50% of splenocytes	(Han et al., 2018; Hou et al., 2021; Sano et al., 2021; Sio et al., 2013; L. Zhao et al., 2018)
	3-50% of bone marrow cells	(Han et al., 2018; Sio et al., 2013; L. Zhao et al., 2018)
	1-30% of liver cells	(Han et al., 2018; L. Zhao et al., 2018)
	60% of blood cells	(L. Zhao et al., 2018)
	1-2% of lymph node cells	(L. Zhao et al., 2018)
	3-8% of live cells in the tumor	(Han et al., 2018; L. Zhao et al., 2018)
Adult anemic mice	30-40% of splenocytes	(Elahi et al., 2013; Grzywa, Sosnowska, et al., 2021; L. Zhao et al., 2018)
	30-40% of bone marrow cells	(Grzywa, Sosnowska, et al., 2021; L. Zhao et al., 2018)
	30% of liver cells	(L. Zhao et al., 2018)
	1-25% of blood cells	(Grzywa, Sosnowska, et al., 2021; L. Zhao et al., 2018)
	1% of lymph node cells	(L. Zhao et al., 2018)
Adult Salmonella-infected mice (15 d postinfection)	50% of splenocytes	(Jackson et al., 2010)
	10% of bone marrow cells[1]	(Jackson et al., 2010)
	3% of liver cells[2]	(Jackson et al., 2010)
Adult LPS-treated mice	40% of splenocytes	(L.-X. Li, Benoun, Weiskopf, Garcia, & McSorley, 2016)
Adult pregnant mice	20-40% of splenocytes[3]	(Delyea et al., 2018; Norton et al., 2009)
	40% of placenta cells[4]	(Delyea et al., 2018)
	10% of uterus cells	(Delyea et al., 2018)
Adult mice after social stress	20% of splenocytes	(McKim et al., 2018)
Adult mice after burn injury	30% of splenocytes	(Hasan, Johnson, Kini, Baldea, & Muthumalaiappan, 2019)
	20% of bone marrow cells[5]	(Hasan et al., 2019)
Adult mice colitis	1% of splenocytes[6]	(Griseri et al., 2012)
	15% of splenocytes[7]	(Shanmugam, Trebicka, & Fu, L.-l., Shi, H. N., & Cherayil, B. J., 2014)
	25% of splenocytes	(Shim, Weliwitigoda, Campbell, Dosanjh, & Johnson, 2021)
	2.5% of bone marrow cells[6]	(Griseri et al., 2012)
Adult mice after UVB exposure	12% of splenocytes	(Sano et al., 2021)
CECs ($CD71^+CD235a^+$) in human		
Human cord blood	50-60% of cells	(Delyea et al., 2018; G. Dunsmore et al., 2017; Elahi et al., 2013; Afshin Namdar et al., 2019; Wynn et al., 2015)
	~10% of cells[8,9]	(Gomez-Lopez et al., 2016)
Peripheral blood of 1-7-day-old neonates	10% of cells	(Elahi et al., 2020)
Peripheral blood of 8-28-day-old neonates	5% of cells	(Elahi et al., 2020)
Peripheral blood of 1-6-month-old infants	2%	(Elahi et al., 2020)
Peripheral blood of 6-12-month-old infants	1%	(Elahi et al., 2020)
Peripheral blood of 1-5-year-old children	1%	(Elahi et al., 2020)
PBMC of healthy donors	0.1-1% of cells	(Delyea et al., 2018; G. Dunsmore et al., 2019; Elahi et al., 2013; Shima Shahbaz et al., 2021; Wynn et al., 2015; L. Zhao et al., 2018)
PBMC of pregnant women	20% of cells[10]	(Delyea et al., 2018; G. Dunsmore et al., 2019)
Human placenta	30-35% of cells	(Delyea et al., 2018; G. Dunsmore et al., 2019; Afshin Namdar et al., 2019)
Human anemia	2-10% of cells	(Grzywa, Sosnowska, et al., 2021; Afshin Namdar et al., 2019)
PBMC of patients with tumors	0.25-5% of cells	(J. Chen et al., 2021; L. Zhao et al., 2018)
PBMC of patients with tumors and mild anemia	2% of cells	(L. Zhao et al., 2018)
PBMC of patients with tumors and moderate or severe anemia	4% of cells	(L. Zhao et al., 2018)
PBMC of patients with anemia	10%	(Grzywa, Sosnowska, et al., 2021)
PBMC of HIV-infected patients	5% of cells	(Afshin Namdar et al., 2019)
PBMC of COVID-19 patients with mild disease[11]	4% of cells	(Shima Shahbaz et al., 2021)
PBMC of COVID-19 patients with moderate disease[11]	20% of cells	(Shima Shahbaz et al., 2021)

(continued on next page)

Table 2 (*continued*)

Model	Frequency	Ref.
PBMC of COVID-19 patients with severe disease[11]	30% of cells	(Shima Shahbaz et al., 2021)
Human HCC tumors	10% of cells	(J. Chen et al., 2021)

[1] No changes compared to healthy controls – 14% of bone marrow cells in controls (Ganz, 2019)
[2] The increase compared to healthy controls – less than 1% of liver cells in controls
[3] The increase of CECs frequency was not observed in the spleen of syngeneic pregnant mice by one team (Delyea et al., 2018) by observed by other (Norton et al., 2009)
[4] The percentage is much lower in syngeneic pregnant mice (about 20% of placenta cells)
[5] Decreased compared to controls – 50% of bone marrow cells in controls (Hasan et al., 2019)
[6] T-cell transfer model of colitis, naive $CD4^+CD25^-CD45RB^{hi}$ T-cells were injected into C57BL/6.*Rag1*$^{-/-}$ recipients
[7] Dextran Sulfate Sodium (DSS)-Induced Colitis
[8] CECs were more abundant in neonates born to women who delivered preterm without labor than in neonates born to women who delivered preterm with spontaneous labor, and then in neonates born to women who delivered at term, regardless of the process of labor
[9] Cells were incubated with a hypotonic lysing solution before staining (Gomez-Lopez et al., 2016) that leads to the lysis of CECs (Wynn et al., 2015)
[10] Increases with the time of pregnancy, highest in the third trimester. Decreases in postpartum.
[11] Increases after 5-7 days after admission to the hospital

inhibited the proliferative response of adult splenocytes to mitogens, however, they did not affect their cytotoxic response (Pavia & Stites, 1979). Further studies found that neonatal splenocytes reduce TNF-α secretion from $CD11b^+$ granulocyte/macrophage cells, $CD11c^+$ dendritic cells, and $B220^+$ lymphocytes (Elahi et al., 2013). Moreover, neonatal splenocytes were shown to suppress the activation of T-cells based on the evaluation of surface expression of T-cell activation markers (e.g. CD69 and CD25) (Elahi et al., 2013).

Table 3
Effects of CECs transfer or CECs depletion *in vivo* in murine models

Model *in vivo*	CECs	Effects	Ref.
Neonatal mice			
Endotoxin challenge	$CD71^+$ cells transfer (increase of 60% over endogenous)	No effect on survival	(Wynn et al., 2015)
	$CD71^+$ cells depletion (decrease to 40% of controls)	No effect on survival	(Wynn et al., 2015)
Polymicrobial sepsis	$CD71^+$ cells depletion (decrease to 40% of controls)	Decreased splenic bacterial load	(Wynn et al., 2015)
	$CD71^+$ cells depletion with or without $CD71^+$ cells transfer	No differences in splenic bacterial load after $CD71^+$ cells transfer	(Wynn et al., 2015)
L. monocytogenes infection	$CD71^+$ cells depletion (decrease to 40% of controls)	Decreased splenic and hepatic bacterial load	(Elahi et al., 2013; Elahi et al., 2020)
		Increased infiltration of the spleen and the liver by $CD11b^+$ and $CD11c^+$ cells	(Elahi et al., 2020)
		Increased activation of $CD11b^+$ cells in the spleen and the liver	(Elahi et al., 2020)
E. coli infection		Decreased splenic and hepatic bacterial load	(Elahi et al., 2013)
Healthy 8-day-old mice		Increased TNF-α production by intestinal $CD11b^+$ and $CD11c^+$ cells	(Elahi et al., 2013)
		Increased level of CD40, CD80 and CD86 in intestinal $CD11b^+$ and $CD11c^+$ cells[1,2]	(Elahi et al., 2013)
B. pertussis infection	$CD71^+$ cells depletion (decrease to 40% of controls)	Decreased lung bacterial load	(G. Dunsmore et al., 2017)
		Decreased lung bacterial load after reinfection	(A. Namdar et al., 2017)
		Increased percentage of TNF-α producing $CD11b^+$ splenocytes	(G. Dunsmore et al., 2017)
		Decreased inflammatory pathological alterations in the lungs	(G. Dunsmore et al., 2017)
		Increased level of CD40, CD80 and CD86 in $CD11b^+$ and $CD11c^+$ cells from lungs	(G. Dunsmore et al., 2017; A. Namdar et al., 2017)
		Increased level of CD40, CD80 and CD86 in $B220^+$ cells	(A. Namdar et al., 2017)
		Increased concentration of IFN-γ, TNF-α, and IL-12 in the lung homogenate	(G. Dunsmore et al., 2017; A. Namdar et al., 2017)
		Increased percentage of NK cells in the lungs	(G. Dunsmore et al., 2017)
		Increased percentage and number of $CD11c^+$ cells in the lungs	(G. Dunsmore et al., 2017)
		Increased percentage and number of $CD11c^+CD8^+$ cells in the lungs	(G. Dunsmore et al., 2017)
		Increased expression of CXCL1 and CXCL2 in the lungs	(G. Dunsmore et al., 2017; A. Namdar et al., 2017)
		Decreased percentage and number of $CD11b^+$ cells in the lungs[3]	(G. Dunsmore et al., 2017)
		Increased percentage of $CD11b^+CD11c^+$ cells in the lungs	(A. Namdar et al., 2017)
		Increased expression of CD25 and CD69 on $CD4^+$ T-cells in the lungs	(G. Dunsmore et al., 2017)
		Increased expression of CD25 on $CD8^+$ T-cells in the lungs	(G. Dunsmore et al., 2017)
		Increased infiltration of $CD4^+$ T-cells in the lungs	(A. Namdar et al., 2017)
		Increased anti-B. pertussis-specific IgG and IgA antibody in the lungs and serum	(A. Namdar et al., 2017)

Table 3 (*continued*)

Model *in vivo*	CECs	Effects	Ref.
Rotavirus infection	$CD71^+$ cells depletion (decrease to 30% of controls in the livers)	Increased infiltration of $CD4^+$ and $CD8^+$ T-cells into the liver of rotavirus-infected mice	(L. Yang et al., 2020)
		Increased activation of NK cells	(L. Yang et al., 2020)
		A lower titer of rotavirus in the liver and extrahepatic bile duct	(L. Yang et al., 2020)
Adult mice			
L. monocytogenes infection	Non-hemolytic anemia (40% CECs of splenocytes)	No differences in splenic and hepatic bacterial load	(Elahi et al., 2013)
Immunization with *E. coli* β-D-galactosidase	Non-hemolytic anemia	Suppression of antibody production by 93.2%	(Conway de Macario & Macario, 1979)
	EPO-injection	Suppression of antibody production by 98.3%	(Conway de Macario & Macario, 1979)
B. pertussis infection	Transfer of neonatal CECs	Increased lung bacterial load	(G. Dunsmore et al., 2017)
		Decreased percentage of TNF-α producing $CD11b^+$ splenocytes	(G. Dunsmore et al., 2017)
Tumor-bearing mice	$CD71^+$ cells depletion (decrease to 5% of controls in the spleens)	Increased proliferation of $CD8^+$ T-cells in response to viral infection	(L. Zhao et al., 2018)
		Decreased tumor growth	(Sano et al., 2021)
	CECs transfer	Decreased proliferation of $CD8^+$ T-cells in response to viral infection	(L. Zhao et al., 2018)
		Increased tumor growth	(L. Zhao et al., 2018)
Pregnancy	$CD71^+$ cells depletion (decrease to 5% of controls in spleens and to about 2.5% in placenta)	Resorption of 100% of fetuses	(Delyea et al., 2018)
		Increased percentage of $CD4^+$ and $CD8^+$ T-cells in the placenta	(Delyea et al., 2018)
		Increased percentage and number of neutrophils in spleen and placenta	(Delyea et al., 2018)
		Increased CXCL1, TNF-α, and IL-6 level in placental tissues	(Delyea et al., 2018)
		Decreased IL-10 and IL-4 level in placental tissues	(Delyea et al., 2018)
		Increased TLR-3, TLR-4, TLR-9, CXCL-1, and IL-6 level in intestinal tissues	(G. Dunsmore et al., 2019)
		Decreased TGF-β level in intestinal tissues	(G. Dunsmore et al., 2019)
		Increased IL-6 and TNF-α production by $CD11c^+$ and $CD11b^+$ intestinal cells	(G. Dunsmore et al., 2019)
		Increased VEGF level in spleen	(G. Dunsmore et al., 2019)
		Increased bacterial load in intestinal tissues	(G. Dunsmore et al., 2019)
		Increased activation of $CD4^+$ and $CD8^+$ T-cells in intestinal tissues[4]	(G. Dunsmore et al., 2019)
T-cell colitis	CECs transfer	Reduced TNF-α production by red pulp macrophages	(Shim, Weliwitigoda, Campbell, Dosanjh, & Johnson, 2021)
		Reduced weight loss	(Shim, Weliwitigoda, Campbell, Dosanjh, & Johnson, 2021)

[1] No differences in $CD11b^+$ and $CD11c^+$ cells in spleen or lung
[2] Lack of the increase in antibiotic-treated mice
[3] The increase of percentage and number of $CD11b^+$ cells in the lung of anti-CD71-treated mice was observed 2 days postinfection (G. Dunsmore et al., 2017; A. Namdar et al., 2017), followed by a decrease (G. Dunsmore et al., 2017)
[4] No differences in the spleens

Within the heterogeneous population of neonatal splenocytes, CECs were identified to be responsible for the immunosuppressive effects. These cells were found to compromise both innate and adaptive immune responses against divert bacteria species, including *L. monocytogenes* (Elahi et al., 2013) and *Bordetella pertussis* (G. Dunsmore et al., 2017; A. Namdar et al., 2017). Depletion of $CD71^+$ CECs in neonatal mice restored the immune response of neonates to numerous pathogens resulting in decreased bacterial load post infection (Table 3) (Elahi et al., 2013; Elahi et al., 2020). Reduction in the number of neonatal CECs enhanced activation of immune cells and production of TNF-α, IL-17, and IFN-γ (Elahi et al., 2013; Elahi et al., 2020; A. Namdar et al., 2017), as well as IgG and IgA antibodies (A. Namdar et al., 2017). Moreover, removal of $CD71^+$ cells from the population of murine neonatal splenocytes eliminated their suppressive effects on the development of immune response (Table 4). In contrast, depletion of immune lineage cells from neonatal splenocytes resulting in over 95% purity of CECs potently exaggerated the suppressive effects (Elahi et al., 2013), further confirming that CECs are the mediators of immunosuppression. Importantly, immunosuppression driven by CECs seems to be rather locally restricted in mice. While the immune response to infection is diminished in many organs in neonates due to the abundance of CECs in the blood, spleen, or intestine, immune cell activation is unaffected in the lymph nodes, where CECs numbers are relatively low (Elahi et al., 2013).

Besides bacterial infections, increased inflammation after administration of depleting anti-CD71 antibodies was also observed in rotavirus-infected neonates (L. Yang et al., 2020). CECs depletion increased the number of $CD4^+$ and $CD8^+$ T lymphocytes as well as infiltration and activation of NK cells in the liver and leads to resistance to rotavirus-induced biliary atresia (L. Yang et al., 2020). Moreover, neonatal hepatic mononuclear cells, which are also abundant in CECs, potently inhibit the pro-inflammatory response in adult hepatic mononuclear cells (L. Yang et al., 2020).

Intriguingly, neonatal CECs did not change the mortality of mice in the endotoxic challenge (Wynn et al., 2015). Moreover, the transfer of CECs did not affect splenic bacterial load nor mortality in the polymicrobial sepsis model (Wynn et al., 2015), in contrast to the depletion of CECs by anti-CD71 antibodies (Elahi et al., 2013). It was proposed that this may result from the disruption of the intestinal barrier by anti-CD71 treatment. Leakage of microbiota from the gut lumen may cause immune priming, that would enhance bacterial clearance during infection (Wynn et al., 2015). These observations would suggest that activation of immune cells does not result from the depletion of CECs, but rather from the contact with bacteria entering murine body through the disturbed intestinal barrier. In pregnant mice, anti-CD71 treatment compromised intestinal integrity and caused increased bacterial load in the intestinal tissues (G. Dunsmore et al., 2019). However, anti-

Table 4
Immunoregulatory effects of CECs *ex vivo*

Cells	Feature	Effect	Ref.
Murine neonatal splenocytes (50% CECs)			
Adult splenocytes	Uptake of tritiated thymidine ([^{3}H] TdR)	↓Decreased	(Pavia & Stites, 1979)
CD11b$^+$ cells	Cytokines production after HKLm stimulation	↓TNF-α ↓G-CSF ↓CXCL5 ↓CXCL1 ↓CCL3 ↓CCL3	(Wynn et al., 2015)
Murine nucleated erythroid cells from neonatal mice			
T-cells	Cytotoxicity against allogenic splenocytes	↓Decreased	(Seledtsova et al., 2004)
B-cells	Proliferation after LPS stimulation or in mixed lymphocytes culture	↓Decreased	(Seledtsova et al., 2004)
Murine CECs from anemic mice			
B-cells	Proliferation after LPS stimulation or in mixed lymphocytes culture	↓Decreased[1]	(Seledtsov et al., 1998; Seledtsova et al., 2004)
T-cells	Cytokines production after stimulation with Concanavalin A	↔ IL-2	(Seledtsov et al., 1998)
	Mixed lymphocytes cytotoxic reaction	↔ cytolysis	(Seledtsov et al., 1998)
Neonatal bone marrow cells (30% of CECs)			
CD11b$^+$ cells	Cytokines production after HKLm stimulation	↓TNF-α ↓G-CSF ↓CXCL5 ↓CXCL1 ↓CCL3 ↓CCL3	(Wynn et al., 2015)
Murine neonatal splenocytes and CD71$^+$ depleted splenocytes			
Splenocytes	Cytokines production after HKBp stimulation	↓ IL-17[2] ↓ IFN-γ[2]	(A. Namdar et al., 2017)
Splenocytes	Cytokines production by T-cells after HKBp stimulation	↓ IL-17 CD4+ T-cells[2] ↓ IFN-γ CD4+ T-cells[2] ↓ IFN-γ CD8+ T-cells[2]	(A. Namdar et al., 2017)
CD11b$^+$ cells	Cytokines production after HKLm stimulation	↓ TNF-α[2]	(Elahi et al., 2013)
CD11c$^+$ cells	Cytokines production after HKLm stimulation	↓ TNF-α[2]	(Elahi et al., 2013)
CD8$^+$ T-cells	Activation after stimulation with anti-CD3	↓ CD69[2]	(Elahi et al., 2013)
B220$^+$ cells	Cytokines production after HKLm stimulation	↓ TNF-α[2]	(Elahi et al., 2013)
Murine neonatal lung cells and CD71$^+$ depleted lung cells			
Lung cells	Cytokines production after HKBp stimulation	↓ IL-17 ↓ IFN-γ	(A. Namdar et al., 2017)
Murine splenocytes of pregnant			
Splenocytes	Cytokines production after stimulation with anti-CD3 and CD28	↓ IFN-γ[3,4]	(Delyea et al., 2018)
Human cord blood cells			
CD11b$^+$ cells	Cytokines production after HKLm stimulation	↓ TNF-α[5] ↓ IL-6[5]	(Elahi et al., 2013)
	Phagocytosis of *B. pertussis*	↓ Decreased to 40%[3]	(G. Dunsmore et al., 2017)
Peripheral blood of human infants			
CD14$^+$ cells	Cytokines production after stimulation with anti-CD3 and CD28	↓ TNF-α[7]	(Elahi et al., 2020)
CD4$^+$ T-cells	Cytokines production after stimulation with anti-CD3 and CD28	↓ IFN-γ[7]	(Elahi et al., 2020)
CD8$^+$ T-cells	Cytokines production after stimulation with anti-CD3 and CD28	↓ IFN-γ[7]	(Elahi et al., 2020)
Murine neonatal CECs cells from the spleen			
CD4$^+$ T-cells	Differentiation into Tregs	↑ Percentage of FoxP3+ CD4+ T-cells[6,8,9]	(S. Shahbaz et al., 2018)
	Activation of crucial signaling pathways	↓ phospho-AKT ↓ phospho-mTOR	(S. Shahbaz et al., 2018)
	Cytokines expression and production after anti-CD3 and anti-CD28 stimulation	↓ IL-2 ↓ IFN-γ ↑ IL-4	(Rincon et al., 2012)
	Proliferation after anti-CD3 and anti-CD28 stimulation	↓ decreased	(Grzywa, Sosnowska, et al., 2021)
CD8$^+$ T-cells	Activation after stimulation with anti-CD3	↓ CD69 ↓ CD25	(Elahi et al., 2013)
B220$^+$ cells	Cytokines production after HKLm stimulation	↓ TNF-α[10]	(Elahi et al., 2013)
CD11b$^+$ cells	Cytokines production after HKLm stimulation	↓ TNF-α[10,11] ↓ IFN-γ	(Elahi et al., 2013)
	Cytokines production after HKBp stimulation	↓ TNF-α ↓ IFN-γ	(G. Dunsmore et al., 2017)
	Cytokines production after brefeldin A stimulation	↓ TNF-α	(G. Dunsmore et al., 2017)
	Phagocytosis of B. pertussis	↓ Decreased to 40%[3]	(G. Dunsmore et al., 2017)
CD11c$^+$ cells	Cytokines production after HKBp stimulation	↓ TNF-α ↓ IFN-γ	(G. Dunsmore et al., 2017)
Splenocytes of a pregnant mouse	Cytokines production by CD11b$^+$ cells after HKLm stimulation	↓ TNF-α	(Delyea et al., 2018)
Placenta cells	Cytokines production by CD11b$^+$ cells after HKLm stimulation	↓ TNF-α	(Delyea et al., 2018)
Splenocytes	Cytokines production by CD11b$^+$ cells after HKLm stimulation	↓ TNF-α[9]	(Delyea et al., 2018)

Table 4 (*continued*)

Cells	Feature	Effect	Ref.
Murine neonatal CECs cells from the liver			
Hepatic mononuclear cells	Cytokines production after anti-CD3 stimulation	↓ TNF-α	(L. Yang et al., 2020)
CECs from anemic mice			
$CD4^+$ T-cells	Proliferation after anti-CD3 and anti-CD28 stimulation	↓ Decreased	(Grzywa, Sosnowska, et al., 2021)
$CD8^+$ T-cells	Proliferation after anti-CD3 and anti-CD28 stimulation	↓ Decreased	(Grzywa, Sosnowska, et al., 2021)
CECs from tumor-bearing mice			
$CD4^+$ T-cells	Cytokines production after peptide stimulation	↓ TNF-α ↓ IFN-γ	(L. Zhao et al., 2018)
	Proliferation	↓ Decreased[7]	(L. Zhao et al., 2018)
$CD8^+$ T-cells	Cytotoxicity of T-cells	↓ killing percentage[7]	(L. Zhao et al., 2018)
	Cytokines production after peptide stimulation	↓ TNF-α ↓ IFN-γ	(L. Zhao et al., 2018)
	Proliferation	↓ Decreased[1]	(L. Zhao et al., 2018)
Murine CECs from the spleen of mice with colitis			
Red pulp macrophages	Cytokines production after LPS stimulation	↓ TNF-α	(Shim, Weliwitigoda, Campbell, Dosanjh, & Johnson, 2021)
Neutrophils		↔TNF-α	(Shim, Weliwitigoda, Campbell, Dosanjh, & Johnson, 2021)
Macrophages		↔TNF-α	(Shim, Weliwitigoda, Campbell, Dosanjh, & Johnson, 2021)
Monocytes		↔TNF-α	(Shim, Weliwitigoda, Campbell, Dosanjh, & Johnson, 2021)
Human cord blood and $CD71^+$ depleted cord blood			
$CD3^+$ T-cells	Proliferation after stimulation with anti-CD3 and CD28	↓ Decreased to 25%[12]	(A. Namdar et al., 2017)
$CD4^+$ T-cells	Proliferation after stimulation with anti-CD3 and anti-CD28	↓ Decreased[12]	(Miller et al., 2018; A. Namdar et al., 2017)
	Percentage of IL-2^+ T-cells	↓ Decreased[12]	(A. Namdar et al., 2017)
	Percentage of IFN-γ T-cells	↓ Decreased[12]	(A. Namdar et al., 2017)
$CD8^+$ T-cells	Activation after stimulation with anti-CD3	↓ CD69[12]	(Elahi et al., 2013)
	Proliferation after stimulation with anti-CD3 and anti-CD28	↓ Decreased[12]	(Miller et al., 2018; A. Namdar et al., 2017)
$CD11b^+$ cells	Cytokines production after HKLm stimulation	↓ TNF-α[12] ↓ IL-6[12]	(Elahi et al., 2013)
	Cytokines production after HKBp stimulation	↓ TNF-α[12]	(G. Dunsmore et al., 2017)
	Phagocytosis of B. pertussis	↓ Decreased to 40%[3,12]	(G. Dunsmore et al., 2017)
$CD19^+$ T-cells	Expression of co-stimulatory signal molecules	↓ CD40[6] ↓ CD86[6]	(A. Namdar et al., 2017)
Human PBMC of pregnant women and $CD71^+$ depleted PBMC			
$CD3^+$ T-cells	Proliferation after stimulation with anti-CD3	↓ Decreased	(G. Dunsmore et al., 2019)
$CD4^+$ T-cells	Proliferation after stimulation with anti-CD3 and anti-CD28	↓ Decreased[13]	(Delyea et al., 2018; G. Dunsmore et al., 2019)
$CD8^+$ T-cells	Proliferation after stimulation with anti-CD3 and anti-CD28	↓ Decreased[13]	(Delyea et al., 2018; G. Dunsmore et al., 2019)
$CD11b^+$ cells	Cytokines production after HKLm stimulation	↓IL-6 ↓TNF-α	(G. Dunsmore et al., 2019)
Human CECs from cord blood			
$CD4^+$ T-cells	Percentage of HIV-infected T-cells	↑Increased[9,14,15]	(Afshin Namdar et al., 2019)
Monocytes	Cytokines production after LPS stimulation	↓ IL-1β ↓TNF-α	(Cui et al., 2016)
Human CECs from anemic individuals			
$CD4^+$ T-cells	Percentage of HIV-infected T-cells	↑Increased	(Afshin Namdar et al., 2019)
Human CECs from PBMC of cancer patients			
$CD8^+$ T-cells	Proliferation after stimulation with anti-CD3 and anti-CD28	↓ Decreased[12]	(L. Zhao et al., 2018)
Human CECs from tumor tissue			
$CD3^+$ T-cells	IFN-γ after stimulation with anti-CD3 and anti-CD28	↓ Decreased	(J. Chen et al., 2021)
$CD4^+$ T-cells	Proliferation after stimulation with anti-CD3 and anti-CD28	↓ Decreased[16]	(J. Chen et al., 2021)
$CD8^+$ T-cells	Proliferation after stimulation with anti-CD3 and anti-CD28	↓ Decreased[16]	(J. Chen et al., 2021)
Human CECs from HIV patients			
$CD4^+$ T-cells	Percentage of HIV-infected T-cells	↑Increased	(Afshin Namdar et al., 2019)
Human PBMC of COVID-19 patients			
$CD3^+$ T-cells	Proliferation after stimulation with SARS-CoV-2 antigens	↓ Decreased	(Shima Shahbaz et al., 2021)
$CD4^+$ T-cells	Cytokines production after stimulation with anti-CD3 and anti-CD28	↓ TNF-α ↓ IFN-γ	(Shima Shahbaz et al., 2021)
	Cytokines production after stimulation with SARS-CoV-2 antigens	↓ TNF-α ↓ IFN-γ	(Shima Shahbaz et al., 2021)
$CD8^+$ T-cells	Cytokines production after stimulation with anti-CD3 and anti-CD28	↓ TNF-α ↓ IFN-γ	(Shima Shahbaz et al., 2021)
	Cytokines production after stimulation with SARS-CoV-2 antigens	↓ TNF-α ↓ IFN-γ	(Shima Shahbaz et al., 2021)
	Degranulation after stimulation with SARS-CoV-2 antigens	↓ Decreased to 70%	(Shima Shahbaz et al., 2021)
Human PBMC of IBD pregnant women and CD71+ depleted PBMC			
$CD4^+$ T-cells	Proliferation after stimulation with anti-CD3 and anti-CD28	↔	(G. Dunsmore et al., 2019)
$CD8^+$ T-cells	Proliferation after stimulation with anti-CD3 and anti-CD28	↔	(G. Dunsmore et al., 2019)
$CD11b^+$ cells	Cytokines production after HKLm stimulation	↔ IL-6 ↔ TNF- α	(G. Dunsmore et al., 2019) (G. Dunsmore et al., 2019)
Murine CD71+ cells from anemic adult mice			
$CD11b^+$ cells	Cytokines production after HKLm stimulation	↔ TNF-α	(Elahi et al., 2013)

(*continued on next page*)

Table 4 (*continued*)

Cells	Feature	Effect	Ref.
Murine adult bone marrow cells (10% of CECs)			
$CD11b^+$ cells	Cytokines production after HKLm stimulation	↓TNF-α ↓G-CSF ↓CXCL5 ↓CXCL1 ↓CCL3 ↓CCL3	(Wynn et al., 2015)

↑ - increased, ↓ - decreased, ↔ - no changes
[1] Dimnished by TGF-β mRNA-specific antisense oligonucleotides or anti-TGF-β antibody
[2] Compared to CD71+ cells depleted neonatal splenocytes
[3] Inhibition diminished by L-Arg supplementation
[4] Inhibition diminished by anti-PDL-1 antibody
[5] Compared to adult PBMC
[6] Higher in T-cells cocultured with $VISTA^+$ CECs than $VISTA^-$ CECs
[7] Reversed by ROSi
[8] Inhibited by TGF-β inhibitor
[9] Not affected by L-Arg supplementation or NAC (ROS scavenger) but diminished by apocynin (NADPH dependent ROS inhibitor)
[10] Inhibition diminished by arginase inhibition (BEC, ABH) and l-arg supplementation
[11] Inhibition did not override by inhibition of myeloperoxidase, NADPH oxidase, superoxide dismutase, heme oxidation, TGF-β receptor or anti-TGF-β antibody as well as ROS scavenger
[12] Compared to $CD71^+$ cells-depleted cord blood cells
[13] Compared to $CD71^+$ cells-depleted PBMC of pregnant women
[14] A similar effect was exerted by incubation of T-cells with CECs before exposure to HIV
[15] Effect not exert by CECs from cord blood of Crohn's disease or ulcerative colitis patients
[16] Diminished by NAC (ROS scavenger), STAT-3 inhibitor, SMADs inhibitor, and NF-κB inhibitor, but not by arginase inhibitor (NOHA) or L-Arg supplementation

CD71 treatment did not compromise intestinal integrity in neonatal mice and did not lead to elevation of serum LPS concentrations (G. Dunsmore et al., 2017). Finally, the immunosuppressive role of CECs was confirmed *in vivo* by the transfer of neonatal CECs into adult mice, which led to the diminished innate immune response against *B. pertussis* (G. Dunsmore et al., 2017).

Among human tissues, cord blood and neonatal spleen are enriched in CECs (Table 2). Moreover, CECs are abundant in the peripheral blood of human neonates and their numbers significantly decline by 4 weeks after birth (Elahi et al., 2020). Similar to murine neonatal splenocytes, human cord blood cells decreased activation and, subsequently, production of TNF-α and IL-6 by adult peripheral blood mononuclear cells (PBMCs) (Table 4) (Elahi et al., 2013) in the cell-to-cell contact independent manner (Cui et al., 2016). Likewise, CECs in the peripheral blood of infants suppressed the production of TNF-α by myeloid cells and IFN-γ by T-cells (Elahi et al., 2020).

The arising question is what is the exact role of CECs in infants. While depletion of CECs in infected murine neonates leads to the improved anti-bacterial response, in healthy neonates depletion of CECs results in aberrant immune cell activation in the intestine. Overactivation of the immune response in the intestine was absent in CECs-depleted germ-free mice or antibiotic-treated mice. Therefore, it was suggested that the role of CECs is to enable colonization of the neonatal body with commensal microbes (Elahi et al., 2013). Since interaction with the microbiome is crucial for the proper development of the immune system (Dzidic, Boix-Amorós, Selma-Royo, Mira, & Collado, 2018; Harbeson, Ben-Othman, Amenyogbe, & Kollmann, 2018), it was proposed that temporal immunosuppression driven by CECs, which results in the susceptibility to infection, is counterbalanced by their protective role (Elahi et al., 2013). In humans, CECs frequency reversely correlates with the percentage of activated T-cells as well as with fecal calprotectin concentrations in meconium (G. Dunsmore et al., 2019), which is a marker of intestinal inflammation (Costa et al., 2003). Therefore, it seems that the critical role of CECs in neonates is to prevent excessive activation of immune cells in response to microorganisms, which would lead to the damage of the intestines (Elahi et al., 2013), liver (L. Yang et al., 2020), and lungs (G. Dunsmore et al., 2017) (Fig. 2).

3.2. CECs and development of feto-maternal tolerance

Complex regulation of the immune system is required for the development of feto-maternal immune tolerance and for the pregnancy maintenance (PrabhuDas et al., 2015). Numerous cell types, including Tregs (Jørgensen, Persson, & Hviid, 2019), tolerogenic dendritic cells (tDCs) (Arck & Hecher, 2013), decidual and uterine NK cells (dNK and uNK, respectively) (Gaynor & Colucci, 2017), as well as MDSCs (Ostrand-Rosenberg et al., 2017; Pan et al., 2016), are involved in the modulation of immune response in pregnant women and in developing fetus.

During pregnancy, mild anemia is physiologic and is caused by an increase in plasma volume, which cannot be rapidly compensated by a proportional increase in the erythrocytes mass (Horowitz, Ingardia, & Borgida, 2013). Increased concentrations of estrogen and 27-hydroxycholesterol, acting *via* estrogen receptor alpha (ERα), favor divisions of HSCs and together with EPO promote erythropoiesis and

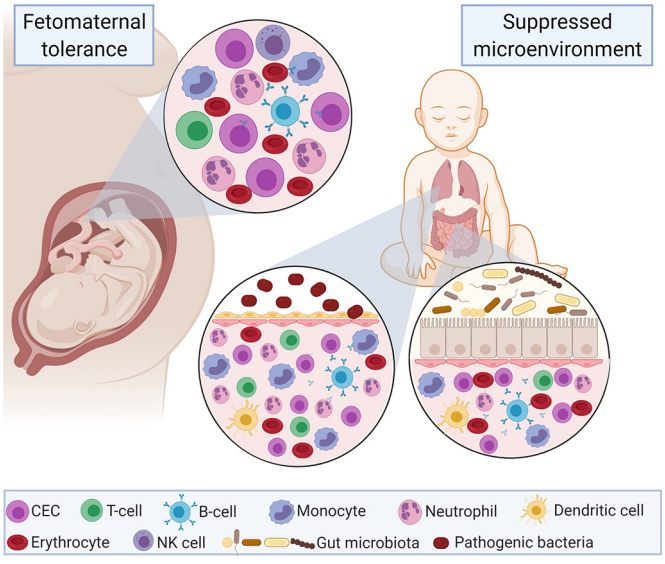

Fig. 2. Erythroid progenitor cells (CECs) regulate the immune system in fetuses, neonates, and pregnant women. CECs are abundant in the fetal spleen, cord blood, placenta, and peripheral blood of pregnant women. CECs suppress the immune response and are responsible for the induction and maintenance of feto-maternal tolerance. In neonates, CECs are abundant in the spleen and peripheral blood and create a suppressed microenvironment that enables gut colonization by commensal microbes. On the other side, CECs are responsible for the susceptibility of neonates to infections.

expansion of CECs in the spleen (Fowler & Nash, 1968; Inra et al., 2015; McMullin, White, Lappin, Reeves, & MacKenzie, 2003; Nakada et al., 2014; Oguro et al., 2017). CECs in the spleen of pregnant mice have increased expression of EPO receptor, which is associated with their increased proliferation and decreased apoptosis (Norton, Fortner, Bizargity, & Bonney, 2009).

It was hypothesized that extramedullary erythropoiesis in pregnancy is induced to support expansion of maternal blood volume (Oguro et al., 2017). However, splenectomy did not affect hematocrit of pregnant mice (Fowler & Nash, 1968), suggesting that erythrocytes production is not the main function of extramedullary erythropoiesis in the spleen of pregnant mice. Moreover, expansion of CECs in the spleen is not observed in syngeneic pregnant mice, in contrast to allogeneic pregnant mice (Delyea et al., 2018), suggesting a more complex extramedullary erythropoiesis role during pregnancy.

The importance of CECs during pregnancy has been revealed only recently. CECs isolated from either spleen of pregnant mice or placenta were found to suppress inflammatory response of myeloid cells *ex vivo* (Delyea et al., 2018), similar to their neonatal counterparts (Table 4). Moreover, depletion of CECs in pregnant mice led to disruption of the feto-maternal tolerance and activation of maternal anti-fetal immune response (Table 3). A decrease in the number of CECs resulted in the increased recruitment of $CD4^+$ and $CD8^+$ T-cells to placenta, which was accompanied by increased production of proinflammatory cytokines, including TNF-α and IL-6 (Delyea et al., 2018). Moreover, depletion of CECs in pregnant mice decreased concentrations of IL-10 and IL-4 (Delyea et al., 2018), cytokines which play a central role in feto-maternal tolerance development (P. Chatterjee, Chiasson, Bounds, & Mitchell, 2014). All these effects were observed only in allogeneic pregnancies and resulted in complete fetal resorption (Delyea et al., 2018), confirming that CECs play a critical role in the development and maintenance of feto-maternal tolerance.

Since CECs regulate gut microbiome in neonates (Elahi et al., 2013), their role in the modulation of gut homeostasis during pregnancy has been also investigated. Alterations in the gut microbiome impact metabolism (Kimura et al., 2020; Koren et al., 2012) and lead to profound changes in both maternal and offspring immunity (D. D. Nyangahu & Jaspan, 2019; Donald D. Nyangahu et al., 2018). Pregnancy induces profound maternal gut microbiota changes, increasing the abundance of Proteobacteria as well as Actinobacteria, but decreasing the number of butyrate-producing bacteria (Koren et al., 2012). Importantly, the highest expansion of CECs in the third trimester coincides with the most profound changes in the maternal gut microbiota (G. Dunsmore et al., 2019; Mesa et al., 2020). Elimination of CECs in pregnant mice leads to substantial changes in the gut microbiome, including an increased abundance of *Clostridium* and members of Bacteroides-Prevotella-Porphyromonas group, but a reduction in *Enterobacteriaceae.* These changes are accompanied by overactivation of dendritic cells, monocytes, and macrophages in the gut (G. Dunsmore et al., 2019).

In pregnant women, the number of CECs in peripheral blood starts to increase in the second trimester, and their number peaks in the third trimester (G. Dunsmore et al., 2019). Moreover, CECs are abundant in both placenta and cord blood (Delyea et al., 2018; G. Dunsmore et al., 2019; Mavrou et al., 2007; Suryawanshi et al., 2018). Human placental CECs exhibit substantial immunomodulatory properties and potently inhibit proliferation of $CD4^+$ and $CD8^+$ T-cells (G. Dunsmore et al., 2019). *Ex vivo,* depletion of CECs from PBMCs of pregnant women increases proliferation of stimulated T-cells (Delyea et al., 2018; G. Dunsmore et al., 2019) and unleashes the inflammatory response of stimulated myeloid cells (G. Dunsmore et al., 2019), confirming the role of CECs in immune response suppression in pregnant women.

The majority of CECs in peripheral blood of pregnant women are of maternal origin (Slunga-Tallberg et al., 1995). However, a meaningful fraction (even up to 30%) of fetal CECs is detected in maternal blood (Byeon, Ki, & Han, 2015; Sohda et al., 1997; Wachtel et al., 1998), which may suggest that fetal CECs take part in induction of antigen-specific immune tolerance (Kahn & Baltimore, 2010; Petroff, 2011). However, direct contact of fetal CECs and non-stimulated maternal PBMC induced secretion of pro-inflammatory cytokines including IL-6, IL-1β, and TNF-α, but reduced production of transforming growth factor β (TGF-β) (Miller et al., 2018). While CECs are generally immunosuppressive, the maternal allogeneic immune response seems to be stronger than suppressive mechanisms of CECs *ex vivo*. Whether fetal CECs are only donors of antigens or actively induce maternal tolerance to fetal antigens remains unknown. However, it was found that cord blood CECs more potently suppress the inflammatory response of stimulated adult peripheral blood than cord blood monocytes (Cui et al., 2016), which suggests that fetal CECs may suppress maternal immune response, especially activation of the innate immune cells, to prevent inflammation and development of detrimental immune response against fetus.

Considering that CECs regulate feto-maternal tolerance in mice, their properties were further studied in preterm and term neonates. Preterm-derived neonatal CECs share a similar transcriptional profile to CECs in term neonates, however, some important differences were detected (Miller et al., 2018). For instance, preterm-born neonate CECs have decreased TGF-β expression (Miller et al., 2018). Intriguingly, preterm neonates have higher numbers of CECs as compared with term neonates (Dulay et al., 2008). In human cord blood, CECs are most abundant in neonates born to women who delivered preterm *via* caesarean section (Gomez-Lopez et al., 2016). It was proposed that in humans, an increase in CECs number in preterm neonates may be a response to inflammatory mediators produced *in utero* (Dulay et al., 2008). Moreover, alterations in the immunoregulatory potential of CECs may contribute to the enhanced immune reactivity against fetus resulting in preterm delivery. On the other side, the delivery method also affects CECs. The cord blood of a caesarean section-delivered newborn had lower CECs frequency, different immunoregulatory phenotype, and more pro-inflammatory milieu than a vaginal-delivered newborn (Garett Dunsmore et al., 2018).

Therefore, CECs arise as an important population of cells that create and maintain feto-maternal tolerance as well as regulate the maternal gut microbiome (Fig. 2). These observations shed new light on the importance of the expansion of CECs and physiological role of pregnancy-associated anemia in pregnant women.

3.3. CECs in cancer

Over one-third of cancer patients develop anemia in the course of the disease (Birgegård et al., 2005; Gilreath & Rodgers, 2020). Importantly, anemia correlates with worse clinical outcomes in many types of cancer (Lanting Liu et al., 2020). CECs are commonly detected in the blood of cancer patients, reaching the highest number in patients with bone marrow metastases (Delsol et al., 1979). The percentage of CECs in blood reversely correlates with hemoglobin concentration (L. Zhao et al., 2018). Increased CECs frequency in the blood of cancer patients is caused by so-called anemia of inflammation (Ganz, 2019), disruption of the HSC niche (Boyd et al., 2017), and impaired differentiation of erythroid progenitors (Grzywa, Justyniarska, Nowis, & Golab, 2021).

Tumor-induced anemia is well recapitulated in murine tumor models (Han et al., 2018; L. Zhao et al., 2018). Hemoglobin concentration and hematocrit gradually decrease with tumor progression. Established tumors create a hypoxic environment, which together with anemia increases serum EPO concentration (Sano et al., 2021). This is accompanied by accumulation of CECs in the extramedullary sites, including spleen and liver, and their expansion in bone marrow (L. Zhao et al., 2018). Of all these sites, the most profound cancer-related changes are observed in the spleen. With tumor progression, frequency of lymphocytes, constituting over 80% of splenocytes in healthy mice, gradually decreases. Conversely, frequencies of macrophages and MDSCs as well as CECs substantially increase (Table 2). In the spleen of late-stage tumors-bearing mice, CECs number exceed

100×10^6, constituting over half of the total spleen-residing cells (L. Zhao et al., 2018). Moreover, CECs infiltrate tumor microenvironment of murine and human tumors localized outside the extramedullary erythropoiesis sites (J. Chen et al., 2021; Sano et al., 2021).

CECs were found to potently suppress antitumor immunity. Adoptive transfer of CECs leads to accelerated tumor growth (L. Zhao et al., 2018), while a decrease in CECs number resulting from administration of either anti-EPO or anti-CD71 antibodies exerts moderate antitumor effects and slows down cancer progression (Sano et al., 2021). *Ex vivo*, CECs inhibit $CD4^+$ T-cell proliferation and differentiation as well as inhibit $CD8^+$ T-cell proliferation and cytotoxicity (Table 4). The same effects were observed for human CECs isolated from PBMC of cancer patients (J. Chen et al., 2021; L. Zhao et al., 2018). Importantly, tumor microenvironment infiltrating CECs were demonstrated to suppressed IFN-γ secretion and proliferation of intratumoral $CD4^+$ and $CD8^+$ T-cells isolated from human liver tumors (J. Chen et al., 2021).

Cancer patients as well as tumor-bearing mice have impaired antiviral and anti-bacterial immunity. Importantly, depletion of CECs not only slows tumor growth, but also leads to the restoration of the immune response to the levels observed in tumor-free mice (L. Zhao et al., 2018). On the contrary, adoptive transfer of CECs decreases T-cell proliferation as well as TNF-α and IFN-γ production in response to chronic lymphocytic choriomeningitis virus (LCMV) (L. Zhao et al., 2018).

As CECs potently impair antiviral and antimicrobial immune response in murine models, they may contribute to the increased risk of infections in cancer patients (Baden et al., 2016). Indeed, hemoglobin concentration inversely correlates with Epstein–Barr virus (EBV) copy number. Moreover, anemic cancer patients have decreased numbers of LMP2- and EBNA1-specific $CD8^+$ T-cells, suggesting that cancer patients with anemia have suppressed immune response compared to the non-anemic individuals (L. Zhao et al., 2018). Therefore, CECs should be considered as a valid population of tumor-induced immunosuppressive cells (Fig. 3).

Besides regulating the immune response, a subset of more mature erythroid cells, named Ter-cells, was found to promote tumor growth in mice. These late-stage CECs are $CD45^-$ and express erythroid lineage markers (CD71 and TER119) and CD44, as well as CD41, is the latter being a marker of megakaryocytic lineage and an indicator of the earliest emerging HSCs (Han et al., 2018). During tumor progression, $CD45^-$ CECs accumulate in the spleen of tumor-bearing mice in the TGF-β and SMAD3 signaling-dependent manner. In contrast to $CD45^+$ CECs, $CD45^-$ CECs have rather poor immunosuppressive properties (Han et al., 2018; L. Zhao et al., 2018). However, transfer of $CD45^-$ CECs also promotes tumor growth, similarly to the transfer of immunosuppressive $CD45^+$ CECs (Han et al., 2018; L. Zhao et al., 2018). Gene expression analysis revealed a high expression of a neurotrophic factor artemin in $CD45^-$ CECs. In murine models, serum artemin concentration increases with tumor progression and correlates with an increase in $CD45^-$ CECs number. $CD45^-$ CECs-secreted artemin has been shown to promote tumor growth *in vitro* and *in vivo* (Han et al., 2018; Hou et al., 2021; T.-J. Li et al., 2021). Accordingly, splenectomy prevents $CD45^-$ CECs expansion and inhibits tumor growth, improving mice survival (Han et al., 2018). Targeting artemin axis leads to the inhibition of tumor growth and increases therapeutic efficacy of both radiotherapy and immunotherapy in murine models (Han et al., 2018; Hou et al., 2021). Artemin-positive cells were also detected in the spleens of cancer patients. High artemin concentration in plasma and increased artemin receptor

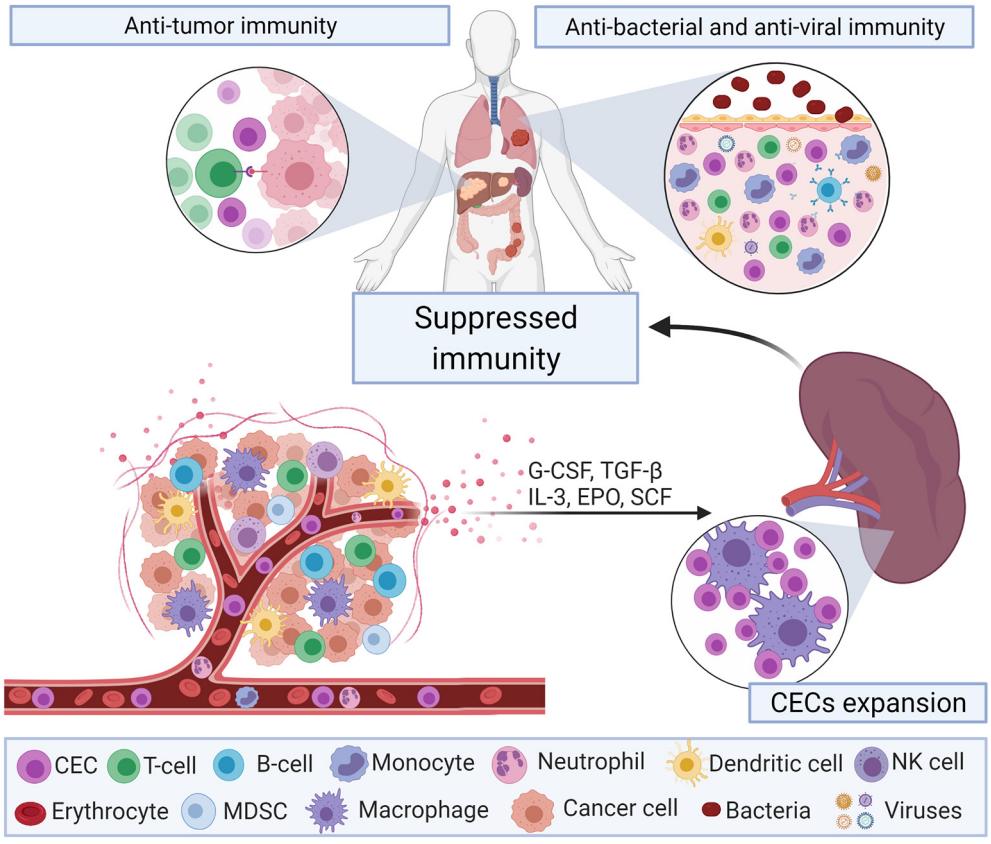

Fig. 3. CECs expand in cancer and suppress anti-tumor as well as anti-bacterial and anti-viral immune response. Cancer cells and immune cells in the tumor microenvironment secrete growth factors, including granulocyte-colony stimulating factor (G-CSF), transforming growth factor β (TGF-β), interleukin 3 (IL-3), erythropoietin (EPO), and stem cell factor (SCF) that induce extramedullary erythropoiesis and CECs expansion in the spleen. CECs suppress immune response leading to impaired anti-tumor immunity. Moreover, CECs accumulation contributes to decreased anti-bacterial and anti-viral immunity.

(GFRα3) expression in tumor tissue correlate with poor prognosis in HCC patients (Han et al., 2018). Moreover, $CD45^-$ CECs number in the spleen correlates with poor prognosis in pancreatic ductal adenocarcinoma patients (T.-J. Li et al., 2021).

Therefore, it seems that while CECs at the earliest stages of their maturation are potent immunosuppressors, more mature CECs lack immunosuppressive capacities, but instead promote tumor growth (Grzywa, Justyniarska, et al., 2021). Whether Ter-cells are a unique population of tumor-induced erythroblast-like cells or can be found in other conditions remains unclear. Transcriptional analyses indicate that they are similar to embryonic CECs (Han et al., 2018). Therefore, an important question is whether Ter-cells retain the ability of normal erythroblasts to differentiate into erythrocytes. If so, this would mean that all CECs have the ability to regulate proliferation and activation of other cell types, making CECs important regulators of tissue hemostasis.

3.4. CECs in infectious diseases

So far, the studies on interactions between antimicrobial immune response and erythroid cells have focused on the inflammation effects on erythropoiesis (Table 5). The decreased life span of erythrocytes and increased erythrophagocytosis during infection lead to the development of anemia (Ohyagi et al., 2013). As a response, erythroid progenitors expand in the spleen, where stress erythropoiesis is induced to compensate for the erythrocytes loss(Baldridge, King, Boles, Weksberg, & Goodell, 2010; Bennett et al., 2019; Gomes et al., 2019). IFN-γ, a central mediator of the immune response during infection, increases the number of reticulocytes in blood and promotes egress of CECs from the bone marrow to circulation (Gomes et al., 2019). *Salmonella* infection, as well as immunization with desiccated, nonviable *Mycobacterium tuberculosis* lead to the induction of extramedullary erythropoiesis and increase the number of erythroid cells in the spleens, while decrease it in the bone marrow (Artinger et al., 2015; Jackson, Nanton, O'Donnell, Akue, & McSorley, 2010). Similarly, in mice, CECs expansion in the spleen is induced by viral infections (Jordan et al., 2013). Moreover, a single injection of bacterial LPS or CpG-oligodeoxynucleotides (toll-like receptor 9 (TLR9) agonists mimicking bacterial DNA), potently increase erythropoiesis rate in the spleen (Einwächter et al., 2020; Fruhman, 1966). Neutralization of EPO prevents infection-related CECs accumulation in the spleen (Einwächter et al., 2020).

The role of CECs expansion during bacterial infection is well-described in neonates. However, the regulation of immune response against pathogens by CECs is less known in adults. Induction of anemia and CECs expansion in adult mice by phlebotomy has no impact on the bacterial load in the liver and spleen in the model of *L. monocytogenes* infection (Elahi et al., 2013). However, neutralization of EPO. that reduces CECs expansion. leads to a decreased bacterial load in the spleen, which may suggest that in adult mice CECs contribute to the suppression of the immune response to *Salmonella* (Jackson et al., 2010). Moreover, in adult tumor-bearing mice, CECs suppress anti-bacterial immune response (L. Zhao et al., 2018). In humans, anemic individuals have impaired immune response and bactericidal activity of leukocytes (Conway de Macario & Macario, 1979). Anemia is also strongly associated with worse outcomes of infection with Gram-positive bacteria (Musher et al., 1994; Musher et al., 2000). However, the exact role of CECs in the anti-bacterial immune response in adults remains unclear.

CECs are also important players in the human immunodeficiency virus (HIV) infections (Afshin Namdar et al., 2019). CECs expand in the peripheral blood of HIV-infected individuals (Table 2) and their frequency correlates with the viral load. CECs from HIV-infected patients enhance infectivity and replication of HIV in $CD4^+$ T-cells. Compared to mature erythrocytes CECs have a higher expression of HIV target molecules, including complement receptor-1 (CD35) and Duffy antigen receptor for chemokines (DARC) (Afshin Namdar et al., 2019), which are known to promote HIV trans-infection and to affect chemokine-driven inflammation (W. He et al., 2008; Horakova et al., 2004; Montefiori, Graham, Zhou, Zhou, & Ahearn, 1994). HIV virions bind to CECs via CD235a, what may result in virus transfer to uninfected T-cells (Afshin Namdar et al., 2019). Importantly, CECs harbor infective HIV particles during antiretroviral therapy (Afshin Namdar et al., 2019). These findings demonstrate that CECs, besides suppressing of immune response against EBV in cancer patients (L. Zhao et al., 2018), also suppress anti-HIV immune response as well as actively promote trans-infection (Afshin Namdar et al., 2019).

Recent studies reveal an important role of CECs in the regulation of antiviral immunity in Coronavirus Disease 2019 (COVID-19). Anemia is observed in over 50% of COVID-19 patients (Chen et al., 2020) and is associated with poor prognosis (Zhou et al., 2020). Moreover, hypoxemia resulting from respiratory failure as well as anemia are independently associated with increased in-hospital COVID-19 patients' mortality (Xie et al., 2020). SARS-CoV-2 infection results in expansion of CECs in peripheral blood (Table 2) (Encabo et al., 2021; Shima Shahbaz et al., 2021). CECs frequency increases during disease progression and reaches the highest values in patients with severe disease

Table 5
Cytokines regulating erythroid cells proliferation and maturation

Cytokine	Effect	Ref.
Pro-inflammatory cytokines		
TNF-α	Direct inhibition of CECs maturation by p55-TNF receptor (TNFR1) *via* NF-κB pathway	(J. J. Liu, Hou, & Shen, 2003; Rusten & Jacobsen, 1995)
	Decreased proliferation of CECs by downregulation of cyclin-dependent kinase 6 (CDK6)	(Dai, Chung, Jiang, Price, & Krantz, 2003)
IL-6	Decreased differentiation, impaired mitochondrial membrane potential, and hemoglobin production in CECs	(McCranor et al., 2014)
IL-33	Decreased differentiation and activation of NK-κB	(Swann et al., 2020)
IFN-β	Decreased growth and differentiation of CFU-E	(Means Jr. & Krantz, 1996)
IFN-α	Decreased growth and differentiation of CFU-E, indirect effect mediated by T-cells	(Means Jr. & Krantz, 1996)
IFN-γ	Decreased growth and differentiation of CECs *via* TWEAK, TRAIL and FasL/CD95L	(Felli et al., 2005; Maciejewski, Selleri, Anderson, & Young, 1995; Zoumbos, Gascon, Djeu, & Young, 1985)
	Disturbed adhesion of progenitor cells to bone marrow stromal cells	(Zeng et al., 2006)
	Decreased expression of EPOR and c-Kit	(Taniguchi, Dai, Price, & Krantz, 1997)
Anti-inflammatory cytokines		
TGF-β	Decreased proliferation but increased differentiation of CECs	(Zermati et al., 2000)
IL-3	Increased self-renewal of BFU-E	(Lewis et al., 1998)
	Increased proliferation of CFU-E	(Umemura, Papayannopoulou, & Stamatoyannopoulos, 1989)
EPO	Decreased apoptosis of CECs	(Koury & Bondurant, 1990; Y. Liu et al., 2006; Silva et al., 1996)
	Increased proliferation and differentiation of CECs	(Klingmüller, 1997)
SCF	Decreased apoptosis of CECs	(Endo et al., 2001)
	Increased proliferation but decreased differentiation of CECs	(K Muta, Krantz, Bondurant, & Dai, 1995)
IGF-1	Decreased apoptosis of CFU-E	(K. Muta & Krantz, 1993)
BMP4	Increased Hb and Bcl-XL expression and decreased GATA2 expression	(Maguer-Satta et al., 2003)
BMP2	Increased Hb, EPOR, Bcl-XL expression and decreased GATA2 expression	(Maguer-Satta et al., 2003)
IL-9	Increased proliferation of CFU-E and BFU-E	(Lu, Leemhuis, Srour, & Yang, 1992)
IL-10	Increased proliferation of BFU-E	(C. Q. Wang, Udupa, & Lipschitz, 1996)

hospitalized in the Intensive Care Units. *Ex vivo,* CECs from COVID-19 patients potently inhibit proliferation as well as cytokine production by $CD4^+$ and $CD8^+$ T-cells after stimulation with either anti-CD3/CD28 or SARS-CoV-2 antigens (Table 4). In COVID-19 patients, the number of CECs negatively correlates with the percentage of T-cells and antibody-secreting plasmablasts (Shima Shahbaz et al., 2021). Moreover, CECs are dominant peripheral blood cells expressing angiotensin-converting enzyme-2 (ACE2), which is bound by viral spike glycoprotein (S protein) (Walls et al., 2020) and serves as cell entry for SARS-CoV-2 virus (Hoffmann et al., 2020). CECs also express CD147 (Shima Shahbaz et al., 2021), a second putative SARS-CoV-2 receptor (K. Wang et al., 2020). Moreover, CECs express cellular serine protease TMPRSS2 (Shima Shahbaz et al., 2021), which is required for S protein priming (Hoffmann et al., 2020). Importantly, infection and subsequent viral RNA release from CECs were confirmed *in vitro* (Encabo et al., 2021; Shima Shahbaz et al., 2021). Dexamethasone used for the treatment of hospitalized COVID-19 patients (Horby et al., 2021) selectively decreases ACE2 and TMPRSS2 expression in CECs, resulting in decreased number of viral RNA copies in CECs after SARS-CoV-2 infection *ex vivo* (Shima Shahbaz et al., 2021). Moreover, it is of great clinical interest whether CECs may affect the development of the immune response to COVID-19 vaccination (J. H. Kim, Marks, & Clemens, 2021), especially since neonatal CECs were found to suppress antibody production against bacterial antigens (A. Namdar et al., 2017) and iron-deficiency anemia (IDA) was associated with decreased vaccine response (Stoffel et al., 2020).

The link between CECs and viral infection was also observed in the analysis of the blood transcriptome of severe respiratory syncytial virus (RSV)-infected patients (Rinchai et al., 2020). The identified A37/erythrocyte signature in the blood transcriptome was caused by the enrichment of CECs in the circulation and was associated with disease severity. Moreover, this specific erythrocyte signature was detected not only in RSV-infected patients but also in liver transplant recipients under maintenance therapy and melanoma patients, confirming previous findings demonstrating the role of CECs in cancer patients (L. Zhao et al., 2018). On the other side, this signature was not enriched in patients suffering from influenza or systemic lupus erythematosus (SLE) (Rinchai et al., 2020).

Taken together, CECs not only suppress cytokine production, proliferation, and degranulation of antigen-specific T cells but also are target cells for viruses and may support trans-infection (Afshin Namdar et al., 2019; Shima Shahbaz et al., 2021) (Fig. 4 and 5).

3.5. CECs in inflammatory diseases

Disruption of homeostasis and predominance of pro-inflammatory over suppressive signals underlie a wide spectrum of inflammatory diseases (Schett & Neurath, 2018). Increased secretion of pro-inflammatory cytokines leads to so-called anemia of inflammation, which is a consequence of both decreased erythropoiesis and reduction of erythrocytes lifespan (Ganz, 2019). Multiple pro-inflammatory cytokines modulate erythroblast maturation in various mechanisms (Table 5). However, whether and how these cytokines influence the immunosuppressive capacities of CECs remains unknown.

Dysregulation of hematopoiesis leading to the skewed granulocyte-monocyte progenitor (GMP) production at the expense of erythroid lineage cells is common in inflammatory diseases (Griseri, McKenzie, Schiering, & Powrie, 2012). Interesting observations were made in the experimental autoimmune encephalomyelitis (EAE) murine model that suggested the inhibitory role of CECs in inflammatory diseases. Mice with iron deficiency anemia do not develop EAE in contrast to non-anemic controls (Grant, Wiesinger, Beard, & Cantorna, 2003). Moreover, administration of EPO ameliorated EAE severity in mice (Savino et al., 2006). Since CECs emerged as immunomodulators of gut homeostasis (Elahi et al., 2013), their role was closely investigated in inflammatory bowel diseases (IBD). A decreased frequency of CECs in the spleen was observed in murine models of colitis (Griseri et al., 2012). Moreover, a recent study demonstrated that CECs suppress colitis in adult mice (Shim, Weliwitigoda, Campbell, Dosanjh, & Johnson, 2021). CECs can reduce TNF-α production by splenic red pulp macrophages in a phagocytosis-dependent manner. Importantly, adoptive transfer of CECs isolated from healthy mice attenuates the wasting syndrome in T-cell-induced colitis and suppresses the pro-inflammatory response of splenic macrophages in mice (Shim, Weliwitigoda, Campbell, Dosanjh, & Johnson, 2021).

In pregnant women with IBD, a decreased CECs frequency was noted in peripheral blood and placenta (G. Dunsmore et al., 2019). Notably, CECs in IBD patients are not only less abundant but also have reduced immunosuppressive capacities. These CECs do not decrease T-cell proliferation as well as fail to regulate the production of inflammatory

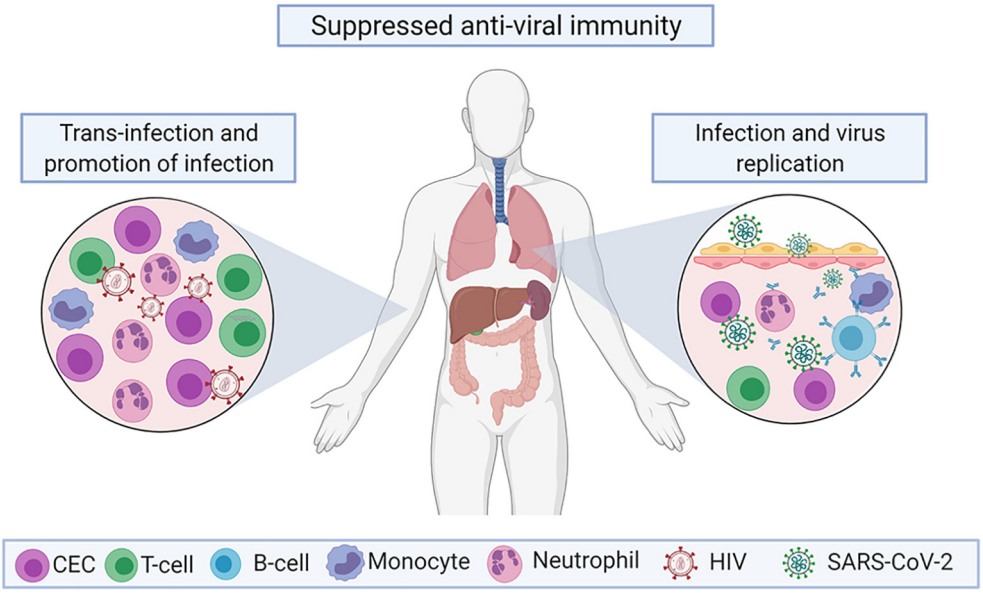

Fig. 4. Erythroid progenitor cells (CECs) suppress the anti-viral immune response. CECs are abundant in the peripheral blood of human immunodeficiency virus (HIV)-infected patients as well as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-infected patients. CECs potently suppress an antigen-specific immune response. Moreover, CECs promote HIV replication in T-cells by producing reactive oxygen species (ROS) and upregulating NF-κB in $CD4^+$ T-cells. CECs also get invaded by HIV and SARS-CoV-2.

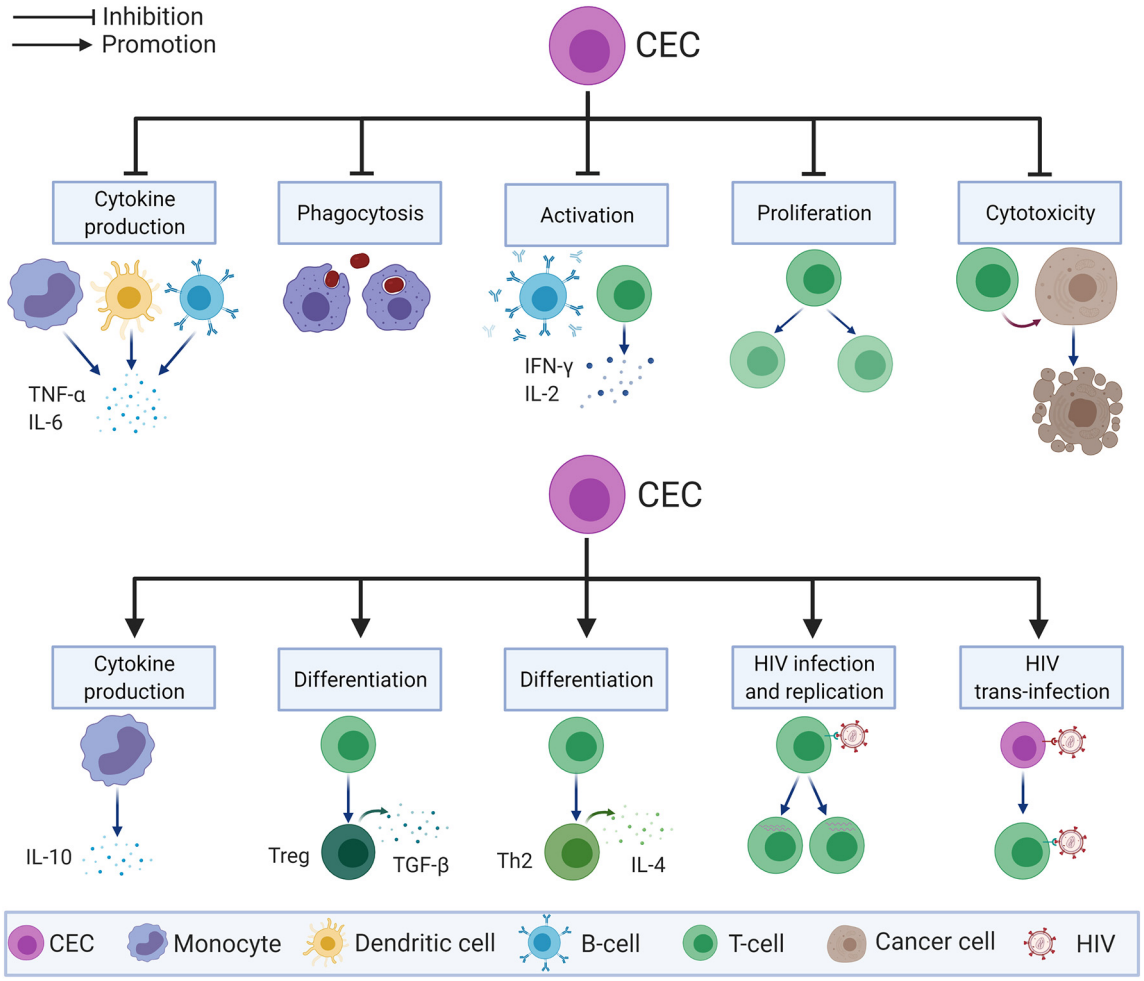

Fig. 5. Effects of CECs on immune cells. CECs suppress the immune response by decreasing the production of pro-inflammatory cytokines by myeloid cells and activation and cytokine secretion by lymphocytes. Effector functions of phagocytes and lymphocytes are inhibited by CECs. On the other side, CECs promote the production of anti-inflammatory cytokines and differentiation of T-cells toward immunosuppressive Tregs and Th2 cells. CECs mediate HIV trans-infection and increase HIV infection and replication in T-cells.

cytokines by PBMC (G. Dunsmore et al., 2019), in contrast to healthy pregnant women (Delyea et al., 2018; G. Dunsmore et al., 2019). Lower frequencies of CECs and their poor immunomodulatory properties in IBD patients predispose them to pro-inflammatory conditions and may contribute to both onset and progression of the disease (G. Dunsmore et al., 2019).

3.6. CECs in anemia

Anemia is a worldwide health problem with global prevalence exceeding 30% (Kassebaum et al., 2014). It is associated with reduced quality of life, especially in the elderly (Wouters et al., 2019), and may predispose to infection (Dunne, Malone, Tracy, Gannon, & Napolitano, 2002; Sales, de Queiroz, & Paiva Ade, 2011). A recent study found that anemia is linked to some alterations of the immune system in children (Hill et al., 2020). Decreased numbers of erythrocytes or total hemoglobin amount in blood lead to tissue hypoxia and, as a consequence, to increased EPO production. The two most common types of anemia are iron-deficiency anemia (IDA) and anemia of inflammation (AI, also known as anemia of chronic disease, ACD) (Camaschella, 2015). Temporary anemia is also observed in patients with acute infections (Jansson, Kling, & Dallman, 1986).

Whether CECs in anemic adults are immunosuppressive was initially unclear. It was suggested that CECs in adult mice do not have significant immunosuppressive capacities (Elahi et al., 2013; L. Zhao et al., 2018). Murine CECs obtained from anemic mice had little impact on T-cell proliferation, but suppressed both IgM and IgG secretion and proliferation of B-cells (Mitasov, Tsyrlova, Kiselev, & Kozlov, 1991; Seledtsov et al., 1998). In contrast, EPO-expanded CECs from anemic mice not only suppress B-cells, but also inhibit T-cells (Seledtsova et al., 2004). A recent report showed that CECs from anemic individuals have a similar role to CECs from HIV-infected patients in the promotion of HIV infectivity of $CD4^+$ T-cells (Afshin Namdar et al., 2019). All above evidence strongly suggest that the immunosuppressive role of CECs in anemic individuals may be underestimated. Indeed, we recently demonstrated that murine and human anemia-induced CECs possess significant suppressive properties (Grzywa et al., 2021). Expansion of CECs in anemic mice leads to creation of an immunosuppressive microenvironment in the spleen leading to impaired T-cells proliferation (Grzywa, Sosnowska, et al., 2021). Moreover, we have observed that ex vivo stimulated T-cells in PBMC of anemic patients produced less IFN-γ compared to T-cells isolated from non-anemic individuals (Grzywa, Sosnowska, et al., 2021).

Moreover, suppression of TNF-α production in myeloid cells is not exclusive to CECs from neonates, pregnant women, or cancer patients. In healthy adult mice other hematopoietic active tissues, including adult bone marrow, also exert this effect to a similar degree as compared with neonatal splenocytes and bone marrow cells (Wynn et al., 2015). Moreover, neonate splenocytes as well as neonatal and adult bone marrow cells compromise production of G-CSF, CXCL5, CCL3, CCL4, but increase secretion of CXCL10 and vascular endothelial growth factor (VEGF) by myeloid cells (Wynn et al., 2015). However, we did not observe impaired myeloid cells function in anemic mice or suppression

of myeloid cells by anemic CECs (Grzywa, Sosnowska, et al., 2021), which suggests that other cell types are responsible for this effect.

4. Mechanisms of CECs-mediated immunosuppression

An increasing number of reports identifies CECs as potent negative regulators of immune response (Fig. 6). Immunomodulation is a common feature of progenitor cells including mesenchymal stem cells (Glenn & Whartenby, 2014) or immature myeloid cells (Grzywa et al., 2020), including immature dendritic cells (Cools et al., 2008). In cancer, the immunosuppressive potential of murine CECs falls between that of Treg cells and MDSCs (L. Zhao et al., 2018), but human CECs seem to more potently suppress immune response than Tregs or MDSCs (J. Chen et al., 2021).

For many years molecular mechanisms of CECs-mediated immunosuppression were unknown. Preliminary studies found that CECs exert immunosuppressive effects against both murine T-cells and B-cells as well as human PBMC *via* heat-stable, proteolysis-resistant, but a neuraminidase-sensitive low-weight factor of unknown identity (Mitasov et al., 1991; Seledtsova et al., 2004). However, further research revealed that CECs use a whole arsenal of mechanisms to modulate the immune response (Table 6). So far, CECs were found to exert immunoregulatory effects by up-regulation of arginase 2 (Elahi et al., 2013), secretion of TGF-β (S. Shahbaz et al., 2018), robust generation of reactive oxygen species (ROS) (L. Zhao et al., 2018), modulation by the immune checkpoint molecules such as VISTA (S. Shahbaz et al., 2018), PD-1 and PDL-1 (Delyea et al., 2018) and other mechanisms that will be discussed below (Fig. 6).

4.1. Depletion of L-arginine by arginase

Two arginase isoforms, cytosolic ARG1 and mitochondrial ARG2 convert L-arginine into L-ornithine and urea as a final step in the urea cycle (Grzywa et al., 2020). L-Arginine is a conditionally essential amino acid that is required for T-cells expansion. L-Arginine depletion by arginase (s) leads to the inhibition of T-cell proliferation and impairment of their effector functions (Grzywa et al., 2020). L-Arginine starvation decreases the levels of CD3ζ, a signal-transducing subunit of TCR-CD3 complex (Rodriguez et al., 2002), inhibits cell-cycle progression (Rodriguez, Quiceno, & Ochoa, 2007), and impairs immune synapse formation (Feldmeyer et al., 2012). Immunoregulation mediated by arginase is a characteristic feature of myeloid cells, including MDSCs of neutrophilic and monocytic origin, monocytes/macrophages as well as mature neutrophils (Grzywa et al., 2020). Arginase activity is also used by cancer cells as a mechanism of tumor immune escape (Czystowska-Kuzmicz et al., 2019).

Inhibition of arginase or supplementation of L-arginine overrides immunosuppressive effects of neonatal CECs on the proinflammatory myeloid cells response (Elahi et al., 2013) and CECs-mediated inhibition of phagocytosis (G. Dunsmore et al., 2017). On the contrary, inhibition of myeloperoxidase, NADPH oxidase, superoxidase dismutase, or ROS neutralization by N-acetylcysteine had no impact on CECs-mediated immunosuppression in these studies. Similarly, inhibitors of indoleamine 2,3-dioxygenase (IDO) and TGF-β, or anti-IL-10 receptor antibodies failed to diminish decreased TNF-α production from myeloid cells co-cultured with neonatal CECs (Elahi et al., 2013), suggesting that murine neonatal CECs immunomodulatory properties rely mainly on arginase activity. Further studies reported that arginase-mediated suppression of immune response is also a feature of fetal CECs and CECs of pregnant mice (Delyea et al., 2018) as well as CECs from adult mice with T-cell-induced colitis (Shim, Weliwitigoda, Campbell, Dosanjh, & Johnson, 2021) and anemia (Grzywa, Sosnowska, et al., 2021).

Interestingly, CECs from human cord blood failed to suppress inflammation in an arginase-dependent manner (Cui et al., 2016), as arginase inhibition did not restore the suppressed secretion of TNF-α and IL-1β, however, it diminished CECs-induced production of IL-10 by monocytes

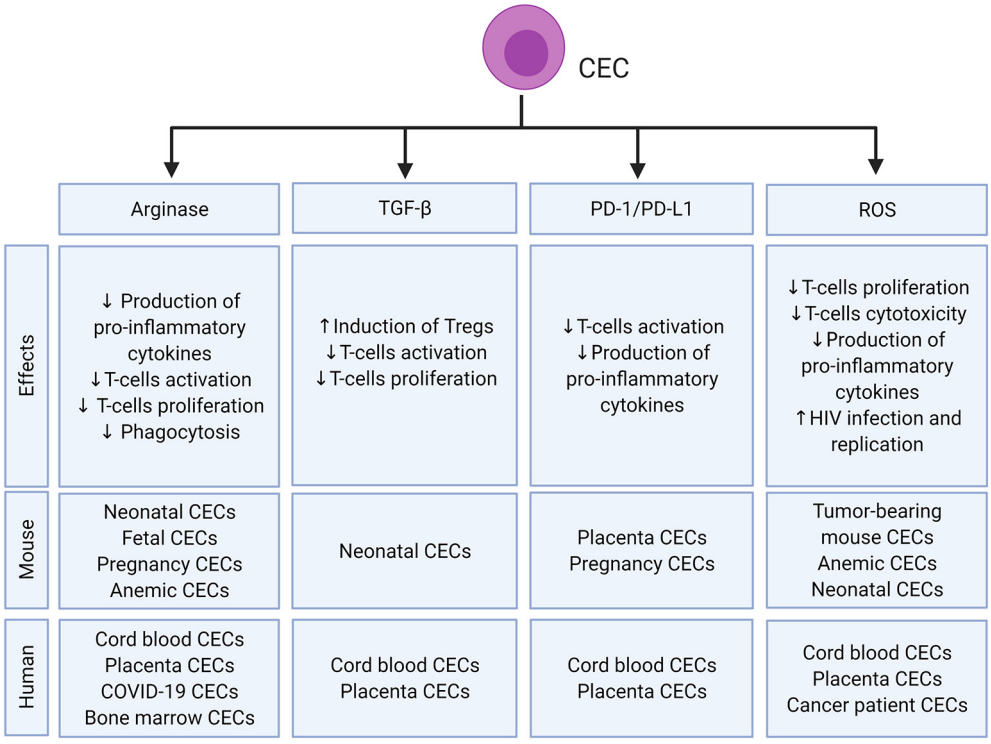

Fig. 6. Mechanisms used by CECs to modulate the immune response. CECs deplete L-arginine *via* arginase, mainly arginase 2, leading to the decreased production of pro-inflammatory cytokines by myeloid cells and decreased T-cells activation and proliferation. The secretion of transforming growth factor β (TGF-β) by CECs decreases T-cells activation and proliferation and induces differentiation towards regulatory T-cells. Expression of programmed death-ligand 1 (PD-L1) on CECs may decrease T-cells activation, production of pro-inflammatory cytokines and leads to T-cells apoptosis. CECs produce a substantial amount of reactive oxygen species (ROS) that suppress T-cells and production of pro-inflammatory cytokines as well as support HIV infection and replication in T-cells. These four mechanisms were found in CECs from different sources in mice and humans.

Table 6
Expression of immunomodulatory molecules in CECs

Source of CECs	Feature	Ref.
Murine fetal liver	↓ARG2[1]	(Delyea et al., 2018)
	No PDL-1 expression	(Delyea et al., 2018)
	No PDL-2 expression	
Neonatal mice - spleens	↑ARG2[2]	(G. Dunsmore et al., 2017; Elahi et al., 2013)
	No PDL-1 expression	(Delyea et al., 2018)
	No PDL-2 expression	
	High VISTA expression (50% of CECs)[3]	(S. Shahbaz et al., 2018)
	Low Galectin-9 expression (5% of CECs)	(S. Shahbaz et al., 2018)
	Low Galectin-1 expression (5% of CECs)	(S. Shahbaz et al., 2018)
	TGF-β expression[4]	(S. Shahbaz et al., 2018)
	Decreasing TLR-1, TLR-2, TLR-4 expression[5]	(S. Shahbaz et al., 2018)
Neonatal mice - bone marrow	High VISTA expression (90%)	(S. Shahbaz et al., 2018)
Pregnant mice	↑ARG2 in placenta	(Delyea et al., 2018)
	↑PDL-1 in placenta	(Delyea et al., 2018)
	↑PDL-2 in placenta	
Tumor-bearing mice - spleen	↑ROS	(L. Zhao et al., 2018)
	↑PD-L1[6]	(Sano et al., 2021)
	↑2B4[6]	(Sano et al., 2021)
Tumor-bearing mice - tumor	↑PD-L1[6,7]	(Sano et al., 2021)
	↑2B4[6,7]	(Sano et al., 2021)
Adult anemic mice	ARG2 expression	(Grzywa, Sosnowska, et al., 2021)
	ROS expression	(Grzywa, Sosnowska, et al., 2021)
Adult mice with colitis	ARG2 expression	(Shim, Weliwitigoda, Campbell, Dosanjh, & Johnson, 2021)
Adult UVB-irradiated mice	↑PD-L1[6]	(Sano et al., 2021)
	↑2B4[6]	
PBMC of healthy donors	↑ROS[8]	(Afshin Namdar et al., 2019)
PBMC of pregnant women	No PDL-1 expression	(Delyea et al., 2018)
	No PDL-2 expression	
Human cord blood	ARG2 expression[9]	(G. Dunsmore et al., 2019)
	ARG1 expression	(Cui et al., 2016)
	↑PDL-1[10]	(Delyea et al., 2018)
	↑PDL-2[10]	
	PD-1H[11]	(G. Dunsmore et al., 2019)
	Low VISTA expression (1% of CECs)	(Elahi et al., 2020; S. Shahbaz et al., 2018)
	TGF-β expression[11]	(G. Dunsmore et al., 2019)
	ROS^{hi} (30% of CECs)[11]	(G. Dunsmore et al., 2019; Afshin Namdar et al., 2019)
	VEGF expression[9]	(G. Dunsmore et al., 2019)
	↑DARC[8]	(Afshin Namdar et al., 2019)
	↑CD35[8]	(Afshin Namdar et al., 2019)
Peripheral blood of human infants	No PDL-1 expression	(Elahi et al., 2020)
	No PDL-2 expression	
	No galectins expression	(Elahi et al., 2020)
	No CD73/CD39 expression	(Elahi et al., 2020)
	High ROS	(Elahi et al., 2020)
	High VISTA	(Elahi et al., 2020)
Human placenta	ARG2 expression[9]	(G. Dunsmore et al., 2019)
	↑PD-1H[11,12]	(S. Shahbaz et al., 2018)
	↑VISTA[12]	(S. Shahbaz et al., 2018)
	↑TGF-β[11]	(S. Shahbaz et al., 2018)
	↑ROS (70% of CECs)[10,13]	(G. Dunsmore et al., 2019; Afshin Namdar et al., 2019)
	VEGF expression[9]	(G. Dunsmore et al., 2019)
	↑DARC[8]	(Afshin Namdar et al., 2019)
	↑CD35[8]	(Afshin Namdar et al., 2019)
Human PBMC of cancer patients	ROS	(L. Zhao et al., 2018)
Human tumors	PD-L1	(Sano et al., 2021)
	ROS	(J. Chen et al., 2021)
	TGF-β	(J. Chen et al., 2021)
	IL-10	(J. Chen et al., 2021)
Human PBMC of HIV-infected	↑DARC[8]	(Afshin Namdar et al., 2019)
	↑CD35[8]	(Afshin Namdar et al., 2019)
Human PBMC of COVID-19 patients	↑ARG1 expression[14]	(Shima Shahbaz et al., 2021)
	↑ARG2 expression[14]	(Shima Shahbaz et al., 2021)
	↑ROS[14]	(Shima Shahbaz et al., 2021)
	No PD-L1/PD-L2 expression	(Shima Shahbaz et al., 2021)
	No VISTA expression	(Shima Shahbaz et al., 2021)
Human PBMC of anemic individuals	ARG1 expression	(Grzywa, Sosnowska, et al., 2021)
	ARG2 expression	(Grzywa, Sosnowska, et al., 2021)
	↑DARC[8]	(Afshin Namdar et al., 2019)
	↑CD35[8]	(Afshin Namdar et al., 2019)

↑ - increased, ↓ - decreased
[1] In fetal liver CECs, compared to CECs in the spleen of adult mice
[2] Compared to adult mice
[3] Highest in 6-9-day-old mice. Higher in bone marrow CECs compared to the spleen.
[4] Highest in $VISTA^+$ CECs
[5] Highest in 1-day-old mice, followed by a potent decrease of the expression
[6] Compared to steady-state bone marrow CECs
[7] Compared to spleen CECs
[8] Compared to mature erythrocytes
[9] Higher in IBD patients
[10] Compared to PBMC of pregnant women as well as compared to $CD11b^+$, $CD11c^+$ or $CD123^+$ cells in human cord blood
[11] Lower in IBD patients
[12] At the protein level
[13] Compared to cord blood
[14] Compared to immune lineage cells

(Cui et al., 2016). It was suggested that while arginase is a key factor in murine CECs, the suppressive effects of human CECs do not rely on arginase activity (Cui et al., 2016). Further studies demonstrated that human cord blood CECs, similarly to neonatal murine CECs, inhibit phagocytosis of *B. pertussis* by neutrophils and macrophages *via* ARG2 (G. Dunsmore et al., 2017). Moreover, human CECs suppress T-cells in an arginase-dependent manner (Grzywa, Sosnowska, et al., 2021). A recent study identified ROS as crucial mediators of suppressive functions of human neonatal CECs (Elahi et al., 2020). However, even a high concentration of ROS inhibitors failed to completely restore production of TNF-α by $CD14^+$ cells and IFN-γ by T-cells that was suppressed by CECs, suggesting cooperation of multiple mechanisms of the suppression (Elahi et al., 2020). Therefore, it seems that arginases mediate immunosuppressive effects of human CECs by regulating selected effector functions of immune cells.

Importantly, the differences in CECs ARG2 expression levels are noted in various models (Table 6). ARG2 is upregulated in neonatal mice as compared with anemic adult mice, and in pregnancy-associated CECs compared with fetal liver CECs. Moreover, in pregnant mice placental CECs have higher expression of ARG2 than CECs in the spleen (Delyea et al., 2018). ARG2 expression is higher in CECs of allogeneic pregnancy compared with syngeneic (Delyea et al., 2018). Arginase activity in placenta as well as in PBMC of pregnant women is crucial for induction of hyporesponsiveness during pregnancy and for maintenance of feto-maternal tolerance (Kropf et al., 2007).

Of notice, there are substantial differences in arginase expression between murine and human CECs, similar to those observed between murine and human myeloid cells (Grzywa et al., 2020). Murine CECs express mitochondrial ARG2 but have low or undetectable cytosolic ARG1 expression (Delyea et al., 2018; Elahi et al., 2013; Grzywa, Sosnowska, et al., 2021). In contrast, human CECs potently express both ARG1 and ARG2 (Grzywa, Sosnowska, et al., 2021; Shima Shahbaz et al., 2021). Moreover, although there is a clear increase in ARG2 levels in CECs in various conditions, the factors regulating arginase expression in CECs remain unknown.

The immune response is regulated not only by the availability of L-arginine but also L-tryptophan, which is metabolized by indoleamine 2,3-dioxygenase (IDO). Various tumor-infiltrating cells express IDO leading to the suppression of T-cells response (Kedia-Mehta & Finlay, 2019). Human erythroid cells express IDO and its expression decreases with their maturation (Sibon et al., 2019). However, whether degradation of L-tryptophan by IDO contributes to CECs-mediated immunoregulation, remains unknown.

4.2. TGF-β and induction of regulatory T-cells

One of the first identified mechanisms of CECs-mediated immunosuppression was TGF-β secretion by these cells (Seledtsov et al., 1998). Cholesterol-modified TGF-β mRNA-specific antisense oligonucleotides, as well as anti-TGF-β antibodies, diminished the suppressed B-cells proliferation induced by CECs-conditioned medium (Seledtsov et al., 1998).

TGF-β is a potent anti-inflammatory cytokine that inhibits differentiation and effector functions of lymphocytes and innate immune cells as well as promotes differentiation of immunosuppressive cells including Tregs (Sanjabi, Oh, & Li, 2017). Moreover, TGF-β itself is a negative regulator of erythropoiesis (Kuhikar et al., 2020; Zermati et al., 2000). TGF-β inhibitors stimulate erythropoiesis mainly by enhancing erythroid progenitors self-renewal (Gao et al., 2016).

CECs secrete relatively high amounts of TGF-β facilitating differentiation of T-cells into Tregs (S. Shahbaz et al., 2018). Importantly, stimulation with heat-killed bacteria or TLR-1 agonists induces TGF-β production in CECs of to (S. Shahbaz et al., 2018). About half of neonatal CECs express inhibitory immune checkpoint VISTA (S. Shahbaz et al., 2018). VISTA is an immunoregulatory receptor, that, unlike other immune checkpoint molecules, is constitutively expressed by most cell subsets of the hematopoietic lineage (ElTanbouly, Schaafsma, Noelle, & Lines, 2020). $VISTA^+$ CECs are more potent immunosuppressors than $VISTA^-$ CECs, produce much more TGF-β and, as a consequence, more potently induce differentiation of T-cells into Tregs (S. Shahbaz et al., 2018). In contrast, $VISTA^-$ CECs as well as CECs isolated from VISTA knock-out mice produce less TGF-β and do not induce significant differentiation of T-cells into Tregs (S. Shahbaz et al., 2018). Moreover, VISTA knock-out newborns have a smaller percentage of Tregs in the spleen as compared with wild-type mice (S. Shahbaz et al., 2018), indicating that VISTA is not only a marker of more immunosuppressive CECs but also takes part in regulation of their immunosuppressive properties.

Peripheral Tregs induction by CECs is mediated by secreted TGF-β and is independent on arginase activity. Importantly, CECs-induced Tregs are more potent immunosuppressors than natural Tregs isolated from murine spleens and robustly inhibit $CD4^+$ effector T-cell proliferation (S. Shahbaz et al., 2018). What is more, in pregnant women, CECs frequency positively correlates with the percentage of Tregs (G. Dunsmore et al., 2019). Similarly, human CECs isolated from human tumors secrete high amounts of TGF-β and suppress T-cell proliferation, which is diminished by STAT3 or SMADs inhibitors (J. Chen et al., 2021).

Nonetheless, TGF-β seems to play a role mainly in CECs-mediated suppression of adaptive immune response, as anti-TGF-β blocking antibody does not affect CECs-mediated suppression of the proinflammatory response of myeloid cells (Cui et al., 2016; Elahi et al., 2013).

4.3. Immunosuppression via immune checkpoints and modulating molecules

Besides modulating immune response by soluble factors, CECs also exert regulatory effects on target cells by direct cell-to-cell contact *via* immune checkpoints.

Programmed cell death protein 1 (PD-1) and programmed death-ligand 1/2 (PD-L1/2) interaction plays a role in regulating the protective immunity and immunopathology to maintain homeostasis and immune tolerance (Qin et al., 2019). PD-1 is expressed on the surface of lymphocytes upon activation, while its ligand, PD-L1, is expressed by many types of hematopoietic cells and some non-hematopoietic cells, especially after stimulation with interferons (Qin et al., 2019). PD-1/PD-L1 interaction attenuates positive signals resulting from TCR activation, inhibits proliferation, and cytokine secretion, as well as promotes T-cells apoptosis (Freeman et al., 2000; Ishida, Agata, Shibahara, & Honjo, 1992).

PD-1/PD-L1 pathway plays a role in the development and maintenance of feto-maternal tolerance (Y. H. Zhang, Tian, Tang, Liu, & Liao, 2015). High expression of PD-L1 and PD-L2 is observed in pregnancy-induced CECs (Delyea et al., 2018). These CECs have higher levels of PD-L1 as compared with myeloid cells (Delyea et al., 2018). The percentage of $PD\text{-}L1^+$ or $PD\text{-}L2^+$ CECs is higher in the placenta than in the spleen of pregnant mice (Delyea et al., 2018). In contrast, neither CECs associated with syngeneic pregnancy nor fetal liver CECs express PD-L1 or PD-L2 (Delyea et al., 2018). PD-L1 blockade, similar to L-arginine supplementation, overrides suppressive effects of pregnancy-induced CECs (Delyea et al., 2018).

High expression of PD-L1 by CECs seems to be a feature of stress erythropoiesis-associated CECs. In mice, ultraviolet B irradiation-induced stress CECs in the spleen have high PD-L1 levels as compared with the bone marrow steady-state CECs (Sano et al., 2021). Moreover, tumor-induced stress CECs have upregulated PD-L1 compared to bone marrow CECs. The highest expression of PD-L1 is observed in tumor-infiltrating CECs (Sano et al., 2021).

Interaction of PD-1 with PD-L1 on erythroid cells prevents at least some autoimmune reactions. There are several described cases of severe anemia during anti-PD-1 antibody (pembrolizumab) therapy, which resulted from pure red-cell aplasia with maturation arrest at the proerythroblast stage or autoimmune hemolytic anemia (Le Aye, Harris, Siddiqi, & Hagiya, 2019; Nair, Gheith, & Nair, 2016; Zhuang et al., 2020).

CECs express high levels of Fas and FasL, which are important in the regulation of erythroblast fate (De Maria, Zeuner, et al., 1999), as well as in the maintenance of peripheral immune tolerance (Yamada, Arakaki, Saito, Kudo, & Ishimaru, 2017). CECs *via* Fas/FasL pathway may induce apoptosis of lymphoblasts (De Maria et al., 1999). Moreover, erythroblasts regulate immune response and contribute to the maintenance of feto-maternal tolerance by secretion of human leukocyte antigen (HLA)-G (Menier et al., 2004). HLA-G is a member of non-classical HLA class I antigens and has immunomodulating functions (Colonna et al., 1998). Its role is best described in the development feto-maternal tolerance during pregnancy (Fuzzi et al., 2002). HLA-G triggers Fas/FasL-mediated apoptosis of activated cytotoxic T-cells (Fournel et al., 2000) and NK cells (Rouas-Freiss, Gonçalves, Menier, Dausset, & Carosella, 1997). CECs in fetal liver and adult bone marrow express and release HLA-G (Menier et al., 2004). Moreover, HLA-G-expressing CECs are also observed in the spleen, trophoblast, and circulation (Menier et al., 2004). Since HLA-G is expressed not only by fetal or neonatal CECs but also by child and adult CECs, it is suggested that HLA-G may either regulate erythropoiesis or contribute to CECs-mediated immunomodulation in various conditions.

CECs also express several other immunomodulating molecules including galectin-1 (S. Shahbaz et al., 2018), anti-inflammatory glycan-binding protein, that suppresses synthesis of proinflammatory cytokines by innate immune cells and serves as a negative regulatory checkpoint for lymphocytes (Sundblad, Morosi, Geffner, & Rabinovich, 2017). Similarly, CECs express galectin-9 (S. Shahbaz et al., 2018), which regulates macrophage differentiation and suppression of the adaptive immune response (Daley et al., 2017). A recent study found a high expression of CD244 (2B4) in CECs, especially in stress-CECs and tumor-infiltrating CECs (Sano et al., 2021). CD244 is an inhibitory receptor in NK cells, T cells, DCs, and MDSCs (Agresta, Hoebe, & Janssen, 2018). Moreover, it was suggested that CECs may act via ectonucleotidases CD39 and CD73 (Elahi, Shahbaz, & Koleva, 2019) that generate immunosuppressive extracellular adenosine (Allard, Longhi, Robson, & Stagg, 2017). On the other side, CECs

also express galectin-3 (S. Shahbaz et al., 2018) that is a pattern-recognition receptor (PRR) and may act as a danger-associated molecular pattern (DAMP) with an important role in the innate immune response against pathogens (Díaz-Alvarez & Ortega, 2017). However, the role of these molecules in CECs-mediated immunoregulation is unknown.

4.4. Immunomodulating cytokines

Erythroid cells secrete numerous cytokines (Table 7). Moreover, they are able to modulate the expression profile of immunomodulatory cytokines in other cells (Cui et al., 2016).

Besides secretion of TGF-β that induces Tregs, neonatal CECs were found to induce differentiation of $CD4^+$ T-cells into IL-4-secreting T_H2 cells (Rincon, Oppenheimer, & Bonney, 2012), which contributes to the observed T_H2 bias in neonatal immunity (Adkins & Du, 1998; H. H. Lee et al., 2008). This effect is diminished in CECs isolated from IL-6 knock-out mice, suggesting that IL-6 derived from the interaction of the CECs and T-cells promotes differentiation toward T_H2 cells (Rincon et al., 2012).

In cancer patients, CECs secrete IL-10, resulting in T-cells suppression (J. Chen et al., 2021). The suppression of T-cell proliferation by CECs can be restored by inhibitors of IL-10 signaling, including inhibitors of NF-κB and STAT3 (J. Chen et al., 2021). Moreover, cord blood CECs can stimulate monocytes to secrete IL-10, which is an autoregulatory mechanism of immunoregulation. Blocking of IL-10 receptor restores production of inflammatory cytokines by myeloid cells in the presence of CECs (Cui et al., 2016). In monocytes, CECs also induce secretion of IL-19, IL-20, and IL-24, cytokines belonging to the IL-10 superfamily (Cui et al., 2016).

4.5. Reactive oxygen species

Erythrocytes are exposed to one of the highest levels of reactive oxygen species (ROS) and thus oxidative stress in the human body (Ghaffari, 2008). Similarly to erythrocytes CECs are prone to oxidative stress. Many sources of oxidants, including oxygen bound to hemoglobin (Ghaffari, 2008) and iron within the prosthetic group of hemoglobin are counterbalanced by antioxidants (Kuhn et al., 2016). High concentrations of antioxidant molecules including glutathione (GSH), ascorbic acid (vitamin C), and α-tocopherol (vitamin E) maintain the reduced state (Kuhn et al., 2016). Moreover, erythroid cells have relatively high levels of superoxide dismutase (SOD), catalase, glutathione peroxidase, and peroxiredoxin II (PRDX2) (Mohanty, Nagababu, & Rifkind, 2014). ROS accumulation is even higher in CECs as compared with mature erythrocytes (Ghaffari, 2008), reaching the highest levels in $CD71^{hi}TER119^{hi}$ cells (B. Zhao, Mei, Yang, & Ji, 2016), and decreases with CECs maturation (S. Chatterjee & Saxena, 2015; Doty et al., 2015; B. Zhao et al., 2016). Excessive ROS accumulation and lipid peroxidation in CECs triggers necroptosis (Canli et al., 2016). ROS are important in regulation of CECs maturation since ROS inhibition promotes enucleation (B. Zhao et al., 2016). They also regulate immune response by modulating T-cell activation, apoptosis, and hyporesponsiveness (X. Chen, Song, Zhang, & Zhang, 2016) as well as polarize macrophages towards an immunosuppressive phenotype (Roux et al., 2019). ROS are used by MDSCs as one of the mechanisms of immunoregulation (Ohl & Tenbrock, 2018).

Transcriptional profiling of CECs from different sources revealed that the ROS pathway genes, including ROS-generating NOX2, are enriched in CECs in tumor-bearing mice (L. Zhao et al., 2018). Moreover, the ROS pathway is enriched in $CD45^+$ CECs from cancer patients compared to $CD45^-$ CECs (J. Chen et al., 2021). Functional assays confirmed that ROS play a dominant role in CECs-mediated immunosuppression in tumor-bearing mice and in cancer patients (J. Chen et al., 2021; L. Zhao et al., 2018). CECs from tumor-bearing mice impair T-cells proliferation as well as cytotoxicity of $CD8^+$ T-cells in a ROS-dependent mechanism, as ROS scavenger, N-acetylcysteine restores T-cells functions (L. Zhao et al., 2018). Likewise, CECs from patients with advanced cancer inhibit T-cells proliferation, which is restored by N-acetylcysteine (L. Zhao et al., 2018). A recent study reported that also human neonatal CECs suppress production of pro-inflammatory cytokines by myeloid cells and T-cells, which is partially restored by ROS inhibitor (Elahi et al., 2020).

CECs express high levels of NADPH oxidase 2 (NOX2), NADPH oxidase, and use mitochondrial ROS to modulate gene expression in T-cells (Afshin Namdar et al., 2019). Among regulated genes, NF-κB is substantially increased in T-cells incubated with CECs. Facilitated HIV infection and replication in $CD4^+$ T-cells by CECs was independent of arginase activity and TGF-β secretion but was diminished by NADPH-dependent ROS inhibitor (Afshin Namdar et al., 2019). By upregulating ROS and NF-κB in T-cells, CECs enhance HIV infection (Afshin Namdar et al., 2019).

4.6. More mature, less immunosuppressive?

The immunosuppressive effects of CECs contrast with a rather pro-inflammatory role of mature erythrocytes. Recently, it was suggested that during maturation, CECs may lose their immunosuppressive capacities (L. Zhao et al., 2018). CECs expressing pan-leukocyte marker CD45, therefore, at the earliest stages of differentiation (Fig. 1), are more potent immunoregulators than late-stage $CD45^-$ CECs (J. Chen et al., 2021; L. Zhao et al., 2018). $CD45^-$ CECs have a different transcriptional profile and downregulated expression of ROS pathway genes compared to $CD45^+$ CECs (L. Zhao et al., 2018). Moreover, human $CD45^+$ have substantially higher ROS levels than $CD45^-$ CECs (Elahi et al., 2020). More mature $CD45^-$ CECs failed to potently inhibit T-cells proliferation in contrast to $CD45^+$ CECs (J. Chen et al., 2021; L. Zhao et al., 2018). The population of more mature $CD45^-$ erythroid cells named Ter-cells also lacks measurable immunosuppressive capacities (Han et al., 2018). These late-stage CECs fail to inhibit host anti-tumor immune response, T-cell proliferation, and Treg induction as well as do not affect dendritic cell activation and IL-12 and IL-6 production (Han et al., 2018). Importantly, in contrast to CECs, more mature Ter-cells do not express immune-related mediators including IL-4, IL-6, IL-10, nor TGF-β (Han et al., 2018). Recently, we demonstrated that CECs possess potent, but transient immunomodulatory properties that disappear during erythroid differentiation (Grzywa, Sosnowska, et al., 2021). However, in the bone marrow of healthy adults, early-stage CECs are very rare as compared to late-stage CECs (Huang et al., 2020). Thus, the regulation of immune response by CECs has the greatest importance in diseases

Table 7
Cytokines expressed or stored in erythroid cells

Cytokine	Murine CECs		Human CECs		Human RBCs
	Neonatal	Adult	Embryonic/cord blood	Adult	Adult
IL-1α, IL-1β	+[1]	+[1]	+[1]	+[2]	-[2]
IL-4	+[1,2]	+[1,-1]	+[1]	+[2]	-[2]
IL-6	+[1,2]	+[1,-1]	+[1]	+[2]	-[2]
GM-CSF	+[1,2]	+[1,2,-1,]	n.d	n.d	+[2]
IFN-γ	+/-[1]	+[1,2]	+[1,2]	+[2]	+[2]
TGF-β	-[1]	+/-[1]	+[1,2]	+[1,2]	n.d.
IL-2	+/-[1]	+/-[1]	+[1]	+[2]	-[2]
IL-3	+/-[1]	+/-[1]	n.d	n.d	+[2]
IL-5	-[1]	-[1]	n.d	n.d	+/-[2]
TNF-α	n.d	n.d	+[1,2]	+[2]	+/-[2]
IL-10	n.d	n.d	+/-[1,6]	+/-[1,2]	-

based on (J. Chen et al., 2021; Han et al., 2018; Karsten, Breen, & Herbert, 2018; Rincon et al., 2012; Sennikov, Eremina, Samarin, Avdeev, & Kozlov, 1996; Sennikov et al., 2004; Sennikov, Krysov, Injelevskaya, Silkov, & Kozlov, 2001)
n.d. – no data,
[1] at the mRNA level
[2] at the protein level

with a significant expansion of CECs with maturation arrest at the earliest stages of differentiation (Grzywa, Justyniarska, et al., 2021).

5. Therapeutic modulation of CECs

Recent years revealed a key role of CECs in the regulation of immune response in multiple conditions. Depending on the condition, CECs may have either beneficial or detrimental effects. Therefore, CECs arose as a promising therapeutic target, and the development of new agents that modulate their functions is of great clinical interest (Fig. 7).

5.1. Promotion of CECs expansion

It seems that the primary role of CECs is to suppress the immune response to minimalize tissue damage and to prevent chronic inflammation. Indeed, induction of stress erythropoiesis and CECs expansion is one of the elements of the inflammatory response (Paulson et al., 2020). However, CECs numbers and their immunomodulatory properties may be impaired in chronic inflammation (G. Dunsmore et al., 2019). Therefore, promotion of CECs expansion and restoration of their immunomodulatory functions may promote resolution of inflammation and thus may be beneficial in inflammatory disorders.

A crucial stimulator of erythropoiesis, EPO, is used for the therapy of chemotherapy induced-anemia and anemia in chronic kidney diseases (Aapro et al., 2019). Moreover, several reports demonstrated that erythropoiesis-stimulating agents (ESAs) are effective for the therapy of inflammatory diseases in preclinical studies. EPO administration ameliorated disease severity in a murine model of colitis (Nairz et al., 2011) and EAE (Weiping Li et al., 2004). However, since EPO may directly regulate immune response (Peng, Kong, Yang, & Ming, 2020), it is unknown to which extent this effect is caused by the expansion of CECs. Other therapeutic strategies that promote CECs expansion include hypoxia-inducible factor (HIF) prolyl hydroxylase inhibitors that lead to HIF stabilization. Roxadustat (FG-4952) was found to increase EPO production and to improve iron availability and is effective for the stimulation of erythropoiesis in patients with anemia of chronic kidney disease (Besarab et al., 2016; Nan Chen et al., 2019; N. Chen et al., 2017). CECs expansion is also promoted by glucocorticoids and PPAR-α agonists (Flygare, Rayon Estrada, Shin, Gupta, & Lodish, 2011; H. Y. Lee et al., 2015; Vignjevic et al., 2015; L. Zhang et al., 2013). Moreover, several drugs, including SGLT2 inhibitor dapagliflozin, were reported to promote erythropoiesis in various mechanisms (Ghanim et al., 2020). However, currently, the most effective treatment strategy of most of the diseases with impaired CECs functions is treating the underlying disease. Nonetheless, new agents that promote CECs expansion may be promising therapeutic strategies. Moreover, it remains to be determined whether and how immunomodulatory properties of CECs can be restored in patients with chronic inflammation.

5.2. Prevention of CECs expansion

When CECs expansion is disproportionate and is associated with enrichment of early-stage CECs, they induce potent immunosuppression. Detrimental effects of CECs have been described in cancer patients

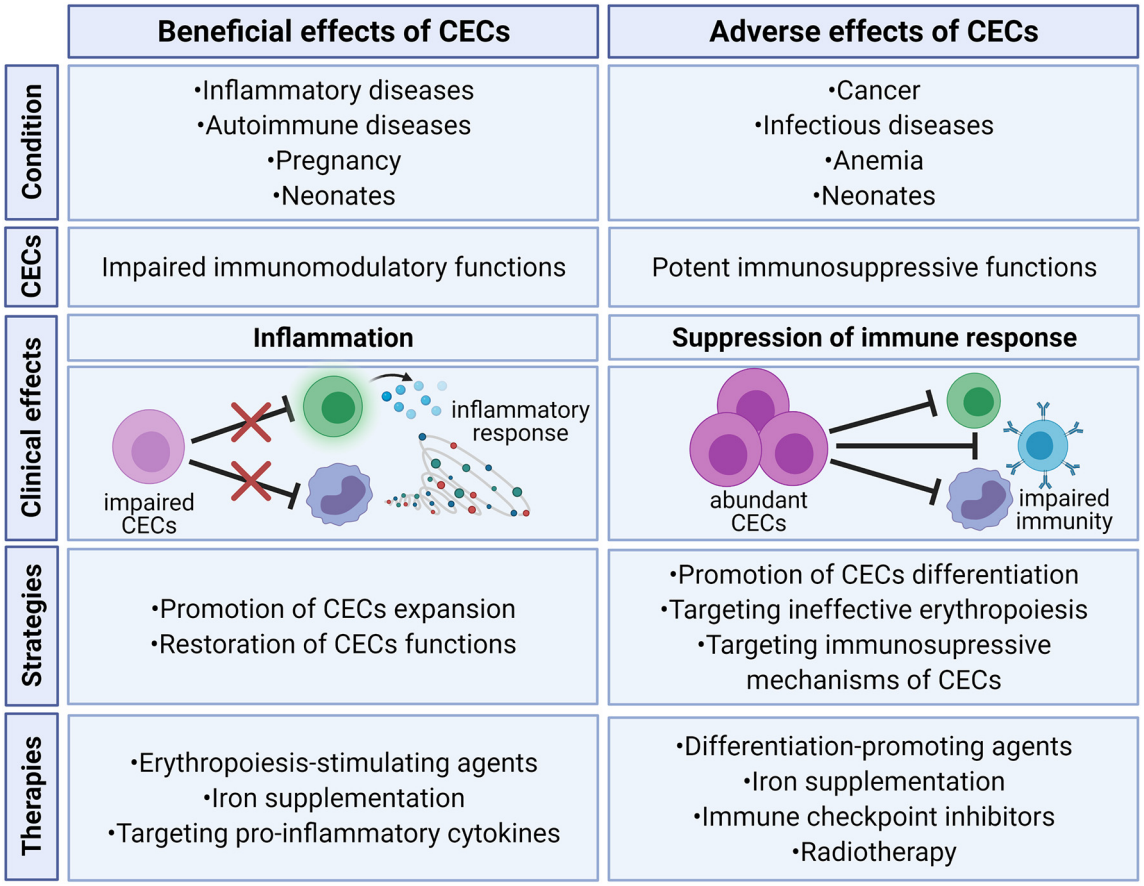

Fig. 7. Therapeutic strategies to target CECs. CECs may have either beneficial or detrimental effects, depending on the condition. CECs may suppress inflammation in inflammatory and autoimmune diseases, during pregnancy, and in neonates. However, their immunomodulatory functions may be impaired which results in an extensive inflammatory response. Thus, the promotion of CECs expansion and restoration of CECs functions are promising therapeutic approaches. Contrary, potent suppression of immune response by CECs has detrimental effects in cancer, infectious diseases, anemia, and neonates. In these cases, promotion of CECs differentiation, targeting ineffective erythropoiesis, and targeting immunosuppressive mechanisms of CECs may diminish suppression of immune response.

(L. Zhao et al., 2018), COVID-19 patients (Shima Shahbaz et al., 2021), HIV patients (Afshin Namdar et al., 2019), and anemic individuals (Grzywa, Sosnowska, et al., 2021). In these cases, the expansion of early-stage CECs suppresses immune response leading to the disease progression and increasing susceptibility to pathogens. Therefore, prevention of CECs accumulation and promotion of their differentiation are promising therapeutic strategies to diminish CECs-induced immunosuppression.

5.2.1. Targeting pro-inflammatory cytokines

Increased levels of pro-inflammatory cytokines including IFN-γ, TNF-α, IL-1, and IL-6 impair erythropoiesis leading to the expansion of CECs and enrichment of early-stage CECs as compensatory mechanisms to anemia (Grzywa, Justyniarska, et al., 2021). Several therapeutics targeting pro-inflammatory cytokine signaling and ameliorating anemia were developed and approved by U.S. Food and Drug Administration (FDA) including anti-IL-6 receptor antibody tocilizumab (Song et al., 2010), anti-IL-6 antibody siltuximab (Casper et al., 2015), anti-TNF-α antibody infliximab (Song et al., 2013), and IL-1 receptor antagonist anakinra (Laskari, Tzioufas, & Moutsopoulos, 2011). Moreover, anakinra as an adjunct to linezolid treatment suppressed inflammation while not affecting host immunity to tuberculosis and restored CECs suppressed by linezolid in preclinical studies (Winchell et al., 2020). Such treatments ameliorate the anemia and rescue CECs differentiation suppressed by inflammation which diminishes the accumulation of early-stage CECs. Nonetheless, more research is required to determine the clinical effects of these strategies on CECs-mediated immune regulation.

5.2.2. Modulation of iron metabolism

Iron a crucial regulator of erythropoiesis (Clara, Antonella, & Laura, 2020). Iron deficiency leads to anemia since its restriction potently impairs CECs differentiation (Bullock et al., 2010; Khalil et al., 2017; S. Liu et al., 2008; Masahiro et al., 2017). Therefore, iron supplementation or modulation of its metabolisms promotes differentiation of CECs decreasing the accumulation of early-stage CECs. However, in many cases, differentiation arrest of CECs is not caused by absolute iron deficiency but by iron sequestration in response to inflammation (Ganz, 2019). Inflammatory stimuli not only directly suppress CECs differentiation, but also induce the production of hepcidin, an inhibitor of the iron-exporting ferroportin resulting in hypoferremia (Nemeth et al., 2004). Thus, therapies that target pro-inflammatory cytokines promote erythropoiesis by increasing iron availability (Song et al., 2010; Song et al., 2013). Furthermore, several new agents that modulate iron metabolism are currently under investigation. These include a monoclonal antibody neutralizing hepcidin (LY2787106) (Vadhan-Raj et al., 2017), and an anti-ferroportin antibody that blocks interaction with hepcidin (LY2928057) (Sheetz et al., 2019). Moreover, Lexaptepid pegol, a pegylated structured L-oligoribonucleotide that binds hepcidin with high affinity was reported to rescue AI in preclinical studies (Schwoebel et al., 2013) and increase serum iron in healthy individuals (Boyce et al., 2016). Similarly, BMPs that stimulate hepcidin production are promising targets of therapy. Anti-BMP5 antibody (LY3113593) restored erythropoiesis in AI leading to the increase in hemoglobin (Sheetz et al., 2019). Moreover, CECs differentiation may be promoted by the regulation of erythroid iron restriction response. It was reported in a preclinical study that isocitrate corrects anemia and erythropoiesis dysregulation induced by inflammation (Richardson et al., 2013).

5.2.3. Promotion of CECs differentiation

Cancer cells and tumor-associated cytokines induce the dysregulation of erythropoiesis and induction of differentiation arrest at the earliest stages of maturation (Grzywa, Justyniarska, et al., 2021). This leads to the anemia and expansion of CECs resulting in the suppression of antitumor immune response and systemic immunity to pathogens (L. Zhao et al., 2018). Thus, promotion of CECs differentiation may diminish immunosuppression, slows disease progression as well as rescues anemia improving the quality of life of cancer patients. Current treatment strategies for cancer-associated anemia that promote CECs differentiation include iron, folic acid, and cyanocobalamin supplementation (Gilreath & Rodgers, 2020). Moreover, several agents were reported to promote CECs differentiation, including p38 kinase inhibitors (P. Hu et al., 2018), mTOR inhibitors (X. Zhang et al., 2014), IDH2 inhibitor enasidenib (Stein et al., 2017), selective serotonin reuptake inhibitor (SSRI) fluoxetine (Sibon et al., 2019), resveratrol (Franco et al., 2014), and vitamin C (Gonzalez-Menendez et al., 2021). However, their clinical effectiveness remains to be determined. In 2020, FDA approved the first agent that selectively promotes erythroid differentiation under maturation arrest. Luspatercept (ACE-536) is a TGF-β ligand-trapping fusion protein that induces late-stage erythropoiesis suppressed by upregulated TGF-β superfamily signaling (Suragani et al., 2014). Luspatercept was shown to be effective in the treatment for anemia caused by ineffective erythropoiesis in patients with myelodysplastic syndromes and β-thalassemia (Cappellini et al., 2020; Fenaux et al., 2020).

5.2.4. Induction of CECs apoptosis

Another strategy to modulate CECs is the induction of their apoptosis leading to their elimination. In murine models, depletion of CECs rescues suppressed immune response and inhibited tumor growth (L. Zhao et al., 2018). However, the specific depletion of CECs in clinical settings is unattainable due to their role as erythrocyte precursors. Nonetheless, a recent study demonstrated that CECs are eliminated as an out-of-field effect of multiple anti-tumor therapies, including radiotherapy and immunotherapy (Hou et al., 2021). Local irradiation or administration of the anti-PD-1 antibody resulted in a decrease in CECs numbers, especially late-stage CECs and Ter-cells, in tumor-bearing mice. Both strategies rely on the activation of adaptive immunity and extensive secretion of IFN-γ by T-cells which induces CECs apoptosis (Hou et al., 2021). Reduction in the number of CECs resulted in a substantial decrease of CECs-secreted artemin in murine serum. Similarly, the concentration of artemin was found to be decreased in cancer patients after radiotherapy (Hou et al., 2021). Thus, the elimination of CECs by radiotherapy and immunotherapy potentially contributes to the therapeutic outcomes in cancer patients.

5.2.5. Inhibition of the mechanisms of CECs-driven immunosuppression

CECs use multiple mechanisms to modulate the immune response, including arginase, ROS, immunomodulatory cytokines, and immune checkpoints. Therefore, it is of great clinical interest to develop therapeutic strategies for the modulation of CECs properties. It was demonstrated that CECs inhibitory effects on immune response may be diminished by arginase inhibitors or L-arginine supplementation *ex vivo* (Delyea et al., 2018; G. Dunsmore et al., 2017; Elahi et al., 2013; Grzywa, Sosnowska, et al., 2021). The availability of modest and selective arginase inhibitors (Abdelkawy, Lack, & Elbarbry, 2017; Grzywa et al., 2020) makes arginases promising therapeutic targets. However, the effects of arginase inhibition on the CECs functions were not investigated *in vivo*. Similar to arginase inhibitors, inhibitors of ROS-generating proteins, including NADPH oxidases NOX), as well as ROS scavenger, N-acetylcysteine, diminish the suppressive effects of CECs on immune cells (Elahi et al., 2020; Grzywa, Sosnowska, et al., 2021; Afshin Namdar et al., 2019; L. Zhao et al., 2018). Moreover, it seems that human CECs rely mostly on ROS, in contrast to murine CECs. Therefore, the available NOX inhibitors are promising agents to modulate CECs functions (E. Cifuentes-Pagano, Meijles, & Pagano, 2014; M. E. Cifuentes-Pagano, Meijles, & Pagano, 2015). Further studies demonstrated that inhibition of immunomodulatory cytokines secreted by CECs, including TGF-β and IL-10, or inhibition of their downstream signaling ameliorate their suppressive effects (J. Chen et al., 2021; Cui et al., 2016; Shima Shahbaz et al., 2021). Moreover, neutralization of TGF-β with monoclonal antibody diminishes the expansion of CECs in

preclinical studies (Han et al., 2018). However, despite promising pharmacological and preclinical studies, anti-TGF-β therapies failed to recapitulate preclinical success in clinical trials (Batlle & Massagué, 2019; Teixeira, ten Dijke, & Zhu, 2020). Similarly, targeting IL-10 in cancer immunotherapy turned out to be less effective than therapy with tumor-targeted or PEGylated IL-10 (Naing et al., 2018; Qiao et al., 2019). Nonetheless, inhibition of STAT3 signaling, which mediates CECs-driven suppression, is currently widely investigated in clinical trials with promising results (Zou et al., 2020). Moreover, recent studies demonstrated that the PD-1/PD-L1 axis is important in the immunoregulatory functions of CECs (Delyea et al., 2018; Sano et al., 2021). Immune checkpoint inhibitors (ICIs), including anti-PD-1/PD-L1 antibodies, revolutionized cancer immunotherapy (Waldman, Fritz, & Lenardo, 2020). Thus, it is of great interest to determine the role of CECs in response to ICIs.

Importantly, despite the identification of multiple mechanisms of immune regulation by CECs, only single therapeutic approaches have been investigated in preclinical studies. Therefore, further research must focus on the identification of rational strategies to modulate CECs functions *in vivo*.

6. Perspectives on CECs research and therapies

Detailed molecular characterization of single cells revealed a continuum of molecular transitions during CEC differentiation to generate mature erythrocyte (Huang et al., 2020; Pellin et al., 2019; Tusi et al., 2018; Xie, Liu, et al., 2020; Zheng, Papalexi, Butler, Stephenson, & Satija, 2018). Thus, CECs are a heterogeneous population of dynamically changing cells. Moreover, embryonic, fetal, and adult erythroid cells substantially differ (Y. Yang et al., 2013). Noteworthy, there are several crucial differences in erythroid cells and their regulation between sexes (Nakada et al., 2014), and between mice and men (An et al., 2014; J. Zhang et al., 2019). Even though most genes are regulated in a similar way, a significant extent of divergence between species was identified including the most highly expressed genes during erythropoiesis (Pishesha et al., 2014). These differences include arginase isoforms, which are the key enzymes in CECs-mediated immunosuppression (Cui et al., 2016; Elahi et al., 2013; Grzywa, Sosnowska, et al., 2021). Finally, CECs features may differ between mouse strains and may be affected by the microbiome and other physiological and environmental factors (S. Shahbaz et al., 2018). All these factors must be taken into account in the studies on CECs role under physiologic and pathologic conditions.

An important unresolved issue is whether (and how) the immunosuppressive properties of CECs are regulated by cytokines and other factors. CECs primed with TLR agonists secrete more TGF-β, enhancing their immunosuppressive phenotype (S. Shahbaz et al., 2018). Moreover, hepatic mononuclear cells from rhesus rotavirus (RRV)-infected neonatal mice compared with healthy neonates more potently inhibits TNF-α production by adult mononuclear cells (L. Yang et al., 2020). CECs isolated from IL-6 knock-out mice failed to induce polarization of T-cells into T_H2 lymphocytes, suggesting that proinflammatory IL-6 may activate the CECs phenotype (Rincon et al., 2012). Moreover, CECs stimulation with heat-killed bacteria or TLR-1 agonists leads to the increased production of TGF-β (S. Shahbaz et al., 2018). In macrophages, TLR agonists induce ARG1 and impair effective immunity against intracellular pathogens (El Kasmi et al., 2008). The potentiation of the immunosuppressive phenotype of mesenchymal stem cells (MSCs) is induced by priming with IFN-γ (D. S. Kim et al., 2018). On the other side, immunosuppressive properties of myeloid cells are regulated by T_H2-type cytokines, IL-10, TGF-β as well as hypoxia, prostanoids, and β2-adrenergic receptor (β2AR)-agonists (Grzywa et al., 2020). In neonates, MDSCs are activated by lactoferrin (Y.-M. He et al., 2018). Uncovering more details of these molecular mechanisms will help in understanding the regulatory mechanisms of the immunosuppressive phenotype of CECs and will explain differences in the features and functions of CECs in different conditions.

The most important questions refer to the role of CECs in the regulation of immune response in humans. CECs are enriched in human neonates, pregnant women, cancer patients, and patients infected with HIV or SARS-CoV-2. Still, only partial evidence for the immunoregulatory role of CECs was provided, mainly due to more complex experimental settings and limitations resulting from ethical issues. Nonetheless, it was shown that human CECs have immunosuppressive effects *ex vivo*. Moreover, it was found that anemic patients have higher EBV copy numbers and decreased numbers of LMP2- and EBNA1-specific $CD8^+$ T-cells in PBMC (L. Zhao et al., 2018). The role of CECs in the complex net of interaction *in vivo* remains elusive. It is also unknown what is the role of CECs in other conditions that are associated with anemia and CECs expansion, including chronic kidney disease (Babitt & Lin, 2012), chronic heart failure (Opasich et al., 2005), chronic obstructive pulmonary disease (Boutou, Hopkinson, & Polkey, 2015), and cystic fibrosis (Fischer, Simmerlein, Huber, Schiffl, & Lang, 2007). Moreover, since neonatal CECs promote polarization of immune response towards T_H2 (Rincon et al., 2012), it is interesting whether CECs contribute to the pathogenesis of chronic T_H2-inflammatory diseases, including asthma and allergy (J. A. Walker & McKenzie, 2018).

Whether modulation of the immunoregulatory properties of CECs is possible in the clinical setting remains unknown. Nonetheless, several agents were reported to promote CECs expansion or CECs differentiation. Moreover, multiple therapeutic strategies modulate erythropoiesis to rescue anemia. However, whether and how these therapies influence CECs-mediated immune regulation remains undetermined. Emerging evidence demonstrates a key role of CECs in various diseases. Thus, the development and clinical testing of therapeutic strategies that rely on the modulation of CECs is of great clinical interest.

7. Conclusions

The experimental and clinical evidence accumulated over the recent years has profoundly changed our view on $CD71^+$ erythroid cells from mere precursors of oxygen transporting cells to cells with a variety of functions, including potent regulation of immune response in different stages of life. Further research on erythroid cells may contribute to a better understanding of the pathogenesis of many diseases and may uncover novel therapeutic targets.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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Review

Tumor Immune Evasion Induced by Dysregulation of Erythroid Progenitor Cells Development

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Simple Summary: Tumor immune evasion is one of the hallmarks of tumor progression that enables tumor growth despite the activity of the host immune system. It is mediated by various types of cells. Recently, immature red blood cells called erythroid progenitor cells (EPCs) were identified as regulators of the immune response in cancer. EPCs expand in cancer as a result of dysregulated erythropoiesis and potently suppress the immune response. Thus, targeting dysregulated EPC differentiation appears to be a promising therapeutic strategy.

Abstract: Cancer cells harness normal cells to facilitate tumor growth and metastasis. Within this complex network of interactions, the establishment and maintenance of immune evasion mechanisms are crucial for cancer progression. The escape from the immune surveillance results from multiple independent mechanisms. Recent studies revealed that besides well-described myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs) or regulatory T-cells (Tregs), erythroid progenitor cells (EPCs) play an important role in the regulation of immune response and tumor progression. EPCs are immature erythroid cells that differentiate into oxygen-transporting red blood cells. They expand in the extramedullary sites, including the spleen, as well as infiltrate tumors. EPCs in cancer produce reactive oxygen species (ROS), transforming growth factor β (TGF-β), interleukin-10 (IL-10) and express programmed death-ligand 1 (PD-L1) and potently suppress T-cells. Thus, EPCs regulate antitumor, antiviral, and antimicrobial immunity, leading to immune suppression. Moreover, EPCs promote tumor growth by the secretion of growth factors, including artemin. The expansion of EPCs in cancer is an effect of the dysregulation of erythropoiesis, leading to the differentiation arrest and enrichment of early-stage EPCs. Therefore, anemia treatment, targeting ineffective erythropoiesis, and the promotion of EPC differentiation are promising strategies to reduce cancer-induced immunosuppression and the tumor-promoting effects of EPCs.

Keywords: immune evasion; erythroid progenitor cells; CD71+ erythroid cells; erythropoiesis; anemia; Ter-cells; ineffective erythropoiesis

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1. Introduction

Cancer immunotherapy has strongly changed the therapeutic landscape in clinical oncology, leading to significant improvements in cancer patients survival [1]. However, despite the induction of durable responses in an unprecedented percentage of cancer patients, the majority still do not respond to the treatment and eventually progress to refractory disease. There are several defined causes of immunotherapy resistance, including low tumor mutational burden [2], impaired antigen presentation by the major histocompatibility complex (MHC) proteins [3], loss of interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) pathway genes [4,5], as well as the development of immunosuppressive tumor microenvironment (TME) [6,7].

TME is composed of many types of cells that regulate tumor growth and progression [8]. The role of regulatory T-cells (Tregs) [9], myeloid-derived suppressor cells (MDSCs) [10], tumor-associated macrophages (TAMs) [11], tumor-associated neutrophils (TANs) [12], and cancer-associated fibroblasts (CAFs) [13] in the regulation of anti-tumor immune response has been established by many years of research (Table 1). Recent reports point to another population of cells, i.e., erythroid progenitor cells (EPCs), that regulate local and systemic immunity in cancer. These cells use similar mechanisms to immune cells and are crucial in the regulation of immune response and cancer progression.

Table 1. Immunomodulatory cells in cancer and their mechanisms of immune regulation.

Cells	Mechanisms	Effects	Ref
Regulatory T-cells (Tregs)	IL-10	T-cell suppression	[14]
	IL-2 consumption	T-cell suppression	[15]
	COX-2 and PGE2	T-cell suppression	[16]
	Adenosine	T-cell suppression	[17]
Myeloid-derived suppressor cells (MDSCs)	ARG1	T-cell suppression	[18]
	IDO	T-cell suppression Tregs induction NK cell suppression	[19,20]
	PD-L1/PD-1	T-cell suppression	[21]
	IL-10	Tregs induction	[22]
	TGF-β	Tregs induction	[22]
	CD40/CD40L	Tregs activation	[23]
	Depletion of cystine and cysteine	T-cell suppression	[24]
	ROS	T-cell suppression	[25]
	Free radical peroxynitrite	Resistance to cytotoxic T-cells	[26]
Tumor associated macrophages (TAMs)	PD-L1/PD-1	Decreased phagocytosis	[27]
	ARG1	T-cell suppression	[28]
	IL-10	T-cell suppression	[29]
	IL-1β	MDSC infiltration Induction of the protumor phenotype	[30,31]
	IL-12	Induction of T-cell response	[32]
	TNF-α	Induction of anti-tumor response	[33]
Tumor associated neutrophils (TANs)	ARG1	T-cell suppression	[18,28]
	NOS	T-cell suppression T-cell apoptosis	[34,35]
	PD-L1/PD-1	T-cell suppression	[36]
Cancer associated fibroblasts (CAFs)	PD-L1/PD-1	T-cell suppression	[37]
	FasL, PD-L2	T-cell suppression	[38]
	IL-6	Induction of PD-L1$^+$ TANs	[39]
	Chemokines	MDSC infiltration	[40]
Erythroid progenitor cells (EPCs)	ROS	T-cell suppression	[41,42]
	IL-10	T-cell suppression	[42]
	PD-L1/PD-1	T-cell suppression	[43]
	TGF-β	T-cell suppression	[42]

ARG1—arginase 1, COX-2—cyclooxygenase-2, FasL—Fas ligand (CD95L, CD178), IDO—Indoleamine-pyrrole 2,3-dioxygenase, IL—interleukin, NK—natural killer, NOS—nitric oxide synthase, PD-1—programmed cell death 1, PD-L1—programmed death-ligand 1, PGE2—Prostaglandin E_2, ROS—reactive oxygen species, TGF-β—transforming growth factor β, TNF-α—tumor necrosis factor α.

In this review, we discuss the role of the dysregulation of erythropoiesis by cancer cells to induce immune evasion and promote cancer progression.

2. Regulation of Erythropoiesis

The differentiation of hematopoietic stem cells (HSCs) to erythroid cells is a stepwise process strictly regulated by multiple intrinsic and extrinsic factors (Table 2), which results in the production of over 2×10^{11} red blood cells (RBCs) per day and allows for the maintenance of erythroid homeostasis [44–48]. This complex net of interactions provides adequate production of RBCs depending on the body's needs. Insufficient oxygen supply to the peripheral tissues resulting in hypoxia is a key trigger of increased erythropoiesis, which is regulated by the increased production of erythropoietin (EPO) in the kidney peritubular fibroblasts and liver interstitial cells and hepatocytes [49].

Table 2. Regulation of erythropoiesis.

Factor	Role in Erythropoiesis	Dysregulation in Cancer	References
SCF	Growth factors regulating early stages of erythropoiesis	Production in TME	[50,51]
G-CSF		Increased serum concentration	[52]
IL-3			[53]
EPO	Growth factors regulating late stages of erythropoiesis	Increased serum concentration	[54]
GDF11		Production in TME	[55]
Activin A		Production in TME	[56]
GATA1	Crucial TFs regulating erythropoiesis	Decreased expression in EPCs in cancer	[57–59]
STAT5		Increased in EPCs in MPNs Decreased in EPCs in iron deficiency	[60,61]
MCL-1 BCL-xL HSP70	Survival factors for erythroid cells		
TGF-β	Negative regulators of erythropoiesis	Production in TME Increased concentration	[62]
SMAD signaling		Increased level in EPCs in cancer	[62]
FasL		High expression on cancer cells	[59,63]
Fas		Increased level in EPCs in cancer	[59,63]
Vitamin B12	Essential vitamins, trace elements, and iron-metabolism proteins	Decreased in a subset of patients	[64]
Folic Acid		Decreased in a subset of patients	[64]
Copper		Increased concentration	[65]
Iron		Decreased in a subset of patients	[66]
Ferritin		Decreased or increased	[66]
Transferrin		Decreased in a subset of patients	[66]
Ferroportin		Decreased expression	[67]
Hepcidin		Increased concentration	[68]

MPN—myeloproliferative neoplasm, TF—transcription factor, TME—tumor microenvironment.

HSCs reside in a unique niche that is created and regulated by various cell types, growth factors, and chemokines [69]. The commitment of HSCs to erythroid lineage begins with the differentiation to a multipotent megakaryocyte–erythroid progenitor cell (MEP), followed by a bust-forming unit-erythroid (BFU-E) and colony-forming unit-erythroid

(CFU-E). During terminal erythropoiesis, CFU-E differentiates into proerythroblasts, basophilic erythroblasts, polychromatic erythroblasts, and orthochromatic erythroblasts that expel their nuclei and generate reticulocytes [70]. Reticulocytes are released to the circulation, where they mature to RBCs within a few days. In healthy humans, erythroblasts represent about 20–30% of nucleated cells in the bone marrow [71,72].

The first steps of erythropoiesis are regulated by hematopoietic cytokines including stem cell factor (SCF), interleukin 3 (IL-3), insulin-like growth factor 1 (IGF-1), and granulocyte-macrophage colony-stimulating factor (GM-CSF) [73–75]. Further erythroid cell differentiation is regulated mainly by EPO [45,76,77]. The impairment of steady-state erythropoiesis triggers stress erythropoiesis that maintains erythroid homeostasis. Stress erythropoiesis is regulated by additional factors including hypoxia, bone morphogenetic protein 4 (BMP4), Hedgehog, glucocorticoids, and peroxisome proliferator-activated receptor α (PPAR-α) [78,79].

Cell lineage specification is regulated through defined transcriptional programs. It is well established that a zinc-finger transcription factor GATA1 is a master transcriptional regulator of differentiation toward erythroid lineage [80]. It is induced at the very early stages of erythropoiesis and is responsible for the regulation of all known erythroid genes [80]. Thus, GATA1 is necessary for erythropoiesis and its lack cannot be compensated as *Gata1* knockout mice fail to generate mature RBCs [81]. Therefore, the cleavage of GATA1 is a key mechanism of erythropoiesis regulation. GATA1 is cleaved by caspases, primarily caspase-3, which is activated in the nucleus of terminally differentiating erythroid cells to enable maturation to RBCs [80,82,83]. Nonetheless, the activation of caspases and GATA1 degradation at earlier stages of differentiation induces differentiation arrest and apoptosis. Therefore, GATA1 is protected from degradation in early-stage EPCs by EPO signaling, $p19^{INK4d}$ cyclin-dependent kinase inhibitor, and HSP70 protein chaperone [76,82,84].

3. Erythroid Progenitor Cells as Immune Regulators

EPCs are predominantly erythroblasts and reticulocytes that differentiate into mature RBCs. EPCs are characterized by the expression of transferrin receptor 1 (CD71) and glycophorin A (CD235a) in humans, and CD71 and TER119 in mice [85]. For many years, EPCs were considered to be solely erythrocytes precursors, without any other significant functions in the human body. However, recent studies revealed the importance of the previously neglected role of EPCs.

Immunomodulatory functions of EPCs were described for the first time in neonates, which are characterized by a physiological enrichment of EPCs in extramedullary sites, including the spleen, liver, and peripheral blood [86]. Neonatal EPCs express arginase-2 (ARG2), $_{L}$-arginine degrading enzyme, and secrete transforming growth factor β (TGF-β), leading to the suppression of cytokine production by myeloid cells [86] and the promotion of T-cell differentiation toward Tregs cells [87]. Despite initial hypotheses that only neonatal EPCs have significant immunoregulatory properties [86], further research expanded our knowledge and revealed that these properties are a general feature of EPCs. The regulation of immune cells by erythroid cells was described for EPCs induced by pregnancy [88], systemic inflammation [89], HIV infection [90], COVID-19 [91], and anemia [92].

EPCs in different conditions modulate immune response via various mechanisms (Table 3). Recent studies also demonstrated that EPCs that expand during cancer progression possess significant immunomodulatory properties and promote tumor growth.

Table 3. Mechanisms of immunomodulatory functions of EPCs.

Source	Mechanism	Effect	Mouse	Humans	Ref.
Neonates	ARG2	↓cytokine production bymyeloid cells	+	+	[86,93]
	TGF-β	↑Tregs differentiation	+	+	[87]
	ROS	↓cytokine production bymyeloid cells ↓cytokine production by T-cells	-	+	[94]
	PD-1/PD-L1	↓cytokine production by T-cells	+	+	[88]
Pregnancy	ARG2	↓cytokine production bymyeloid cells	+	+	[24,93]
	TGF-β	↑Tregs differentiation	n.d.	+	[93]
	ROS	↓cytokine production bymyeloid cells ↓cytokine production by T-cells	n.d.	+	[93]
	PD-1/PD-L1	↓cytokine production by T-cells	+	+	[88]
Inflammatory diseases	EPCs phagocytosis	↓cytokine production byred pulp macrophages	+	n.d.	[89]
HIV-infected patients	ROS	↑HIV replication in T-cells ↑HIV trans-infection	n.d.	+	[90]
COVID-19 patients	ARG1	↓cytokine production by T-cells ↓T-cell proliferation	n.d.	+	[95]
	ARG2	↓cytokine production by T-cells ↓T-cell proliferation	n.d.	+	[95]
	ROS	↓cytokine production by T-cells ↓T-cell proliferation	n.d.	+	[95]
Anemia	ARG1	↓cytokine production by T-cells ↓T-cell proliferation	-	+	[92]
	ARG2	↓cytokine production by T-cells ↓T-cell proliferation	+	+	[92]
	ROS	↓cytokine production by T-cells ↓T-cell proliferation	+	+	[92]
Cancer	TGF-β	↓T-cells proliferation↓cytokine production by T-cells	n.d.	+	[42]
	ROS	↓T-cell proliferation↓cytokine production by T-cells	+	+	[41,42]
	PD-L1/PD-1	↓cytokine production by T-cells	+	+	[43]
	IL-10	↓T-cell proliferation↓cytokine production by T-cells	n.d.	+	[42]

↑—promoted, ↓—suppressed, n.d.—no data, - — no role, +—reported mechanism.

4. The Role of Erythroid Progenitor Cells in Cancer

Cancer progression is associated with the suppression of immune response that enables tumor growth and leads to increased susceptibility to infections in patients with advanced disease [96]. It is caused by the remodeling of the immune cell landscape that impairs not only a local anti-tumor response, but also systemic antibacterial and antiviral immunity [97]. Cancer cells and tumor-associated stromal cells reprogram hematopoiesis and promote the polarization of immune cells toward suppressive phenotypes. In cancer, the spleen is a key organ of extramedullary hematopoiesis and is responsible for the production of suppressive immune cells [98]. It is well established that cancer dysregulates hematopoiesis to generate MDSCs that suppress antitumor response [10,99]. However, during tumor progression, immune cells in the murine spleen are vastly outnumbered by another type of cells, EPCs [41,62]. Moreover, substantial EPC expansion is observed in the peripheral blood and the liver of tumor-bearing mice and cancer patients. EPCs also infiltrate murine and human tumors, and their frequency in TME is much higher than that of MDSCs or Treg cells [41–43,62].

Similar to neonatal counterparts, EPCs induced by cancer were found to potently suppress immune response (Figure 1). The proliferation and cytotoxicity of $CD8^+$ T-cells, as well as the proliferation of $CD4^+$ T-cells and T_H1 differentiation, are inhibited by tumor-

induced murine EPCs [41]. In murine models, the depletion of EPCs with anti-CD71 antibody inhibits tumor growth [43]. Likewise, EPCs isolated from peripheral blood of cancer patients or human tumors potently suppress T-cell proliferation and the production of IFN-γ via paracrine and direct cell-to-cell contact manner [41,42].

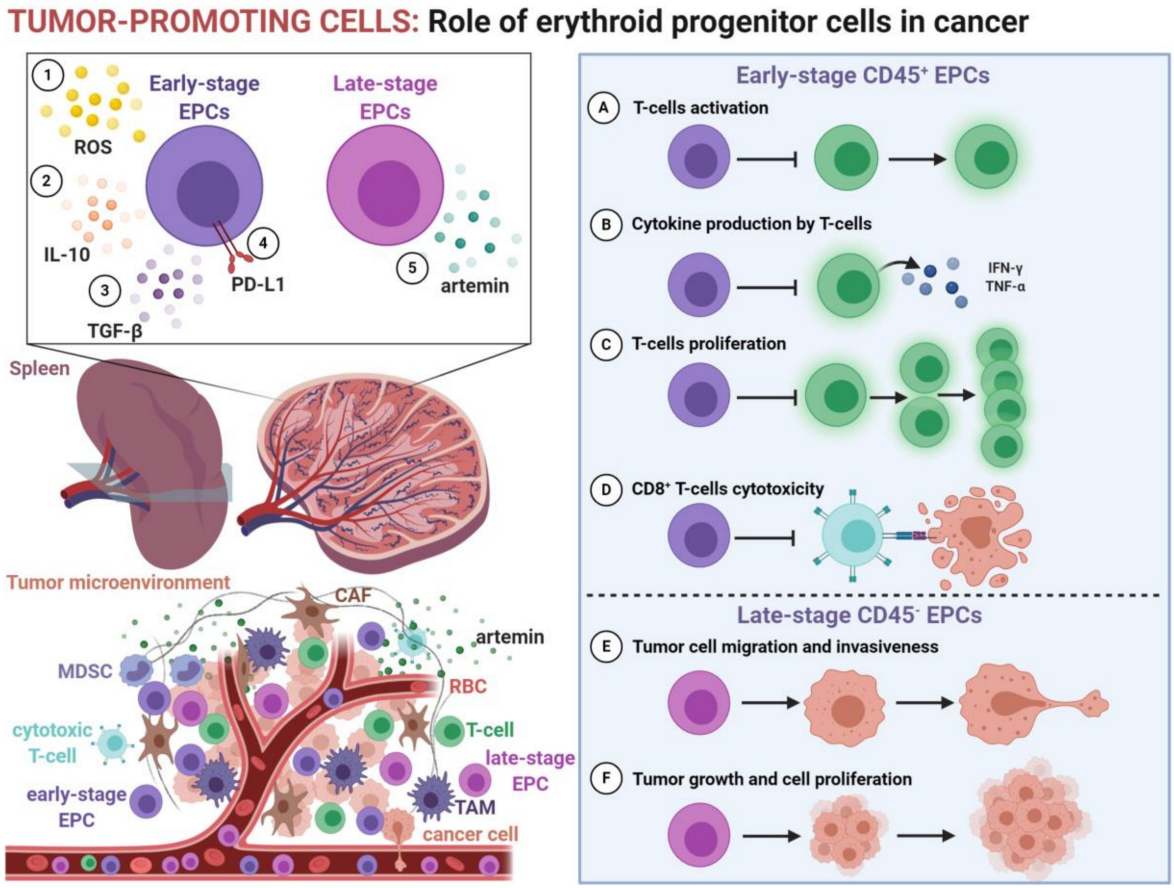

Figure 1. The role of erythroid progenitor cells (EPCs) in cancer. During disease progression, EPCs expand in the extramedullary sites, including the spleen. Moreover, EPCs are abundant in the peripheral blood of cancer patients and infiltrate the tumor microenvironment. Early-stage CD45$^+$ EPCs use (**1**) reactive oxygen species (ROS), (**2**) interleukin-10 (IL-10), (**3**) transforming growth factor β (TGF-β), and (**4**) programmed death-ligand 1 (PD-L1) to modulate the immune response. EPCs inhibit (**A**) T-cell activation, (**B**) production of interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α), (**C**) T-cell proliferation, and (**D**) cytotoxicity of CD8$^+$ T-cells. More mature CD45$^-$ EPCs regulate cancer progression by (**5**) secretion of a neurotropic factor, artemin. These late-stage EPCs, called Ter-cells, promote (**E**) tumor cell migration and invasiveness as well as (**F**) tumor growth and cell proliferation.

Erythropoiesis is a continuous process by which erythroid cells change their characteristics to differentiate into specialized oxygen-transporting RBCs. The transcriptional profile [41,42,100–107] and cell proteome [106,108–111] substantially change during erythroid maturation. Growing evidence indicates that the role of EPCs in cancer changes with maturation (Table 4). During differentiation, EPCs lose expression of CD45, a pan-leukocyte marker [112]. Therefore, CD45 may be used as a marker of early-stage EPCs [41]. In tumor-bearing mice, CD45$^+$ EPCs constitute over 40% of EPCs and are predominantly responsible for the immunosuppressive effects of EPCs [41]. These early-stage EPCs were found to potently suppress T-cells, in contrast to more mature CD45$^-$ erythroid cells [41,62]. In mice, the suppressive capacity of CD45$^+$ EPCs falls between Tregs and MDSCs [41], but in humans, CD45$^+$ EPCs are even more potent immunosuppressors than both Tregs and MDSCs [42].

Table 4. Differences in immune-related mediators between early-stage and late-stage EPCs [41–43,62].

Feature	Early-Stage EPCs ($CD45^+$)	Late-Stage EPCs ($CD45^-$)
ROS level	↑	↓
IL-10	↑	↓
TGF-β	↑	↓
ROS pathway	↑	↓
IL-10 pathway	↑	↓
TGF-β pathway	↑	↓
PD-1/PD-L1	n.d.	n.d.
ARG2	n.d.	n.d.

↑—increased, ↓—decreased, n.d.—no data.

Transcriptional analysis revealed a close resemblance between $CD45^+$ EPCs and MDSCs and enrichment in the reactive oxygen species (ROS) pathway in $CD45^+$ EPCs [41]. Early stage $CD45^+$ EPCs have upregulated expression of NADPH oxidase (NOX) family members [41,42], crucial ROS-generating NADPH oxidases [113]. As a result, they have increased ROS levels compared to $CD45^-$ counterparts [41,42]. Although ROS are required for T-cell activation, excessive ROS levels impair T-cell immunity [114]. Thus, ROS are a well-established mechanism of T-cell suppression by MDSCs [115]. Similarly, EPCs were found to suppress T-cells in a ROS-dependent manner. Apocynin, an NADPH oxidase inhibitor, as well as N-acetylcysteine, an ROS scavenger, diminished the suppressive effects of EPCs [41,42,116]. The infiltration of EPCs to TME probably contributes to the high ROS levels triggering oxidative stress. High ROS levels in TME impair the functions of tumor-infiltrating lymphocytes and dendritic cells, while promoting the recruitment and accumulation of Tregs and MDSCs [117]. However, ROS inhibition did not restore T-cell functions completely [41]. Further studies revealed that $CD45^+$ EPCs induced by cancer use multiple additional immunoregulatory mechanisms, including IL-10, TGF-β, and PD-1/PD-L1 [42,43]. Thus, the immunoregulatory functions of EPCs rely on many mechanisms identified for immunosuppressive cells in TME (Table 1).

It seems that EPCs impair both anti-tumor immunity and systemic immune response to pathogens. In mice, $CD45^+$ EPCs potently inhibit the antigen-specific response of tumor-infiltrating cytotoxic T-cells [41]. The transfer of $CD45^+$ EPCs into tumor-bearing mice accelerated tumor growth [41], confirming the suppression of anti-tumor response by EPCs. Likewise, $CD45^+$ EPCs suppressed proliferation and cytokine production by tumor-infiltrating T-cells from cancer patients [42].

Importantly, the expansion of EPCs in cancer is most remarkable in the spleen (Table 3), which is the largest secondary lymphoid organ involved in the development of systemic immune response to blood-borne antigens [118]. Similarly to neonates that are also characterized by the expansion of EPCs in the spleen [86], adult tumor-bearing mice have increased susceptibility to viral and bacterial infections compared to healthy mice [41]. Ex vivo, EPCs suppressed antigen-specific cytotoxic T-cells [41]. In vivo, the depletion of EPCs with anti-CD71 antibody rescued the suppressed proliferation of virus-specific $CD8^+$ T-cells, restoring anti-viral immunity in tumor-bearing mice while the transfer of $CD45^+$ EPCs potentiated the suppression of immune response [41]. In humans, anemic cancer patients have higher EPC numbers and increased Epstein–Barr viral (EBV) load due to suppressed anti-viral immunity [41]. These latter findings suggest that as in mice, EPCs suppress a systemic immune response in cancer patients.

It was suggested that the suppressive properties of EPCs may be restricted to stress erythropoiesis-induced EPCs. However, $CD45^+$ EPCs isolated from the spleen, liver as well as bone marrow of the tumor-bearing mice suppressed T-cells to a similar extent [41]. Moreover, steady-state EPCs from human bone marrow also suppress T-cells [92]. Nonetheless, there are significant differences in the expression of immunomodulatory molecules, including PD-L1, between EPCs isolated from the bone marrow, spleen, and TME [43]. This suggests that the differences in EPC properties may result from stimulation with some

factors, presumably cytokines or TME components, which may enhance or diminish the immunosuppressive properties of EPCs.

Tumor-Promoting Role of CD45$^-$ EPCs

The majority of tumor-induced EPCs are CD45$^-$ [41,62]. While early-stage CD45$^+$ EPCs potently suppress the immune response, more mature CD45$^-$ EPCs lack this capacity (Table 4). However, the transfer of CD45$^-$ EPCs also promotes tumor growth and decreases the survival of tumor-bearing mice [62].

These tumor-induced splenic CD45$^-$ EPCs were called Ter-Cells [62]. They are a population of late-stage EPCs as they have a high nucleus/cytoplasm ratio, scant cytoplasm, dense chromatin, and few organelles, as well as lacking the expression of the major histocompatibility complex (MHC) class I [62], a marker of mature erythroid cells [119]. In contrast to early-stage EPCs, CD45$^-$ EPCs do not influence T-cell proliferation, dendritic cell activation, and cytokine secretion as well as fail to induce Tregs [62]. Moreover, CD45$^-$ EPCs have very low or undetectable levels of immune-related mediators, including IL-10, TGF-β, IL-4, prostaglandin E2 (PGE-2), and ROS [41,42,62]. Therefore, CD45$^-$ EPCs do not promote tumor growth by inhibiting the anti-tumor response.

Transcriptional analysis revealed marked overexpression of a neurotrophic factor artemin in CD45$^-$ EPCs [62]. The physiological role of artemin involves the regulation of neuronal survival, maintenance, and differentiation [120]. Artemin also has protumorigenic activity and promotes cancer cell survival, proliferation, migration, and invasiveness [62,121–123]. In murine models, it promotes tumor growth and accelerates disease progression [62]. Artemin activates the glial cell-derived neurotrophic factor (GDNF) family receptor alpha-3 (GFRα3) and its co-receptor RET on cancer cells. Downstream signaling of artemin promotes the phosphorylation of extracellular signal-regulated kinase (ERK), protein kinase B (AKT), and caspase-9, promoting proliferation and invasiveness, while preventing apoptosis in tumor cells, even induced by the therapy [62]. The same effects are exerted by artemin-secreting CD45$^-$ EPCs. Thus, the reduction in EPC expansion reduces the increase in the artemin concentration in the serum and decreases tumor growth [62]. Artemin-expressing CD45$^-$ EPCs were also detected in the spleens of patients with hepatocellular carcinoma (HCC) and pancreatic ductal adenocarcinoma (PDAC) [62,123], which suggests their role in cancer patients.

Moreover, these differences are also manifested by their localization. While immunomodulatory early-stage EPCs accumulate in the spleen and intensively infiltrate TME, tumor-promoting late-stage EPCs are detected mainly in the spleen where they secrete artemin into circulation [41,42,62,123]. Collectively, early-stage and late-stage EPCs differ substantially regarding their gene expression profile, level of immunomodulatory mediators, and their role in promoting cancer progression (Table 5).

Table 5. Different role of early-stage and late-stage EPCs in cancer [41–43,62,116,123].

Process	Early-Stage EPCs (CD45$^+$)	Late-Stage EPCs (CD45$^-$)
T-cell proliferation	↓ suppressed	↔ no effect
Production of IFN-γ by T-cells	↓ suppressed	↔ no effect
Production of TNF-α by T-cells	↓ suppressed	↔ no effect
CD8$^+$ T-cells cytotoxicity	↓ suppressed	↔ no effect
Dendritic cells activation	n.d.	↔ no effect
Production of IL-6 and IL-12 by dendritic cells	n.d.	↔ no effect
Tregs induction	n.d.	↔ no effect
Anti-tumor immune response	↓ suppressed	↔ no effect
Activation of signaling pathways in tumor cells	n.d.	↑ promoted
Regulation of cancer cell metabolism	↑ promoted	n.d.
Tumor cells proliferation	n.d.	↑ promoted
Tumor cells invasiveness	n.d.	↑ promoted
Tumor growth	↑ promoted	↑ promoted

↑—promoted, ↓—suppressed, ↔—no effect, n.d.—no data.

5. Expansion of Erythroid Progenitor Cells

EPCs predominantly occupy niches in the bone marrow where they differentiate into RBCs. However, EPC frequency in the steady-state bone marrow is relatively low, especially when compared to mature erythrocytes. In healthy individuals, EPCs are not detected in extramedullary sites, besides a small percentage of reticulocytes in peripheral blood [124]. However, under several conditions, EPCs substantially expand in the bone marrow as well as in extramedullary sites.

The expansion of EPCs is physiological in neonates and during pregnancy [88,93,125,126]. In neonates, EPCs accumulate in the extramedullary sites due to insufficient bone marrow erythropoiesis during the first days of life [125,126]. During pregnancy, extramedullary erythropoiesis enables the production of sufficient numbers of erythrocytes [88]. Moreover, the expansion of EPCs is also observed in anemic patients as a mechanism increasing oxygen transport [92,127]. Recent studies revealed that extramedullary erythropoiesis and EPC expansion may also be a part of the inflammatory response [128–130]. A recent analysis of blood transcriptome revealed that the signature of immature erythroid cells is also associated with severe respiratory syncytial virus (RSV) infection, pharmacological immunosuppression, and late-stage cancer [131].

In tumor-bearing mice, EPCs expand during tumor progression in many extramedullary organs (Table 6), predominantly the spleen, liver, and peripheral blood, as well as infiltrate tumors [41,43,62]. In humans, EPCs were detected in the spleen, TME, and peripheral blood of cancer patients [41,62,123,131]. In general, anemia severity correlates with the frequency of EPCs [41]. In some cases, the expansion of EPCs in peripheral blood is so substantial that it causes a so-called leukoerythroblastic reaction [132,133].

Table 6. The frequency of EPCs in tumor-bearing mice and cancer patients.

Organ	Mice		Humans		Ref.
	Healthy	Tumor-Bearing	Healthy	Cancer Patient	
Peripheral blood	5%	60%	0.13%	2–4.25%	[41,42]
Spleen	5%	20–50%	0.02%	0.15%	[41,43,62,123]
Bone marrow	15–20%	55%	14%	n.d.	[41,43,134]
Liver	10%	2–30%	2.5%	10%	[41,42,62]
Lymph node	1%	1%	n.d	n.d	[41,62]
Tumor	-	2–10%	-	10%	[41,42,62]

n.d.—no data, - — not applicable.

6. Cancer-Induced Dysregulation of Erythropoiesis

The main cause of EPC expansion is the increase in EPO concentrations in response to anemia. However, the mechanisms of EPC induction by cancer are complex and rely on multiple components that dysregulate erythropoiesis (Figure 2), leading to ineffective erythropoiesis, characterized by erythroid differentiation arrest and increased apoptosis of erythroid cells, and is a feature of various diseases, including β-thalassemia [135]. Importantly, emerging evidence suggests that cancers not only induce potent EPC expansion, but also arrest their development at the earliest stages of differentiation. This leads to the suppression of immune response driven by EPCs, which are potent but physiologically transient immunosuppressors [92].

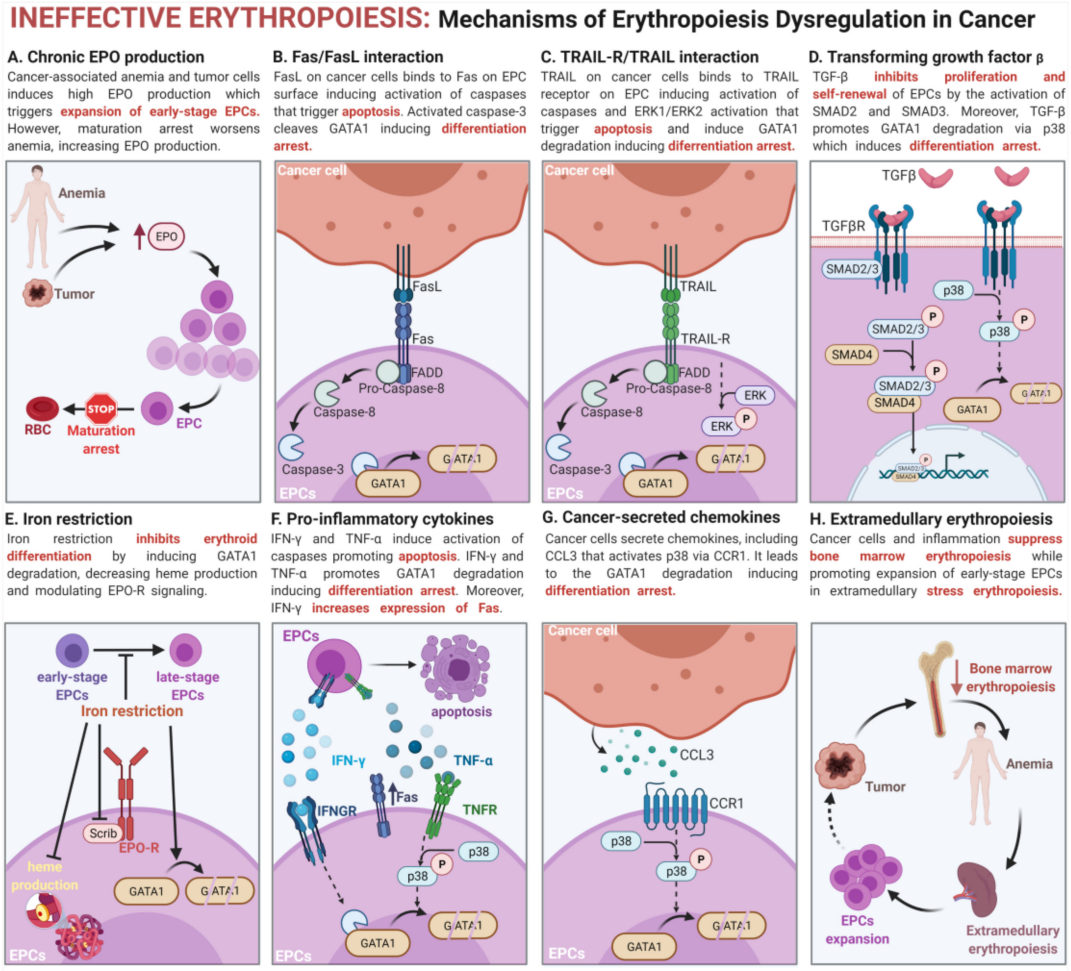

Figure 2. Mechanisms of erythropoiesis dysregulation in cancer. Expansion of early-stage EPCs is caused by (**A**) chronic erythropoietin (EPO) production. However, EPCs are unable to generate mature red blood cells (RBCs) due to increased apoptosis and differentiation arrest. EPCs apoptosis is triggered by (**B**) FasL/Fas and (**C**) TRAIL-TRAIL-R interaction between EPCs and cancer cells. Differentiation arrest of early-stage EPCs is caused by (**D**) transforming growth factor β (TGF-β), (**E**) iron restriction, (**F**) pro-inflammatory cytokines, and (**G**) cancer-secreted chemokines. Inhibited maturation is an effect of GATA1 degradation mediated by caspase-3 or p38 activation. (**H**) Bone marrow steady-state erythropoiesis is suppressed by inflammation and triggers stress erythropoiesis and expansion of EPCs in extramedullary sites.

6.1. Dysregulation of Hematopoietic Stem and Progenitor Cells Differentiation

The dysregulation of erythropoiesis by cancer begins at the first stage of hematopoiesis. Malignant hematological cells suppress hematopoietic stem and progenitor cells (HSPCs) in the bone marrow, limiting their differentiation and inducing a quiescent state. This suppression is mediated by various mechanisms, including SCF [51], TNF-α [136], arginase [137], TGF-β [138–140], and stanniocalcin 1 [141]. On the other site, HSPCs are enriched in the extramedullary sites, predominantly the spleen and the circulation of cancer patients, and are myeloid-biased to generate suppressive myeloid cells [142–146]. Cytokines and growth factors secreted by cancer cells and TME force hematopoiesis to the generation and maintenance of immunosuppressive cells that promote tumor growth [144]. Increased numbers of HSPCs in the circulation correlate with advanced tumor stage and decreased progression-free survival in cancer patients [142,146]. In cancer, TNF-α secreted by activated T-cells increases the proliferation of HSPCs and induces emergency myelopoiesis in the bone marrow [143]. Nonetheless, despite strong myeloid polarization, HSPCs in the extramedullary sites, including the spleen, also exhibit increased capacity to differentiate into BFU-E in tumor-bearing mice compared to healthy mice [144].

6.2. Disruption of Hematopoietic Stem and Progenitor Cells Niche

Cancer cells also directly impair HSPCs' maintenance and differentiation by disrupting their niche, resulting in the loss of quiescence and stemness of HSPCs [51,147]. This phenomenon is the most prominent for hematological malignancies that primarily develop in the bone marrow and outcompete native HSPC niches [148]. Nonetheless, solid tumors were also reported to disrupt the HSPCs' niche. Melanoma cells secrete vascular endothelial growth factor (VEGF), which reduces available vascular niches in bone marrow, promoting HSC mobilization [149]. Moreover, tumor-secreted exosomes educate bone marrow cells toward a pro-metastatic phenotype [150] and promote the production of pro-inflammatory cytokines by mesenchymal stem cells to support cancer cell growth while suppressing HSPCs [151].

6.3. Suppression of Erythroid Differentiation of Hematopoiesis Stem Cells

The differentiation of HSCs is skewed towards myelopoiesis by cancer. Thus, the potential of erythroid differentiation of HSCs is commonly suppressed, especially in the bone marrow. EPC precursors, MEPs, are the most suppressed progenitor cells in the bone marrow of mice with hematological malignancies [57,58,136,140,152–154]. In plasma cell myeloma, malignant cell infiltration correlates negatively with hemoglobin concentration, but not with leukocytes or platelet counts, which suggests the selective impairment of erythropoiesis by malignant cells [155]. This suppression is partially compensated by the increased proliferation of early-stage EPCs in cancer [57]. Moreover, erythroid progenitors are activated in extramedullary sites, including the spleen [154].

6.4. Chronic Erythropoietin Production

EPC expansion is triggered primarily by EPO, which promotes the survival, proliferation, and differentiation of EPCs [156]. EPO induces the expansion of highly proliferative early-stage EPCs [157]. Normally, this response is rapid and EPO concentration quickly decreases after the induction of EPC expansion, which results in RBC generation [158]. However, when the erythropoietic response is insufficient to rescue anemia, EPO is produced constantly. This results in the substantial expansion of early-stage EPCs in the bone marrow as well as in the extramedullary sites. In cancer, EPO is secreted predominantly in response to tissue hypoxia resulting from anemia.

Moreover, tumors may directly trigger the upregulation of EPO production in a vascular endothelial growth factor (VEGF)-dependent mechanism. Vascular endothelial growth factor (VEGF) is a growth factor produced by malignant and stromal cells in TME to induce neovascularization, vessel remodeling [159], and to modulate antitumor immune response [160]. VEGF concentration is substantially increased in the plasma of cancer patients [161]. Importantly, VEGF stimulates EPO secretion by splenic stromal cells expressing platelet-derived growth factor receptor β (PDGFR-β) [162]. Increased VEGF concentration in plasma leads to the increased reticulocyte index in the circulation and expansion of early-stage EPCs in the bone marrow and the spleen [163].

6.5. Induction of Erythroid Cell Apoptosis

Another mechanism of erythropoiesis dysregulation is the direct induction of erythroid cell apoptosis by cancer cells. Apoptosis induced by death receptor Fas (CD95) and Fas ligand (FasL, CD95L, or CD178) interaction is a critical negative regulatory axis of erythropoiesis [76,82]. These negative signals can be overcome by high EPO concentrations that promote EPC expansion during erythropoietic stress response [164].

Cancer cells secrete multiple factors that induce the expression of death receptors on EPCs, increasing their susceptibility to apoptosis [59,63]. Thus, EPCs from cancer patients have significantly upregulated Fas receptor [59,63]. Moreover, ligands for cell death receptors are commonly overexpressed by malignant cells [59,63,165]. The activation of death receptors triggers the activation of caspases that cleave GATA1 transcription factors, leading to the maturation arrest or apoptosis of EPCs [166]. Maturation arrest

caused by GATA1 degradation results in the accumulation of EPCs at the earliest stages of differentiation [155,166]. Moreover, GATA1 downregulation decreases the induction of anti-apoptotic proteins, including Bcl-xL and Bcl-2 [167,168]. The enhanced loss of erythroid precursors due to apoptosis leads to compensatory mechanisms and, consequently, higher percentages of early erythroblasts in the bone marrow of cancer patients [59].

Similar to FasL, malignant cells have an increased level of TNF-related apoptosis-inducing ligand (TRAIL) [59,63,169]. EPCs are characterized by the physiological expression of TRAIL receptors [170]. Their stimulation by TRAIL on cancer cells induces differentiation arrest caused by the activation of caspases and the induction of ERK1/ERK2 signaling [82,170,171].

6.6. Transforming Growth Factor β

TGF-β is an important cytokine that promotes tumor growth and immune evasion [172]. Its concentration is substantially increased in the TME and serum of cancer patients [62,140,172–174]. Overactivation of the TGF-β pathway affects not only cells in TME, but also hematopoietic cells. Indeed, in cancer patients TGF-β signaling is the most dysregulated signaling pathway in HSPCs, which leads to impaired hematopoiesis, especially erythropoiesis [140]. In erythroid cells, TGF-β potently inhibits proliferation and self-renewal, but at a low concentration it may accelerate the differentiation of late-stage EPCs by promoting mitophagy [83,175–178].

TGF-β induces the maturation arrest of early-stage EPCs by noncanonical activation of p38, which in turn triggers GATA1 degradation [57,58,140]. Moreover, TGF-β activates SMAD2 and SMAD3 via the type III TGF-β receptor, which is transiently upregulated in early-stage EPCs [178]. Indeed, EPCs in tumor-bearing mice have overactivated SMAD2 and SMAD3 [62]. Accordingly, tumor-induced expansion of EPCs is substantially reduced in Smad3-deficient mice [62]. Moreover, EPC expansion may be prevented by the treatment with neutralizing antibody against TGF-β [62]. The ability to induce EPCs is decreased in mice bearing TGF-β-deficient tumor cells; however, not completely [62]. These findings confirm that TGF-β secreted by tumor cells and also by non-malignant cells in TME is a key factor inducing EPC expansion in cancer via SMAD signaling.

Another mechanism by which TGF-β impairs erythropoiesis involves IL-33, a member of the IL-1 superfamily of cytokines. Tumor-secreted TGF-β induces the expression of IL-33 in TME [179]. Indeed, an increased concentration of IL-33 has been reported in different types of cancer and often correlates with poor prognosis [180]. Notably, IL-33 potently inhibits the differentiation of EPCs at early stages by NF-κB activation and the inhibition of signaling pathways downstream of erythropoietin receptor (EPO-R) [181].

Other members of the TGF-β superfamily, including growth differentiation factor 11 (GDF11, also known as BMP11) and GDF15, have a similar role in the regulation of erythropoiesis. GDF11 induces the differentiation arrest of early-stage EPCs by the activation of SMAD2 and SMAD3 pathways, inhibiting terminal differentiation [182–184]. In myelodysplastic syndrome (MDS) patients, GDF11 serum concentration is negatively correlated with late erythropoiesis [185]. Erythropoiesis is also suppressed by GDF15, which modulates iron metabolism [186].

On the other side, some members of the BMP pathway, including BMP4, are crucial regulators of stress erythropoiesis and initiate the differentiation and expansion of EPCs, enabling erythropoietic response [128,187,188].

6.7. Iron Restriction

Iron is an important trace element required for many biological processes, including the heme synthesis [78,189]. Thus, its metabolism is regulated by multiple proteins including iron-transporting transferrin, iron-storing ferritin, and ferroportin responsible for iron export from the cell [190]. Absolute iron deficiency is detected in over 40% of cancer patients [191]. Notably, iron restriction selectively impairs erythroid cell differentiation, but not granulocytic nor megakaryocytic progenitors [61,192–194]. Iron is a metabolic

checkpoint that restrains the expansion of EPCs triggered by EPO in the case of insufficient iron availability. Iron restriction downregulates the crucial control element of the EPO receptor, Scribble, preventing further EPC maturation [61]. Moreover, iron control of EPC differentiation is mediated by an aconitase-associated regulatory pathway that compromises heme production and modulates EPO signaling [194]. This results in profound changes in the gene expression profile, including the downregulation of GATA1 and its target genes, leading to the impairment of EPC maturation with the differentiation arrest of early EPCs [192–195].

EPCs can obtain and concentrate iron with exceptional efficacy [196]. Nonetheless, cancer cells and nonmalignant cells in TME are also characterized by increased iron metabolism [197,198]. Cancer cells overexpress CD71 and compete with the EPCs for transferrin-bound iron [199]. Moreover, cells in TME, especially macrophages, accumulate iron, leading to its sequestration from EPCs and exaggerating iron deficiency [165].

6.8. Pro-Inflammatory Cytokine-Driven Erythropoiesis Impairment

Anemia of inflammation (also referred to as anemia of chronic disease) is associated with systemic inflammation, which is one of the hallmarks of cancer and is primarily caused by altered iron distribution [200,201]. Inflammation activates the inflammasome, which triggers enzymatic activation of caspases [202]. Inflammasome assembly in HSPCs leads to the GATA1 cleavage by caspase-1, which favors myelopoiesis over erythropoiesis and suppresses terminal erythropoiesis, leading to the maturation arrest of EPCs [203]. In mice expressing active $Kras^{G12D}$, the activation of inflammasome leads to myeloproliferation and anemia with a compensatory expansion of EPCs in peripheral blood [204]. In this model, anemia as well as EPC expansion are reduced after pharmacological inflammasome inhibition [204].

Chronic inflammation inhibits the late-stage differentiation of EPCs, leading to the maturation arrest of the early-stage EPCs, which is mediated by various cytokines [205]. One of the critical mediators of inflammation is interferon γ (IFN-γ) [206], which also potently impairs erythropoiesis, leading to anemia [207]. Erythroid cells stimulated with IFN-γ have increased levels of pro-apoptotic caspases, induced differentiation arrest, and triggered apoptosis [208,209]. Moreover, IFN-γ upregulates the expression of Fas on EPCs, increasing their susceptibility to apoptosis in vivo [210]. Additionally, IFN-γ induces the expression of a key regulator of myeloid differentiation, PU.1, in EPCs [207]. During physiological erythropoiesis, the expression of PU.1 is downregulated due to the inhibitory effects on GATA1 functions and erythroid cell differentiation [211–213]. Thus, chronic IFN-γ production results in decreased erythropoietic activity in the bone marrow, but increased myelopoietic activity [207]. Moreover, IFN-γ reduces RBC life span and increases macrophage erythrophagocytosis, aggravating anemia and stimulating EPC expansion [207].

Similar suppressive effects on erythropoiesis have been described for another pro-inflammatory cytokine, TNF-α. Cancer patients are characterized by the chronic production of TNF-α, which promotes immune escape and tumor progression [214]. TNF-α induces the maturation arrest of early-stage EPCs and promotes their apoptosis [82,215–218]. This effect is mediated by the p55 TNF receptor and the activation of caspases [82,215]. TNF-α also upregulates p38 MAPK in EPCs, which phosphorylates acetylated GATA1, promoting its degradation [219,220]. Moreover, TNF-α upregulates PU.1 and GATA2 in HSPCs, which antagonize erythroid cell differentiation [221].

Likewise, the maturation arrest of EPCs at early stages and the inhibition of EPC proliferation are also triggered by other proinflammatory cytokines that are overexpressed in cancer, including IL-1 [222], IL-6 [223] or IL-12 [224].

6.9. Cancer-Secreted Chemokines

Erythropoiesis is also influenced by dysregulated chemokine profiles in the bone marrow plasma and serum of cancer patients. One of these chemokines is CCL3, which

is upregulated in the majority of patients with hematopoietic malignancies [57,58] and a subset of patients with solid tumors [225]. CCL3 suppresses erythroid differentiation by p38 activation via CCR1, and this leads to the degradation of GATA1 [57,58,226]. On the other side, chemokines may also promote erythropoiesis by recruiting monocyte-derived macrophages to create erythroblastic islands in the extramedullary sites [227].

6.10. Induction of Extramedullary Stress Erythropoiesis

Suppressed bone marrow steady-state erythropoiesis is a hallmark of inflammation and is caused by the production of pro-inflammatory cytokines and iron sequestration [130,207,228,229]. Suppression steady-state erythropoiesis is often observed in patients with hematological malignancies [136,140,152,153] and solid tumors [230]. As a consequence, stress-erythropoiesis is activated in extramedullary sites to maintain erythroid homeostasis [129]. EPO secreted in response to anemia promotes the formation of erythroblastic islands in the spleen followed by the extensive proliferation of erythroid cells [228,231]. Multiple inflammatory cytokines that suppress erythropoiesis in the bone marrow simultaneously induce stress erythropoiesis. This effect was reported for IFN-γ [207], TNF-α [229], IL-1β [229,232], IL-6 [223], and G-CSF [233]. Notably, extramedullary stress erythropoiesis may be also induced by other factors, including ultraviolet B (UVB) exposure, tumor-promoting environmental stress [43], and chronic stress [234,235], which often accompany cancer.

Importantly, EPCs in extramedullary sites may still exhibit differentiation arrest that results in the enrichment of early-stage EPCs [207]. Thus, early-stage EPC fraction is increased in the spleen of tumor-bearing mice compared with acute anemic mice that also have induced stress erythropoiesis [41].

6.11. Chemotherapy-Induced Impairment of Erythropoiesis

The myelosuppressive effects of chemotherapy are another cause of anemia in cancer [166]. Importantly, early-stage EPCs are especially sensitive to the cytotoxic effects of chemotherapeutic agents, while late-stage EPCs are more resistant [236]. EPC apoptosis triggered by chemotherapy is induced by caspase activation and can be prevented by the SCF-mediated up-regulation of anti-apoptotic proteins Bcl-2 and Bcl-X_L [236]. Therefore, it was suggested that SCF may be used in the supportive therapy of chemotherapy-treated cancer patients [237]. This approach may diminish the development of anemia, which would cause the extensive expansion of EPCs after treatment.

7. Modulation of EPCs to Inhibit Their Tumor-Promoting Effects

The development of strategies that modulate the immune response in cancer patients is of great clinical interest. The modulation of immunosuppressive and tumor growth-promoting EPC mechanisms is a promising approach to diminish their negative role. Moreover, treating anemia to prevent EPC expansion as well as targeting ineffective erythropoiesis may causally decrease the tumor-promoting effects of EPCs.

7.1. Modulation of EPCs Immunosuppressive Mechanisms

7.1.1. Reactive Oxygen Species

The production of ROS by EPCs is a key mechanism of immune suppression as ROS scavengers substantially rescue T-cell function suppressed by both murine and human EPCs [41,42,116]. Several antioxidant-based therapies were demonstrated to have potent antitumor effects in preclinical studies [238]. However, further studies revealed that antioxidants may accelerate tumor progression and promote metastasis [239,240]. Therefore, current studies focus on increasing rather than decreasing ROS levels in TME due to increased vulnerability to oxidative stress-induced apoptosis [241]. Indeed, ROS-generating agents or inhibitors of antioxidant systems are efficient in preclinical studies; however, they are without satisfactory results in clinical trials [242]. Thus, more research is required to determine the clinical utility of ROS-based therapies in cancer.

7.1.2. IL-10

IL-10 was considered for many years as a potent anti-inflammatory cytokine. Accordingly, EPCs were found to secrete IL-10, which suppresses T-cells [42]. However, many studies in this field demonstrated that its role in cancer is more complex than initially envisioned [243–246]. Intriguingly, IL-10-based therapy, including pegylated IL-10 (Pegilodecakin), is much more efficient than therapies neutralizing IL-10 effects [246–248].

7.1.3. PD-L1/PD-1 Axis

Targeting immune checkpoints has revolutionized clinical oncology. Monoclonal antibodies targeting PD-L1 or PD-1 reverse the inhibitory signals triggered by the PD-L1/PD-1 axis and enhance antitumor immune response [249–251]. PD-L1 is also expressed by murine and human tumor-induced EPCs [43]. Interestingly, the expression of PD-L1 is higher in stress erythropoiesis EPCs compared to steady-state EPCs in the bone marrow, and it reaches the highest levels in tumor-infiltrating EPCs [43]. Although an exact role of the PD-L1/PD-1 axis in the EPC-mediated suppression of immune response was not assessed, it seems that immune checkpoint inhibitors may at least partially diminish their tumor-promoting effects.

7.1.4. TGF-β

The production of TGF-β in the TME is crucial to induce and maintain its immunosuppressive character [172]. TGF-β is produced by various types of cells, including EPCs [42]. The inhibition of SMAD signaling induced by TGF-β rescues T-cell proliferation and the production of IFN-γ suppressed by EPCs [42]. Therefore, modulating TGF-β signaling is a promising strategy to attenuate immune evasion induced by tumor-associated cells, including EPCs. Indeed, several anti-TGF-β-based immunotherapies were shown to be effective in preclinical studies, especially in combination with immune checkpoint inhibitors [252–255]. Therefore, targeting TGF-β signaling is a promising approach to suppress the tumor-promoting effects of EPCs.

7.2. Anti-Artemin Therapy

Late-stage EPCs promote tumor growth and invasiveness via the secretion of artemin [62,123]. Anti-artemin neutralizing antibody inhibits tumor growth and increases the survival of tumor-bearing mice [62]. Anti-artemin therapy is also currently tested for the treatment of cystitis-induced bladder hyperalgesia [256]. However, the clinical utility of targeting artemin or its signaling as the modulation of tumor growth-promoting effects of EPCs is unknown.

7.3. Treating Anemia to Prevent EPC Expansion

Since the modulation of EPCs' tumor-promoting mechanisms is rather ineffective, a decrease in EPC expansion and the induction of their differentiation is a promising strategy. The correction of anemia in cancer patients is one of the strategies to prevent EPC expansion. Most anemic patients have iron deficiency (ID) [191]; therefore, the determination of iron status and treatment is recommended for cancer patients according to the guidelines [66,257,258]. Current European Society for Medical Oncology (ESMO) guidelines of anemia management in cancer patients are presented in Figure 3.

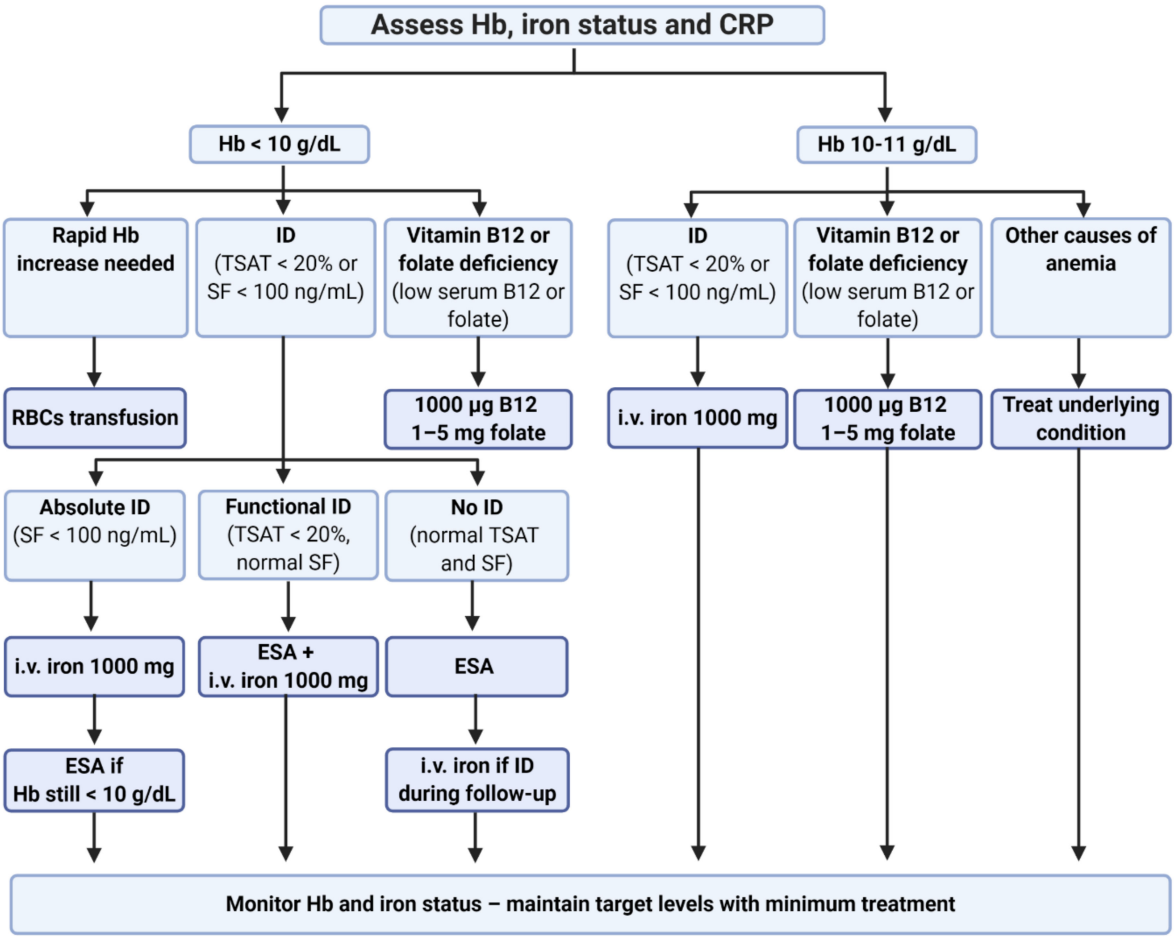

Figure 3. Management of anemia in cancer patients according to ESMO guidelines [258]. Additionally to TSAT and SF, the percentage of hypochromic cells (%HYPO) > 5% and reticulocytes hemoglobin content (CHr) < 28 pg can be used to determine impaired iron status. ID can be treated with oral iron only if ferritin < 30 ng/mL and CRP < 5 mg/L. CRP—C-reactive protein, ESA—erythropoiesis-stimulating agent, Hb—hemoglobin, i.v.—intravenous, ID—iron deficiency, RBCs—red blood cells, SF—serum ferritin, TSAT—transferrin saturation.

Impaired iron status can be diagnosed by total iron-binding capacity (TIBC), transferrin saturation (TSAT), or serum ferritin (SF) levels tests. TSAT enables the determination of iron status available for erythropoiesis, and its low levels together with high SF (>100 ng/mL) suggest functional iron deficiency (FID) [66]. Iron should be supplemented intravenously or orally for patients with low ferritin and without anemia of inflammation (CRP < 5 mg/L) [66].

However, anemia in cancer is not commonly caused by absolute ID, but rather results from iron sequestration (functional ID) [66,200,201]. In this group of patients, iron-replenishing strategies may not be effective. Therefore, targeting iron metabolisms with hepcidin antagonists to modulate the hepcidin-ferroportin axis is a promising treatment option [66,259]. Moreover, several novel therapies are currently under investigation for cancer-associated anemia, including ascorbic acid, androgens, BMP2 and BMP6 antagonists, as well as activin traps [66].

7.4. Targeting Ineffective Erythropoiesis to Decrease EPC Expansion

Modulating EPC expansion by promoting EPC differentiation is a novel therapeutic strategy to diminish the tumor-promoting effects of EPCs (Figure 4).

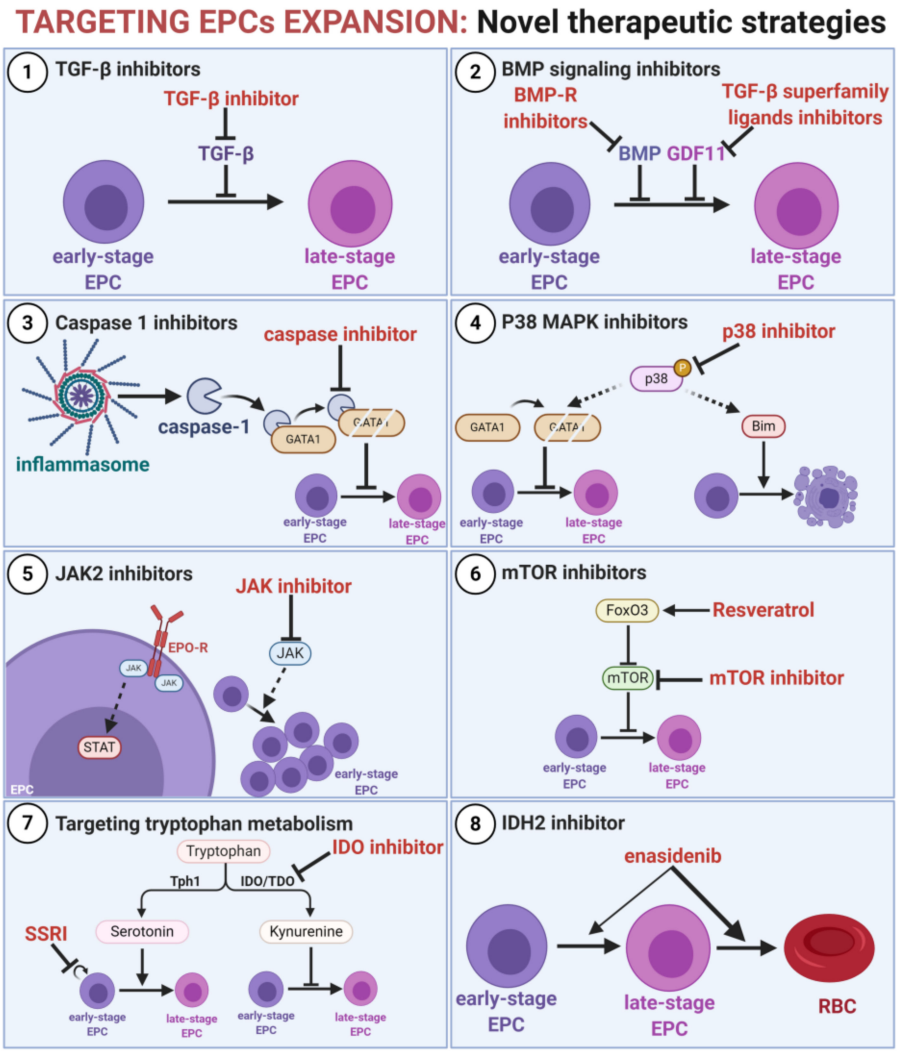

Figure 4. Targeting EPC expansion as a novel therapeutic strategy. Cancer-induced dysregulation of erythropoiesis resulting in the differentiation arrest of EPCs and their expansion may be diminished by different agents. Inhibitors of (**1**) transforming growth factor β (TGF-β) and (**2**) BMP signaling rescue maturation arrest. (**3**) Caspases inhibitors inhibit GATA1 cleavage triggered by the inflammasome. (**4**) P38 inhibitors promote differentiation and decrease the apoptosis of EPCs. (**5**) JAK inhibitors decrease activation of EPO-induced signaling, which decreases EPCs expansion. (**6**) mTOR inhibitors and inducers of FoxO3 promote differentiation of early-stage EPCs. (**7**) Increasing serotonin (5-HT) concentration with either selective serotonin reuptake inhibitors (SSRIs) or inhibitors of the kynurenine metabolism pathway promotes differentiation of EPCs. (**8**) Enasidenib, an inhibitor of mutated IDH2 promotes terminal differentiation of EPCs.

TGF-β is a key negative regulator of erythropoiesis, which triggers differentiation arrest and promotes the expansion of EPCs [62]. TGF-β inhibitors stimulate EPC differentiation in vitro [178,260]. In murine models, the anti-TGF-β antibody inhibits tumor growth and prevents the expansion of $CD45^{-}$ EPCs [62]. Moreover, SMAD inhibitors rescue T-cell proliferation and IFN-γ production suppressed by EPCs [42]. Therefore, the modulation of TGF-β and SMAD2/3 signaling is a promising approach to promote erythroid cell maturation and to diminish their suppressive effects [261], despite the lack of potent antitumor effects of monotherapy in clinical trials [262].

Myelodysplastic syndromes (MDS) are characterized by the impairment of erythroid cell differentiation with maturation arrest at the early stage [263,264]. In murine MDS models, TGF-β superfamily ligand-trapping fusion protein ACE-536 promotes erythroid

maturation by binding GDF11, inhibiting SMAD2/3 signaling, and promoting late-stage erythropoiesis, reducing rescue anemia [182]. Similar effects are exerted by the compound ACE-011, which promotes terminal erythropoiesis and prevents EPC expansion in β-thalassemia [184]. In clinical trials, luspatercept (ACE-536) and sotatercept (ACE-011) reduced the severity of anemia in patients with MDS and β-thalassemia [265–267]. Clinical trials of sotatercept in cancer patients showed that it may also be effective for the treatment of chemotherapy-induced anemia [268].

Moreover, it was suggested that targeting BMP signaling may be beneficial for anemia of inflammation [269]. The inhibition of BMP downregulates IL-6 signaling and decreases hepcidin levels, resulting in the restoration of erythropoiesis suppressed by inflammation [270,271].

Caspase-1 activation by inflammasome is one of the mechanisms skewing the differentiation of HSPCs toward myeloid cells [203]. Thus, the inhibition of caspase-1 results in the upregulation of GATA1 and the rescue of inflammation-induced anemia [203]. Moreover, caspase inhibitors trigger the differentiation of EPCs after the induction of maturation arrest by FasL or TNF-α [82]. Several caspase-1 inhibitors are available, including a potent and selective inhibitor, VX-765 [272]. Therefore, it is of great interest to evaluate the effects of caspase inhibitors on EPC expansion and differentiation in cancer.

P38 MAPK is an important pathway regulating erythropoiesis. P38 is activated by multiple inflammatory signals and restrains EPC differentiation by GATA1 degradation [220] and by suppressing the silencing of Bim, which triggers apoptosis [273]. Moreover, p38 functions as an oncogenic kinase with a complex role in cancer [274]. Therefore, p38 inhibition may simultaneously have an antitumor effect and promote EPC maturation. Notably, p38 inhibition enhances EPC maturation in an EPO-independent manner [273], but also decreases the production of endogenous EPO under stress conditions [275], suggesting that p38 inhibitor therapy should probably be combined with ESAs.

In erythroid cells, EPO triggers EPO-R and association with cytoplasmic Janus kinase 2 (JAK2), a crucial signal transducer [276]. The overactivation of JAK2, most commonly caused by V617F mutation, is associated with myeloproliferative neoplasms, including polycythemia vera [277]. Nonetheless, increased JAK2 activity may also be caused by high levels of EPO caused by anemia and chronic hypoxia, a state observed in β-thalassemia and cancer. In β-thalassemia, JAK2 inhibitors decrease ineffective erythropoiesis, prevent the expansion of EPCs, and reduce splenomegaly [278]. JAK2 inhibitors, including ruxolitinib and fedratinib, are approved for the treatment of patients with MPNs [279]. Moreover, targeting JAKs is a promising therapeutic strategy for the treatment of different types of cancer [280]. Whether JAKs inhibitors may decrease the expansion of EPCs in cancer patients remains unknown.

The mechanistic target of rapamycin (mTOR) is a central protein kinase orchestrating cell growth, metabolism, and immune response. Thus, mTOR is widely tested in clinical trials as a target for cancer therapy [281]. Importantly, mTOR inhibition rescues EPC differentiation under ineffective erythropoiesis by inducing the cell cycle exit of early-stage EPCs [282]. Moreover, mTOR inhibition in EPCs may be triggered by Forkhead-box-class-O3 (FoxO3) [282]. The activation of FoxO3 by resveratrol induces early erythroid maturation and decreases their proliferation, resulting in a reduction in ineffective erythropoiesis [283].

Erythropoiesis is also regulated by serotonin (5-HT) [284,285]. Dysregulated tryptophan metabolism with an enhanced kynurenine pathway is common in cancer, and is increasingly being recognized as a viable metabolic pathway regulating immune response [286]. A skew towards the kynurenine pathway leads to decreased serotonin (5-HT) concentrations, resulting in the impaired differentiation and decreased survival of EPCs [284,285]. The upregulation of 5-HT triggered by EPO is crucial to protect EPCs from apoptosis at the CFU-E-to-proerythroblast transition checkpoint [284]. Pharmacological increase in 5-HT with fluoxetine, a selective serotonin reuptake inhibitor (SSRI), rescues

anemia [284]. Therefore, targeting the 5-HT axis in EPCs with either SSRI or kynurenine pathway inhibitors may diminish the tumor-promoting role of immature erythroid cells.

Enasidenib is an Food and Drug Administration (FDA)-approved, first-in-class preferential inhibitor of mutated isocitrate dehydrogenase 2 (IDH2) that promotes the differentiation of acute myeloid leukemia blasts [287]. Interestingly, enasidenib was found to act independently of IDH2 on EPCs. Enasidenib potently promotes erythroid differentiation through the modulation of protoporphyrin IX (PPIX) accumulation and hemoglobin production in late-stage EPCs [288]. As a result, increased hemoglobin concentration and RBC transfusion independence were reported for enasidenib-treated patients [287,289].

Some studies suggested that natural compounds may decrease EPC expansion. Dangguibuxue decoction (DGBX), a traditional Chinese medicine, abolishes EPC accumulation, promotes their differentiation, and rescues anemia, leading to the activation of anti-tumor immune response and a decrease in tumor growth [290]. Moreover, a recent study revealed that vitamin C has a critical role in the regulation of late-stage erythropoiesis and is able to rescue ineffective erythropoiesis [291].

7.5. Splenectomy

In cancer, the spleen becomes a central organ of extramedullary hematopoiesis, responsible for the generation of suppressive cells including EPCs and myeloid cells [292]. Therefore, it was suggested that splenectomy could be beneficial for cancer patients. In preclinical models, splenectomy inhibits tumor growth and prolongs the survival of tumor-bearing mice [62]. It also abolishes the induction of EPC expansion in extramedullary sites [62]. Similarly, splenectomy leads to the depletion of MDSCs, enhancing the activation of antitumor immunity [293]. Intriguingly, splenectomy before tumor inoculation or during tumor progression attenuates the decrease in RBC count and hemoglobin concentration, alleviating anemia [62].

However, clinical data are much less promising. Randomized trials showed that splenectomy in cancer patients not only has no advantages, but is also associated with increased perioperative morbidity [294–296]. Therefore, more preclinical and clinical studies are required to evaluate the effects of splenectomy in cancer.

8. Clinical Consequences of Tumor-Induced Anemia and EPC Expansion

Anemia is very common in cancer patients. Its prevalence differs from 30–90% depending on the type of cancer as well as the diagnostic criteria. It substantially decreases the quality of life of cancer patients [296,297]. Moreover, anemia is associated with shorter survival for patients with different types of cancer and a 65% overall increase in the risk of mortality compared to non-anemic cancer patients [191,298,299]. Importantly, severe anemia is associated with hypoxia in the TME of both primary and metastatic tumors [132], which is a known driver of aggressive tumor phenotype [300].

Clinical outcomes of EPC expansion are still unclear. In cancer patients, EPC expansion is the most prominent in individuals with moderate or severe anemia [41]. Moreover, the expansion of $CD45^+$ EPCs correlates with a higher EBV load and suppressed T-cell response against the major antigenic EBV proteins, LMP2 and EBNA1 [41]. A recent study also demonstrated that $CD45^-$ EPCs may have clinical significance. In PDAC patients, the counts of $CD45^-$ EPCs in the spleen are increased compared with noncancerous pancreatic tumors or benign pancreatic masses [123]. High $CD45^-$ EPC counts predicted poor prognosis and were associated with larger tumor size and lymph node metastases [123]. Moreover, increased serum artemin concentrations, as well as increased expression of its receptors, correlate with poor prognosis in cancer patients [62,123]. Collectively, these observations demonstrate that in cancer patients, early-stage $CD45^+$ EPCs may suppress the immune response, and late-stage $CD45^-$ EPCs may promote tumor growth by the secretion of artemin. Nonetheless, more research is required to accurately dissect the clinical role of EPCs in cancer patients.

9. Conclusions

In recent years, we have expanded our knowledge regarding the mechanisms of tumor evasion induced by dysregulation in hematopoiesis. The initial assumption that cancer only significantly regulates myelopoiesis turned out to be an oversimplification. Emerging evidence demonstrates that harnessing erythroid lineage cells together with megakaryocytes and platelets [301,302] is critical for cancer progression and immune evasion. EPCs promote tumor growth by either suppressing anti-tumor immune response or secreting growth factors, depending on the developmental stage. EPCs share many similarities with well-described suppressive cells of the immune system and use the same mechanisms to regulate the immune response.

However, several issues remain unclear and need to be investigated. First, despite differences in the expression of the immunomodulatory molecules [43], factors regulating the immunosuppressive properties of EPCs are unknown. Presumably, tumor-secreted cytokines and TME may potentiate the tumor-promoting role of EPCs. Moreover, it remains elusive whether and, if so, what factors promote the recruitment of EPCs to TME. Additionally, it is not known what are the interactions between EPCs and other cells in TME, including MDSCs, TAMs or NK cells, and comprehensive studies on the role of EPCs in TME are currently limited by technological advances. Recently, transcriptional profiling at a single-cell level has revolutionized our understanding of the complexity of cell interactions in TME, and indicates further research directions [303]. However, most of the protocols involve extensive hypotonic lysis of red blood cells [304], which drastically reduces the number of EPCs [305], excluding them from analyses of TME networks. Therefore, there is a great need to define the whole landscape of TME that includes EPCs.

Future research should focus on the comprehensive characterization of immunomodulatory mechanisms of EPCs and their regulation to better understand their function in tumor immune evasion and to enable targeting them in immunotherapy. It remains unknown whether EPCs in cancer may induce Treg differentiation, similar to their neonatal counterparts [87]. Similarly, regardless of a well-established role of EPCs in neonates [86,94], the regulation of myeloid cell response by EPCs in cancer is unknown. Moreover, erythroid cells were reported to produce IL-1β, IL-2, IL-4, IL-6, IFN-γ, and TNF-α [306]; however, their role in immune regulation by EPCs remains unknown.

It needs to be determined whether and how EPCs contribute to the clinical outcome of cancer patients undergoing various types of treatment, including immunotherapy. It was reported that EPCs may contribute to the drug resistance of cancer cells [116]. Therefore, targeting EPCs or their effector mechanisms in combination with other therapies may improve therapeutic effectiveness. Finally, the development and clinical testing of agents that could rescue erythroid maturation under cancer-induced EPC differentiation arrest are of great interest. Until then, anemia treatment is the best strategy to reduce EPC expansion and differentiation arrest, as well as to minimalize the tumor-promoting role of EPCs and to improve the survival and quality of life of cancer patients.

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ARTICLE

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Potent but transient immunosuppression of T-cells is a general feature of CD71^{+} erythroid cells

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CD71^{+} erythroid cells (CECs) have been recently recognized in both neonates and cancer patients as potent immunoregulatory cells. Here, we show that in mice early-stage CECs expand in anemia, have high levels of arginase 2 (ARG2) and reactive oxygen species (ROS). In the spleens of anemic mice, CECs expansion-induced $_{L}$-arginine depletion suppresses T-cell responses. In humans with anemia, CECs expand and express ARG1 and ARG2 that suppress T-cells IFN-γ production. Moreover, bone marrow CECs from healthy human donors suppress T-cells proliferation. CECs differentiated from peripheral blood mononuclear cells potently suppress T-cell activation, proliferation, and IFN-γ production in an ARG- and ROS-dependent manner. These effects are the most prominent for early-stage CECs (CD71^{high}CD235a^{dim} cells). The suppressive properties disappear during erythroid differentiation as more differentiated CECs and mature erythrocytes lack significant immunoregulatory properties. Our studies provide a novel insight into the role of CECs in the immune response regulation.

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CD71$^+$ erythroid cells (CECs) normally reside in the bone marrow and are progenitors and precursors to over 2×10^{11} of oxygen-transporting red blood cells (RBCs) generated per day[1]. In mice, when steady-state erythropoiesis becomes insufficient to meet increased tissue oxygen demands, CECs are released from the bone marrow to the circulation and expand in the extramedullary hematopoietic sites. In humans, increased RBCs damage or loss of blood is compensated by increased erythropoietic activity in the bone marrow. Recent studies revealed an unexpected complexity of CEC functions. CECs arose as a relevant population of cells regulating immunity[2–5]. Initially, CECs were reported to suppress both innate and humoral immune responses in neonates[4,6,7] and it was suggested that their immunomodulatory functions are restricted to early life events[4]. However, further studies revealed a crucial role of CECs in the regulation of multiple phenomena such as fetomaternal tolerance[8], immune response in cancer patients[9,10], systemic inflammation in colitis[11], and anti-viral response in human immunodeficiency virus (HIV) infection[12], as well as SARS-CoV-2-induced disease (COVID-19)[13]. It has been reported that CD45$^+$ CECs induced by advanced tumors inhibit CD8$^+$ and CD4$^+$ T cell proliferation and impair antimicrobial immunity[10]. Interestingly, the authors demonstrated that CECs from mice with acute hemolytic anemia (HA), induced by systemic phenylhydrazine (PHZ) administration, are not immunosuppressive as compared with CECs from tumor-bearing mice[10]. This could lead to the conclusion that only CECs in newborns and patients with advanced cancer have robust immunosuppressive properties. In this study, we provide evidence that CECs in anemic mice do have immunoregulatory properties, but PHZ used to induce hemolysis affects the mechanisms of immune suppression used by these cells masking their phenotype. Moreover, we comprehensively elucidate the role of CECs in the regulation of immune response in both mice and humans and demonstrate that immunomodulatory properties of CECs are robust but transient and disappear during their maturation.

Results

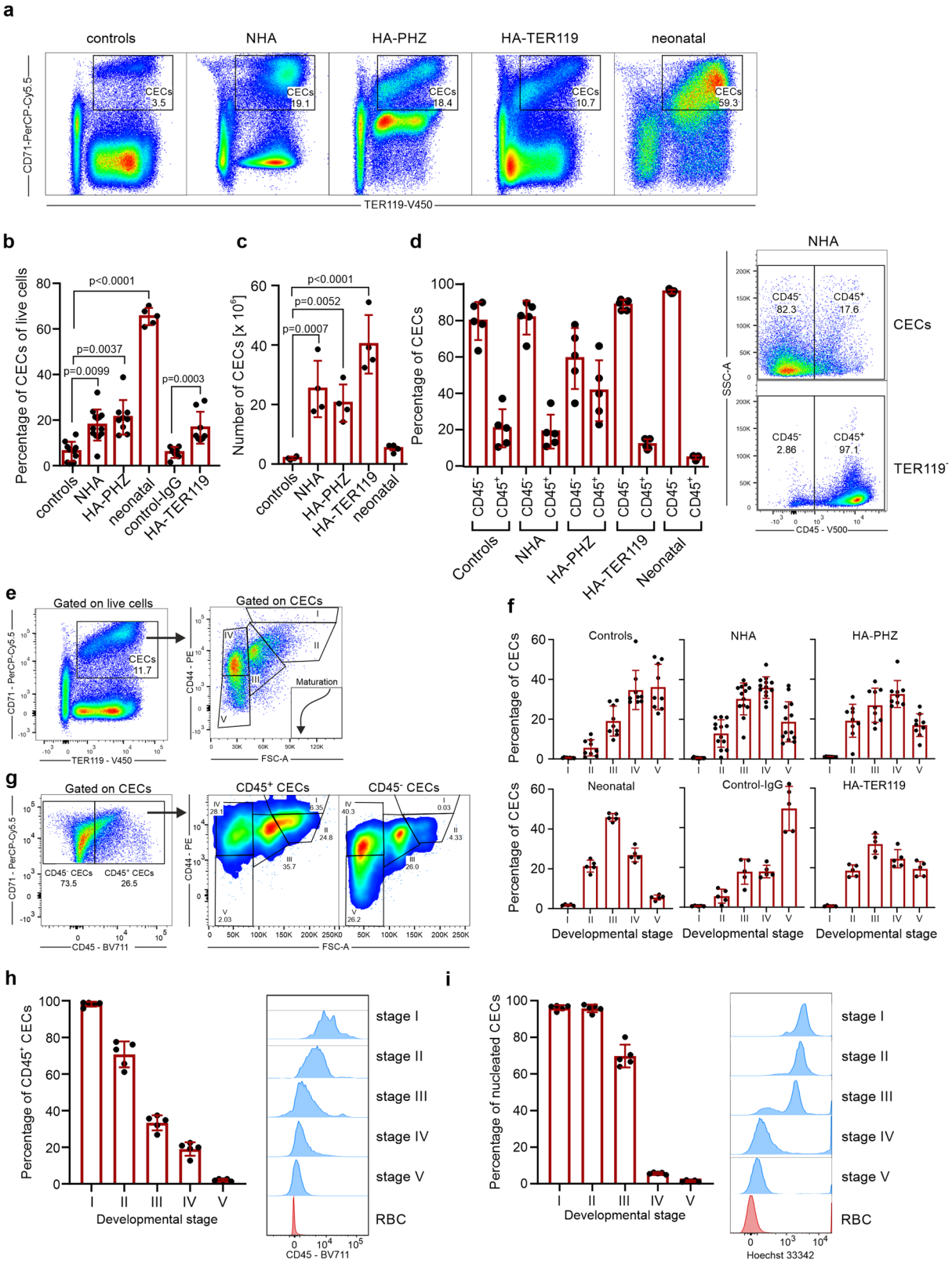
Early-stage CECs expand in the spleens of anemic mice. We initially compared the expansion of CECs in 3-day-old neonatal and adult anemic mice (Fig. 1a). Non-hemolytic anemia (NHA) was induced by phlebotomy and HA was induced either by administration of PHZ (HA-PHZ) or anti-TER119 antibodies (HA-TER119) (see Supplementary Fig. 1 for hematological parameters of these mice). Since in mice stress erythropoiesis rely on the erythropoietic activity of the spleen[14,15], we assessed CECs expansion in this organ. CECs expanded in the spleens of anemic mice as compared with controls but were significantly less frequent than in neonatal mice (Fig. 1b). However, CEC numbers in the spleen were substantially higher in anemic mice than in neonates or controls (Fig. 1c). The percentage of CECs increased also in the blood of anemic mice (Supplementary Fig. 2a) but remained unchanged in the bone marrow (Supplementary Fig. 2b). Recent studies indicated that CECs at the earliest stages of differentiation express CD45 and more potently suppress immune response[9,10]. The proportion of CD45$^+$ to CD45$^-$ CECs was the highest in HA-PHZ mice and the lowest in neonatal mice (Fig. 1d). Analysis of developmental stages of CECs based on cell size and CD44 levels (Fig. 1e)[16] revealed enrichment of less differentiated CECs in anemic mice compared to non-anemic controls (Fig. 1f and Supplementary Fig. 2c). These early-stage CECs expressed CD45 (Fig. 1g, h) and were predominantly erythroid progenitors before enucleation (Fig. 1i).

The T cell immune response is impaired in anemic mice. Next, we sought to determine whether the expansion of early-stage CECs induced by anemia might impair the function of the immune system. To this end, we assessed selected functionalities of myeloid cells, B cells, and T cells in control and anemic mice. In contrast to neonatal mice[4,6], production of tumor necrosis factor-α (TNF-α) by splenic CD11b$^+$ cells after stimulation with heat-killed *E. coli* (HKEc) (Supplementary Fig. 3a, b) or the concentration of anti-ovalbumin (OVA) IgG antibodies after OVA-ALUM immunization (Supplementary Fig. 3c, d) was unimpaired in adult anemic mice as compared with healthy controls. Intriguingly, we found that the proliferation of adoptively transferred SIINFEKL-specific OT-I T cells in response to OVA stimulation was decreased in the spleen of NHA mice compared to healthy controls (Fig. 2a, b).

Since the expansion of CD71$^+$ cells was the most substantial in the spleens of anemic mice (Supplementary Fig. 3e) and the ratio of CECs number to T cells number was significantly increased in anemia (Supplementary Fig. 3f, g), we hypothesized that CECs might be responsible for T cells suppression. Indeed, CECs isolated from the spleens of both HA and NHA anemic mice (Fig. 2c and Supplementary Fig. 4a) suppressed the proliferation of CD4$^+$ T cells that were activated with anti-CD3/CD28 beads (Fig. 2d). Altogether, these data document a rather selective impairment of T cell response by CECs in anemic mice.

Murine CECs have high ROS levels and express ARG2. Both reactive oxygen species (ROS) generation and expression of L-Arg-degrading enzymes were previously identified as the effectors of the immunoregulatory activity of neonatal CECs[4,17]. Accordingly, we found that both cytoplasmic and nuclear ROS levels were higher in anemia-induced CECs as compared with mature RBCs (Fig. 3a and Supplementary Fig. 5a, b). ROS reached the highest values in the CECs at the earliest stages of their maturation (Supplementary Fig. 5c, d), i.e. in CD45$^+$ CECs (Supplementary Fig. 5e, f). Interestingly, in contrast to human CECs[13], ROS levels in murine CECs, including CD45$^+$ CECs, were significantly lower than in the cells of non-erythroid lineages such as myeloid cells or T cells (Fig. 3b).

Murine CECs expressed ARG2, a mitochondrial arginase isoform (Fig. 3c, d), but had almost undetectable cytosolic ARG1 based on intracellular staining (Fig. 3e) as well as enhanced yellow fluorescent protein (eYFP) signal in reporter B6.129S4-Arg1^{tm1Lky}/J mice that express eYFP under *Arg1* promoter (Fig. 3f, g). Similar to ROS, the levels of ARG1 and ARG2 were the highest in early-stage CECs and consequently decreased during maturation (Supplementary Fig. 6a–g). Intriguingly, while the level of ARG2 (Fig. 3c), as well as the percentage of ARG2$^+$ CECs, were similar in all groups (Fig. 3d), the fraction of ARG1$^+$ cells was substantially higher in HA-PHZ mice as determined by intracellular staining (Fig. 3e). This finding seems counter-intuitive considering that ARG-dependent degradation of L-arginine leads to T cell suppression[18,19], and we did not observe the suppression of T cells in HA-PHZ mice in vivo (Fig. 2b). Moreover, CECs from HA-PHZ mice exerted the weakest suppressive effects on T cell proliferation (Fig. 2d). Increased expression of ARG1 in HA-PHZ CECs was further confirmed by ARG1 mRNA detection (Supplementary Fig. 7a) and in reporter B6.129S4-Arg1^{tm1Lky}/J mice (Fig. 3g, h) indicating that flow cytometry findings were not artifactual. HA-PHZ CECs had increased expression of ARG2 mRNA as compared with NHA CECs (Supplementary Fig. 7b), but no increase in ARG2 protein levels was observed (Fig. 3d). Surprisingly, despite robust upregulation of ARG1 levels, total arginase activity in both CECs isolated from HA-PHZ mice and CECs-conditioned medium was lower even than that in CECs from NHA mice



(Fig. 3i, j). Moreover, CECs cultured ex vivo in the presence of PHZ strongly upregulated ARG1 expression (Fig. 3k, l). Then, we sought to confirm whether PHZ is responsible for the attenuation of CECs immunoregulatory properties. Indeed, we found that CECs isolated from NHA lose their suppressive effects on T cells proliferation in the presence of PHZ (Supplementary Fig. 8a, b).

PHZ targets arginase and suppresses its activity. Increased expression with a concomitant decrease in arginase activity suggested an interaction between PHZ and arginase. Further studies showed that indeed PHZ inhibits the activity of recombinant human ARG1 and ARG2, with an IC_{50} of 1017 and 61 μM, respectively (Fig. 4a). However, PHZ did not affect the production of nitric oxide (NO) by nitric oxide synthase, which is also

Fig. 1 Anemia induces CECs expansion in the spleen. a Representative plots of CD71^{+}TER119^{+} CECs of total live cells in the spleens of control, anemic, and 3-day-old neonatal mice. **b** The frequency of CD71^{+}TER119^{+} CECs of total splenocytes in control ($n = 10$), control-IgG ($n = 7$), anemic (NHA, $n = 13$; HA-PHZ, $n = 9$; HA-TER119, $n = 8$), and 3-day-old neonatal mice ($n = 5$). *P* values were calculated with Kruskal–Wallis test with Dunn's post hoc test. **c** Numbers of CD71^{+}TER119^{+} CECs in the spleens of control ($n = 4$), anemic (NHA, $n = 4$; HA-PHZ, $n = 4$; HA-TER119, $n = 4$), and neonatal ($n = 4$) mice. *P* values were calculated with one-way ANOVA with Dunnet's post hoc test. **d** Percentages of CD45.2^{-} and CD45.2^{+} cells within CECs (CD71^{+}TER119^{+}) population ($n = 5$). Representative plot of CD45 levels in CECs and TER119^{-} cells in the spleen of NHA mouse. **e** Gating strategy for CECs developmental stages based on CD44 expression and cells size[16]. **f** Developmental stages of CECs in control mice ($n = 9$), NHA mice ($n = 13$), HA-PHZ ($n = 9$), HA-TER119 ($n = 5$), and neonatal mice ($n = 5$). **g** Representative plot of CD71 and CD45 levels in CECs in the spleen of NHA mouse and analysis of developmental stages of CD45^{+} CECs and CD45^{-} CECs. **h** Percentages of CD45^{+} CECs in different developmental stages in the spleens of NHA mice ($n = 5$). Histograms show the fluorescence of CD45-BV711. Red blood cells (RBCs) are presented as a negative control. **i** Percentages of nucleated CECs (Hoechst 33342^{+}) in different developmental stages in the spleens of NHA mice (n = 5). Histograms show the fluorescence of Hoechst 33342-INDO-1. Red blood cells (RBCs) are presented as a negative control. Data show means ± SD. Each point in **b**–**d**, **h**, **i** represent data from individual mice. *n* values are the numbers of mice used to obtain the data. The source data underlying **b**–**d**, **f**, **h**, **i** are provided as a Supplementary Data 2 file.

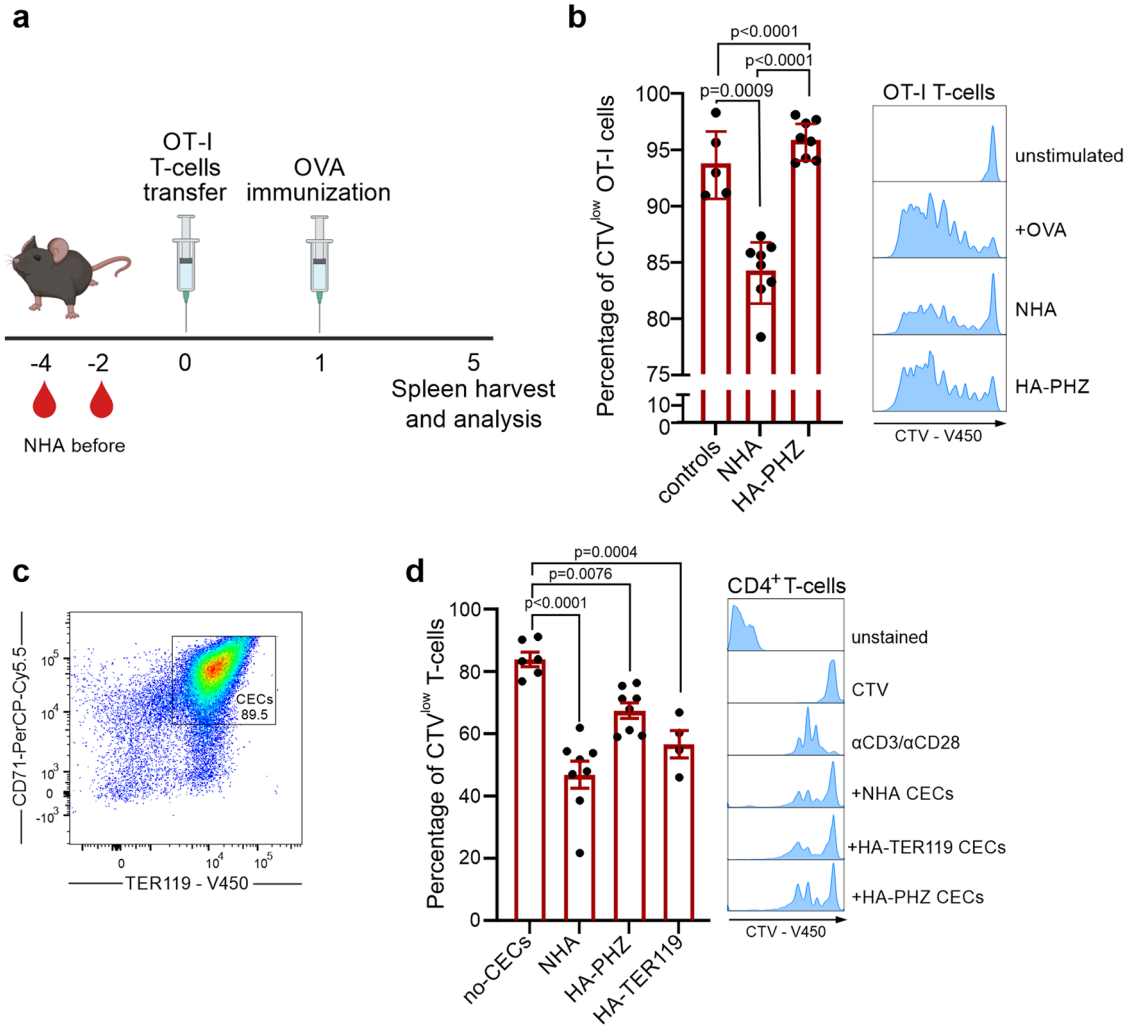
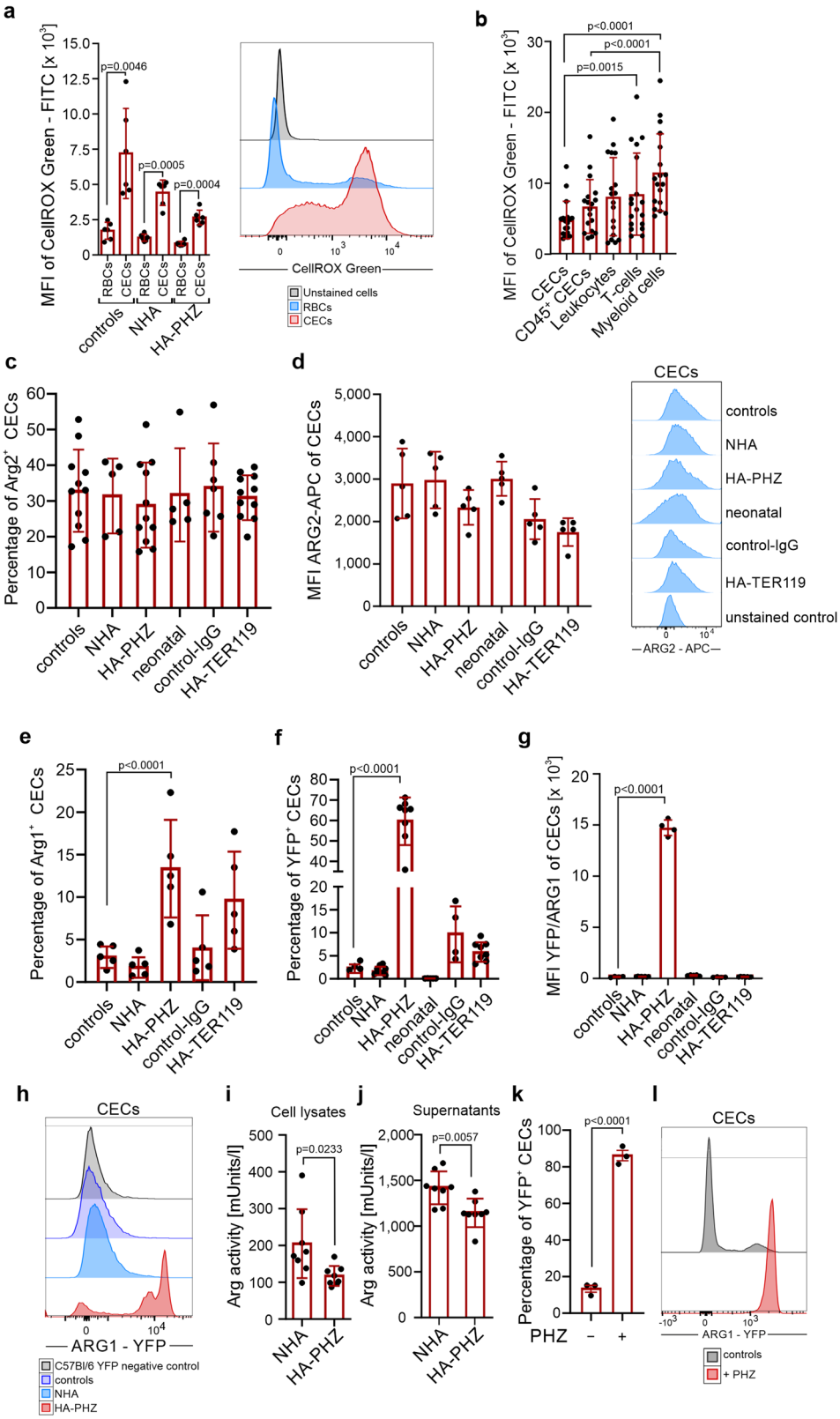


Fig. 2 Anemic mice have impaired T cell immune response. a Schematic presentation of the experimental setting. T cells isolated from OT-I mice were labeled with CellTraceViolet (CTV) and adoptively transferred to anemic and healthy control mice and stimulated with OVA. Scheme created using BioRender.com. **b** Percentage of proliferating (CTV^{low}) OT-I T cells in the spleen of NHA mice ($n = 8$), HA-PHZ mice ($n = 8$), and healthy controls ($n = 5$). Histograms show the fluorescence of CTV (CellTraceViolet)-V450 of OT-I T cells. *P* values were calculated with one-way ANOVA with Tukey's post hoc test. **c** Representative plot of CD71 and TER119 levels in isolated CECs. Additional plots are presented in Supplementary Fig. 4. **d** Proliferation triggered by αCD3/αCD28 in CTV-labeled CD4^{+} T cells co-cultured with CECs isolated from the spleens of NHA ($n = 8$), HA-PHZ ($n = 8$), or HA-TER119 ($n = 4$) mice. T cell:CECs ratio was 1:2. Representative proliferation histograms of αCD3/αCD28-stimulated CD4^{+} T cells co-cultured with CECs. Histograms show the fluorescence of CTV (CellTraceViolet)-V450. *P* values were calculated with one-way ANOVA with Dunnet's post hoc test. Data show means ± SD. Each point in **b**, **d** represents data from individual mice. *n* values are the numbers of mice. The source data underlying **b**, **d** are provided as a Supplementary Data 2 file.



using L-arginine as a substrate (Fig. 4b). To elucidate how PHZ interacts with ARG1 and ARG2 a molecular docking simulation was carried out with PHZ, L-arginine, as well as 2-amino-6-borono-2-(2-(piperidin-1-yl)ethyl)hexanoic acid (ABH) that is a strong ARG1 inhibitor[20]. PHZ binds to the active sites of all arginases, where it forms several polar interactions involving D128, D232, or T246 (Supplementary Fig. 9a). Thus, it may block the entry of other molecules to the active site. However, predicted binding energies suggest that among the tested ligands PHZ has the weakest affinity for arginases, and thus a significant concentration of this compound may be required to induce any biological effect, which indeed is the case in vivo. The transient nature of interactions between PHZ and arginases was also confirmed by a short 100 ns MD simulation (Supplementary

Fig. 3 CECs from anemic mice express ARG2 and have high levels of ROS. a Mean fluorescence intensity (MFI) of CellROX Green-FITC in CECs ($CD71^+TER119^+$) and RBCs ($CD71^-TER119^+$) of control ($n = 6$), NHA ($n = 6$), and HA-PHZ ($n = 6$) mice. Histograms show representative fluorescence of CellROX Green-FITC in CECs and RBCs from the spleens of the NHA mouse. *P* values were calculated with unpaired *t*-test. **b** MFI of CellROX Green-FITC in CECs ($CD71^+TER119^+$), $CD45^+$ CECs ($CD45^+CD71^+TER119^+$), leukocytes ($CD45^+TER119^-$), T cells ($CD45^+CD3e^+$), and myeloid cells ($CD45^+CD11b^+$) ($n = 18$). *P* values were calculated with Friedman's test with Dunn's post hoc test. **c** Percentages of $ARG2^+$ CECs in control mice ($n = 11$), anemic mice (NHA, $n = 5$; HA-PHZ, $n = 11$; HA-TER119, $n = 11$), neonatal mice ($n = 5$), and isotype control-IgG-treated mice (control-IgG, $n = 7$) based on intracellular staining. **d** MFI of ARG2-APC in CECs from control mice, anemic mice (NHA, HA-PHZ, HA-TER119), neonatal mice, and isotype control-IgG-treated mice (each group $n = 5$). Histograms show the representative fluorescence of ARG2-APC in CECs in different groups and in anti-ARG2-unstained controls. **e** Percentages of $ARG1^+$ CECs based on intracellular staining ($n = 5$). *P* values were calculated with one-way ANOVA with Dunnet's post hoc test and with an unpaired *t*-test for HA-TER119. **f** Percentages of YFP^+ CECs in reporter B6.129S4-$Arg1^{tm1Lky}$/J mice (controls $n = 4$, NHA $n = 8$, HA-PHZ $n = 8$, neonatal $n = 5$, control-IgG $n = 4$, HA-TER119 $n = 8$). *P* values were calculated with one-way ANOVA with Dunnet's post hoc test and with an unpaired *t*-test for HA-TER119. **g** MFI of YFP-FITC in CECs of reporter B6.129S4-$Arg1^{tm1Lky}$/J mice in control mice ($n = 4$), anemic (NHA $n = 8$, HA-PHZ $n = 4$, HA-TER119 $n = 8$), neonatal mice ($n = 5$), and isotype control-IgG-treated mice ($n = 4$). *P* values were calculated with one-way ANOVA with Dunnet's post hoc test. **h** Representative fluorescence of ARG1-YFP in CECs in reporter B6.129S4-$Arg1^{tm1Lky}$/J control mice and anemic mice (NHA, HA-PHZ). Background fluorescence of YFP in CECs from wild-type C57Bl/6 mice presented as a negative YFP control. **i**, **j** Total arginase activity in CECs lysates (**i** $n = 8$) or in the supernatants from CECs cultures (**j** $n = 8$). *P* values were calculated with one-way ANOVA with unpaired *t*-test. **k**, **l** Percentages of $ARG1^+$ CECs (**k**) isolated from the spleens of B6.129S4-$Arg1^{tm1Lky}$/J incubated with diluent or PHZ (100 μM for 24 h) ($n = 3$) and representative fluorescence of ARG1-YFP (**l**). *P* values were calculated with unpaired *t*-test. Data show means ± SD. Each point in **a**–**g**, **i**–**k** represents data from individual mice. *n* values are the numbers of mice used to obtain the data. The source data underlying **a**–**g**, **i**–**k** are provided as a Supplementary Data 2 file.

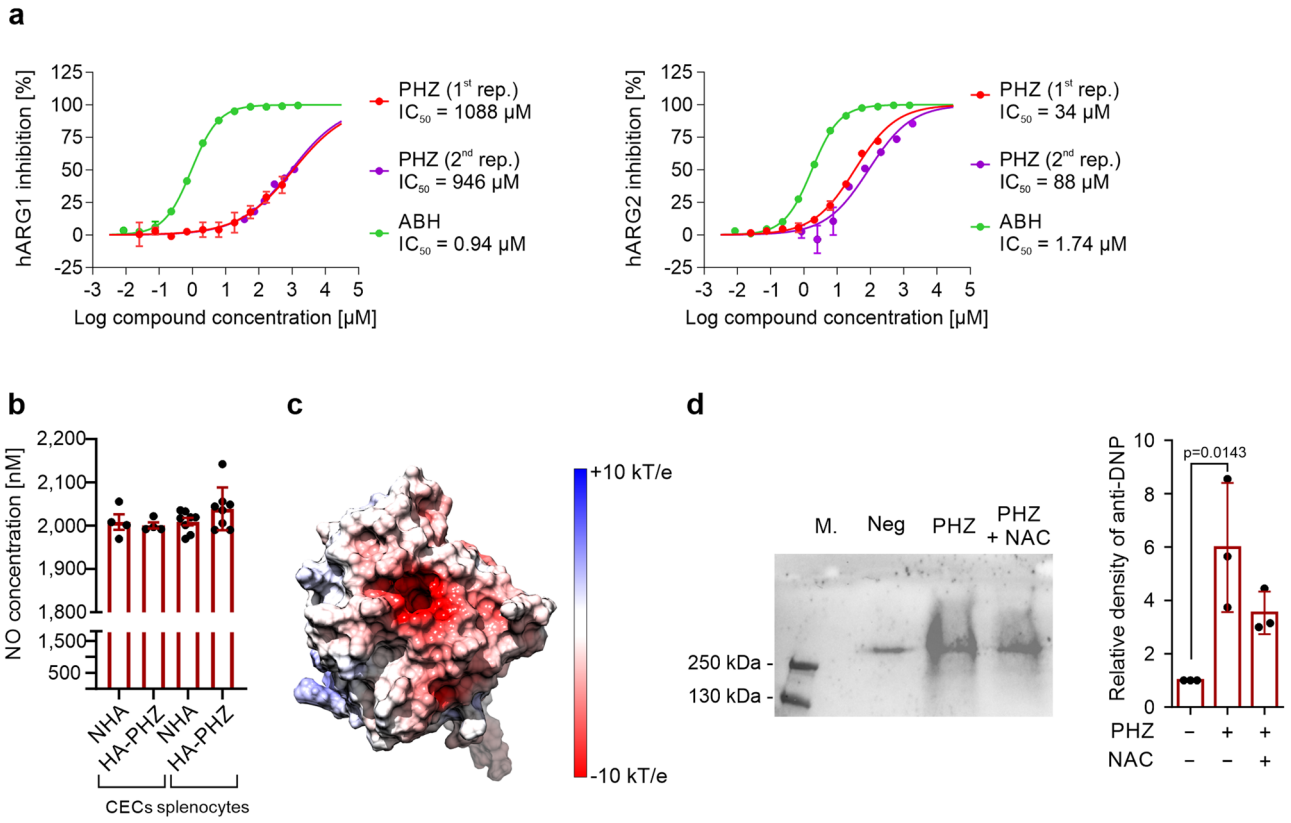


Fig. 4 Phenylhydrazine targets arginase, inhibits its activity, and induces oxidative damage. a Inhibition curves for recombinant human ARG1 and ARG2, and IC_{50} values for PHZ ($n = 2$) and 2(*S*)-amino-6-boronohexanoic acid (ABH). **b** NO production from CECs and whole splenocytes population isolated from NHA ($n = 4$) and HA-PHZ ($n = 4$) mice. *P* values were calculated with an unpaired *t*-test. **c** The electrostatic surface potential of the human ARG1. The potential was calculated with APBS and projected onto the molecular surface of the protein. The figure was prepared with UCSF Chimera. **d** Carbonylation of ARG1 in the presence of PHZ ($n = 3$). Representative blot (left) and densitometric analysis done with ImageJ software (right). The negative lane represents ARG1 precipitates treated with 2 N HCl alone. *P* values were calculated with ordinary one-way ANOVA with Dunnett's multiple comparisons test. Data show means ± SD. Each point in **b** represents data from individual mice. *n* values are the numbers of mice used to obtain the data or the number of biological replicates of in vitro experiments. The source data underlying **a**, **b**, **d** are provided as a Supplementary Data 2 file.

Fig. 9b, c). The ligand remained bound to the active site for only 15–30% of the simulation time, despite its initial placement inside the ligand-binding pocket. The analysis of electrostatic surface potential revealed the presence of a large, negatively charged area around the substrate-binding pocket of ARG1 that likely plays a role in attracting positively charged L-arginine to the catalytic site (Fig. 4c). Since PHZ in the presence of oxygen leads to the formation of free radicals and hydrogen peroxide[21], we hypothesized that decreased ARG activity in CECs from HA-PHZ mice might emerge due to non-specific non-covalent interactions of PHZ

with the catalytic pocket of ARG1 that leads to oxidative changes in the enzyme, decreased activity, and compensatory increase in its expression. Indeed, the incubation of recombinant ARG1 with PHZ in the presence of oxygen led to a significant increase in the carbonylation of the enzyme. However, this effect was only slightly reduced by concomitant incubation with *N*-acetylcysteine (ROSi) (Fig. 4d). Moreover, ROS scavengers did not prevent ARG1 induction by PHZ in vivo (Supplementary Fig. 10a, b) nor in vitro (Supplementary Fig. 10c, d). Thus, we demonstrated that PHZ targets ARG leading to the diminishment of CECs immunoregulatory properties; however, the exact mechanism that would explain PHZ-mediated inhibition of ARG activity remains elusive.

CECs degrade L-Arg and produce ROS leading to the suppression of T cells. Due to the interaction between PHZ and arginases, we chose NHA as a model of anemia-induced CECs for further studies. We found that $CD4^+$ T cells stimulated with anti-CD3/CD28 beads in the presence of CECs showed down-regulation of activation markers CD25 and CD69, which was less pronounced for CD62L (Fig. 5a). Both arginase inhibitor (ARGi, OAT-1746, a membrane-permeable, potent inhibitor of both arginase isoforms[22–24]) and ROS inhibitor (ROSi, *N*-acetylcysteine) nearly completely restored the proliferation of T cells that was inhibited by co-culture with CECs isolated from NHA mice (Fig. 5b), similar to CECs isolated from neonates (Supplementary Fig. 11). Likewise, CEC-conditioned medium had a suppressive effect on T cell proliferation, and supplementation with either of L-arginine or ARGi restored T cell proliferation to percentages akin to the control group (Fig. 5c).

To confirm that early-stage CECs that have the highest ROS levels (Supplementary Fig. 5) and ARG expression (Supplementary Fig. 6) have the most potent suppressive effects on T cells, we isolated the fraction of nucleated cells (erythroid progenitors, developmental stages I–III, Fig. 1i) from CECs using density-gradient centrifugation. We found that the whole CEC population suppressed $CD4^+$ T cells proliferation by 43% while isolated nucleated CECs (nCECs) completely inhibited it (Fig. 5d), confirming that they are responsible for the suppressive effects.

At a 1:10 of T cells to CECs ratio, similar to that observed in anemia (Supplementary Fig. 3f, g), CECs completely suppressed the proliferation of $CD4^+$ (Fig. 5e) and $CD8^+$ T cells (Fig. 5f). Further studies revealed that the expansion of CECs in anemic mice leads to the substantial increase of the total arginase activity (Fig. 5g). This effect was caused by an increased ARG2 but not ARG1 levels in the spleen (Fig. 5h–j). Even though the concentration of L-arginine was only slightly decreased in the serum of anemic mice (Supplementary Fig. 12), their splenic $CD4^+$ T cells and $CD8^+$ T cells had decreased levels of CD3ζ (Fig. 5k, l), a marker of L-arginine T cell starvation[22,25]. Accordingly, ex vivo stimulation of T cells with anti-CD3/CD28 beads in the presence of CECs resulted in a decrease in CD3ζ, which was prevented by ARGi and completely restored by the combination of ARGi and ROSi (Fig. 5m, n). Noteworthy, the decrease in CD3ζ was not observed in the lymph nodes of anemic mice, where CECs are a relatively rare population (Supplementary Fig. 13a–c). Altogether, these results show that CECs suppress T cells response in anemic mice via both arginase and ROS and their local accumulation in the spleen impairs T cell immunity.

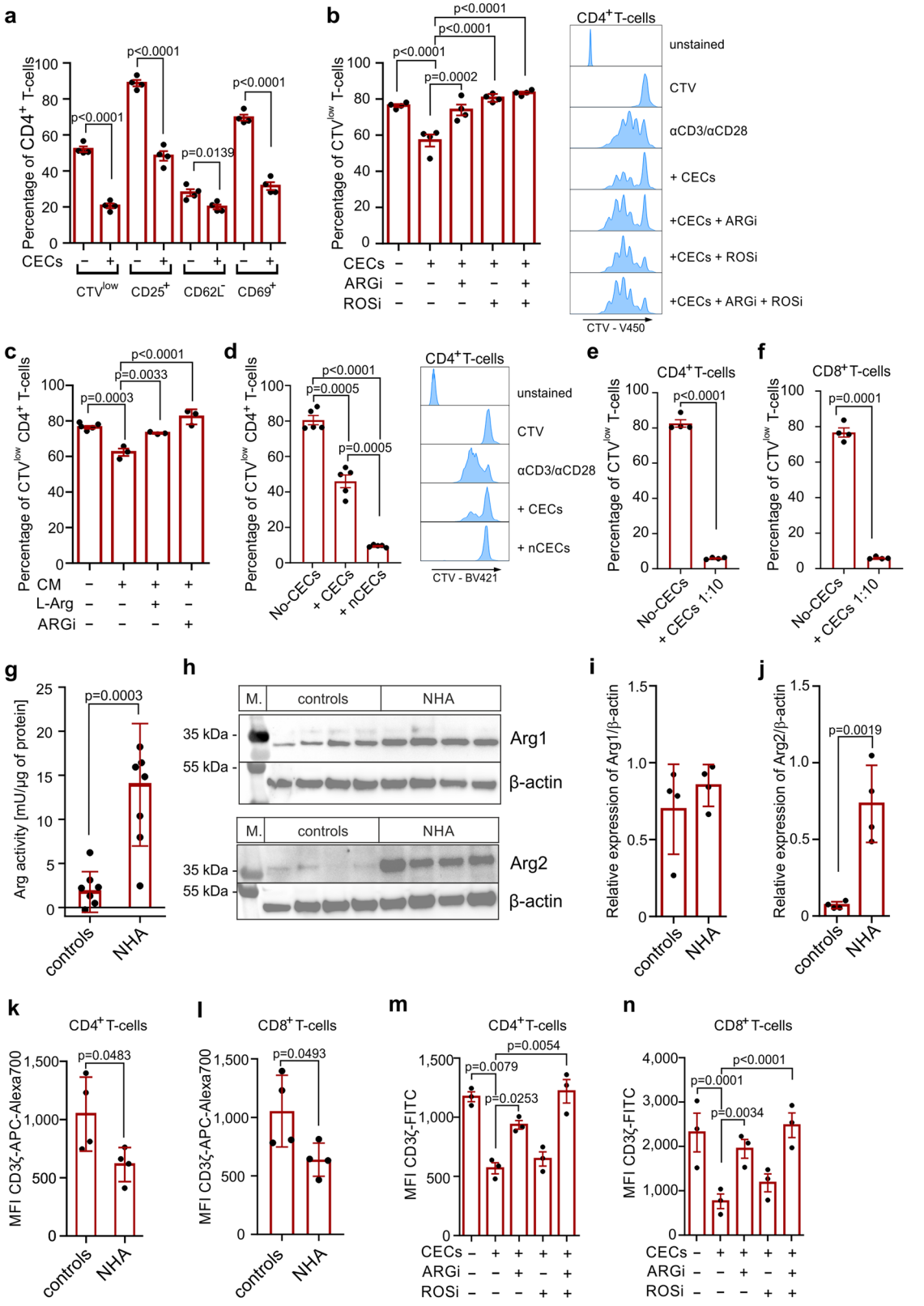
To further study the role of ARG2 in the modulation of immune response by CECs, we assessed the suppressive effects of CECs isolated from anemic mice lacking functional *Arg2* gene ($Arg2^{-/-}$, $Arg2^{tm1Weo}$/J mice[26]). $Arg2^{-/-}$ mice had effective stress erythropoiesis (Supplementary Fig. 14a). Despite a slightly increased percentage of $ARG1^+$ CECs compared to wild-type mice ($Arg2^{+/+}$) (Supplementary Fig. 14b), no significant changes in total ARG1 levels were observed in these cells (Supplementary Fig. 14c). In contrast to wild-type mice, expansion of CECs in the spleen of anemic $Arg2^{-/-}$ mice was not associated with a significant decrease in CD3ζ in T cells (Fig. 6a, b). Moreover, CECs isolated from $Arg2^{-/-}$ mice had substantially diminished suppressive effects on T cell proliferation as compared with $Arg2^{+/+}$ CECs (Fig. 6c), confirming a critical role of ARG2 in the regulation of T cells function by murine CECs.

CECs expand in the blood of anemic individuals and suppress IFN-γ production by T cells. Then, we sought to investigate the role of CECs in anemic patients (Supplementary Table 1 and Supplementary Data 1). The percentages of CECs ($CD71^+CD235a^+$) in the peripheral blood were substantially increased in anemic patients compared to non-anemic control individuals (Fig. 7a, b). The number of CECs in the blood (Fig. 7c) reversely correlated with hemoglobin concentration (Fig. 7d) and was the highest in patients with moderate and severe anemia (Fig. 7e).

In anemic patients, CECs constituted a substantial fraction of peripheral blood mononuclear cells (PBMCs) (Fig. 7f, g) and were predominantly at the latest stages of differentiation with a very small percentage of $CD45^+$ CECs (Supplementary Fig. 15a, b). We found that the production of IFN-γ in response to CD3/CD28 stimulation was suppressed in T cells from anemic individuals when compared to non-anemic controls (Fig. 7h, i). However, T cells proliferation (Supplementary Fig. 15c, d) or the production of TNF-α by myeloid cells in response to killed bacteria (Supplementary Fig. 15e) were comparable in anemic patients and control individuals.

CECs from human bone marrow suppress T cells proliferation. Since the expansion of CECs in the peripheral blood of anemic individuals was not associated with the suppression of T cells proliferation, we investigated the immunoregulatory properties of CECs from the healthy human bone marrow (Fig. 8a). CECs in the bone marrow are enriched with early-stage CECs (Supplementary Fig. 16a, b) and are predominantly $CD45^+$ (Supplementary Fig. 16c, d). Similar to murine CECs, their counterparts in the human bone marrow express ARG2 (Fig. 8b–d). Importantly, human erythroid cells also express ARG1 (Fig. 8b–d). The expression of both ARG isoforms was higher in $CD45^+$ than in $CD45^-$ CECs (Supplementary Fig. 16e, f). CECs isolated from human bone marrow (Supplementary Fig. 16g) significantly suppressed proliferation of both $CD4^+$ and $CD8^+$ T cells (Fig. 8e, f). These effects were diminished by the ARGi, confirming arginase-dependent effects of human CECs.

Erythroleukemia-derived erythroid cell lines suppress T cells in an ARG- and ROS-dependent mechanism. Further, we investigated the immunoregulatory properties of model human erythroleukemia-derived erythroid cell lines: K562, HEL92.1.7, and TF-I. These cells express multiple erythroid-lineage markers, including CD71 and CD235a (Fig. 9a), and spontaneously differentiate into erythroblast-like cells. We found that similarly to primary CECs, erythroid cells have substantial arginase activity (Supplementary Fig. 17a), express both ARG1 (Supplementary Fig. 17b) and ARG2 (Supplementary Fig. 17c), and have high ROS levels (Supplementary Fig. 17d). Notably, all examined types of erythroid cells potently suppressed proliferation of human $CD4^+$ (Fig. 9b–e) and $CD8^+$ (Fig. 9f–i) T cells in an ARG- and ROS-dependent manner (Fig. 9e, i and Supplementary Fig. 18a, b).



Suppression of T cell function is a general feature of erythroid cells which disappears during their maturation. Our results demonstrated that T cell suppression is a common feature of both murine and human CECs. Apparently the immunoregulatory properties were the most potent at the earliest stages of differentiation when the levels of ROS, ARG1, and ARG2 are the highest. Therefore, we next sought to establish a model of ex vivo differentiation of erythroid cells. To this end, CECs were expanded and differentiated from PBMCs of healthy human donors (Supplementary Fig. 19a). PBMC-derived CECs expressed erythroid markers, including CD71, CD235a, CD36, and CD49d, and had high expression of CD44 and CD45 (Supplementary

Fig. 5 CECs degrade L-Arg and produce ROS leading to the suppression of T cells. a Proliferation and surface markers in αCD3/αCD28-stimulated $CD4^+$ T cells co-cultured with CECs isolated from NHA mice ($n = 4$) at a ratio 1:2 (T cells:CECs). *P* value was calculated with an unpaired *t*-test. **b** Effects of ARGi (OAT-1746, 500 nM) and ROSi (*N*-acetylcysteine, 100 μM) on the proliferation of αCD3/αCD28-stimulated $CD4^+$ T cells co-cultured with CECs isolated from the spleens of NHA mice ($n = 4$) at a ratio 1:2 (T cells:CECs). Representative proliferation histograms of αCD3/αCD28-stimulated $CD4^+$ T cells co-cultured with CECs in the presence of ARGi or ROSi. Histograms show the fluorescence of CTV (CellTraceViolet)-V450. *P* value was calculated with one-way ANOVA with Bonferroni's post hoc test. **c** Effects of L-arginine supplementation (1000 μM) or ARGi (OAT-1746, 500 nM) on the proliferation of αCD3/αCD28-stimulated $CD4^+$ T cells cultured in full medium or in CECs-conditioned medium (CM) ($n = 3$). *P* value was calculated with one-way ANOVA with Bonferroni's post hoc test. **d** Proliferation of αCD3/αCD28-stimulated $CD4^+$ T cells co-cultured with total CECs population or with nucleated CECs (nCECs) isolated using density-gradient centrifugation from NHA mice ($n = 5$) at a ratio 1:2 (T cells:CECs). Histograms show the fluorescence of CTV (CellTraceViolet)-V450. *P* value was calculated with repeated measures ANOVA with Holm–Sidak's post hoc test. **e**, **f** Proliferation of αCD3/αCD28-stimulated $CD4^+$ T cells (**e**) or $CD8^+$ T cells (**f**) co-cultured with CECs isolated from NHA mice ($n = 4$) at a ratio 1:10 (T cells:CECs). *P* value was calculated with paired *t*-test. **g** Arginase activity of the splenocytes lysate of control and anemic mice calculated per μg of total protein based on bicinchoninic acid (BCA) protein assay. *P* value was calculated with an unpaired *t*-test. **h** The level of ARG1 and ARG2 in the splenocytes lysate of control ($n = 4$) and anemic mice ($n = 4$). β-Actin showed as a loading control. **i**, **j** Relative density of ARG1 (**i**) and ARG2 (**j**) compared to β-actin. *P* value was calculated with an unpaired *t*-test. **k**, **l** The level of CD3ζ in $CD4^+$ (**k**) and $CD8^+$ (**l**) T cells in the spleen of control ($n = 4$) and anemic mice ($n = 4$) based on intracellular staining. *P* value was calculated with an unpaired *t*-test. **m**, **n** The levels of CD3ζ in $CD4^+$ (**m**) and $CD8^+$ (**n**) αCD3/αCD28-stimulated T cells in the presence of CECs isolated from NHA mice ($n = 4$) based on intracellular staining. *P* value was calculated with one-way ANOVA with Bonferroni's post hoc test. Data show means ± SEM (**a**–**f**, **m**, **n**) or means ± SD (**h**, **i**–**l**). Each point in **a**–**g**, **i**–**n** represents data from individual mice. *n* values are the numbers of mice used to obtain the data or the number of biological replicates in in vitro experiments. The source data underlying **a**–**n** are provided as a Supplementary Data 2 file.

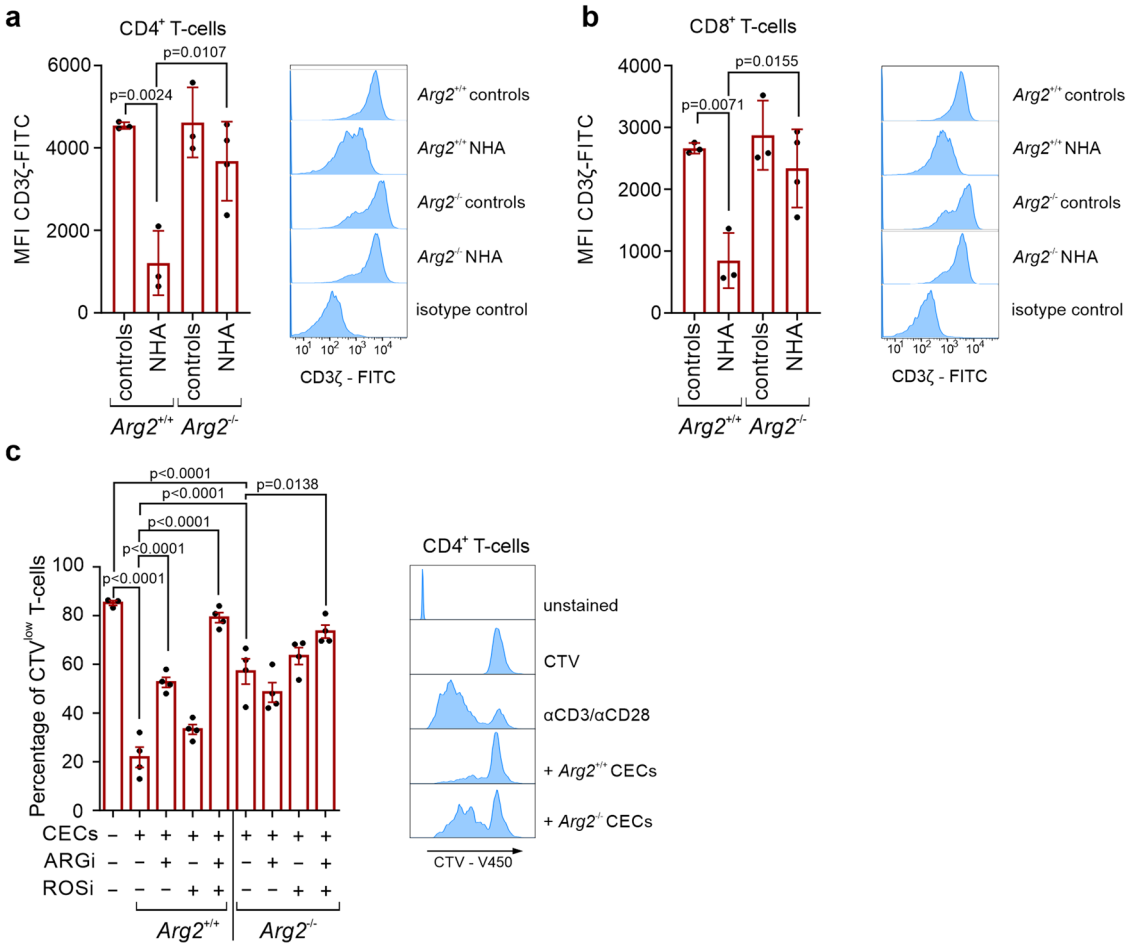


Fig. 6 CECs from *Arg2*$^{-/-}$ mice have impaired immunoregulatory properties. a The levels of CD3ζ in spleen $CD4^+$ (**a**) and $CD8^+$ (**b**) T cells in control ($n = 4$) and anemic mice ($n = 4$) based on intracellular staining. Histograms show the fluorescence of CD3ζ-FITC in $CD4^+$ (**a**) and $CD8^+$ (**b**) T cells. *P* values were calculated with one-way ANOVA with Tukey's post hoc test. **c** Proliferation of αCD3/αCD28-stimulated $CD4^+$ T cells co-cultured with CECs isolated from NHA *Arg2*$^{-/-}$ mice or NHA wild-type *Arg2*$^{+/+}$ mice at a 1:4 ratio (T cells:CECs). Representative proliferation histograms of αCD3/αCD28-stimulated $CD4^+$ T cells co-cultured with CECs isolated from *Arg2*$^{-/-}$ mice or wild-type *Arg2*$^{+/+}$ mice in the presence of ARGi or ROSi. Histograms show the fluorescence of CTV (CellTraceViolet)-V450. *P* values were calculated with one-way ANOVA with Bonferroni's post hoc test. Data show means ± SD (**a**, **b**) or means ± SEM (**c**). Each point in **a**–**c** represents data from individual mice. *n* values are the numbers of mice used to obtain the data or the number of biological replicates in in vitro experiments. The source data underlying **a**–**c** are provided as a Supplementary Data 2 file.

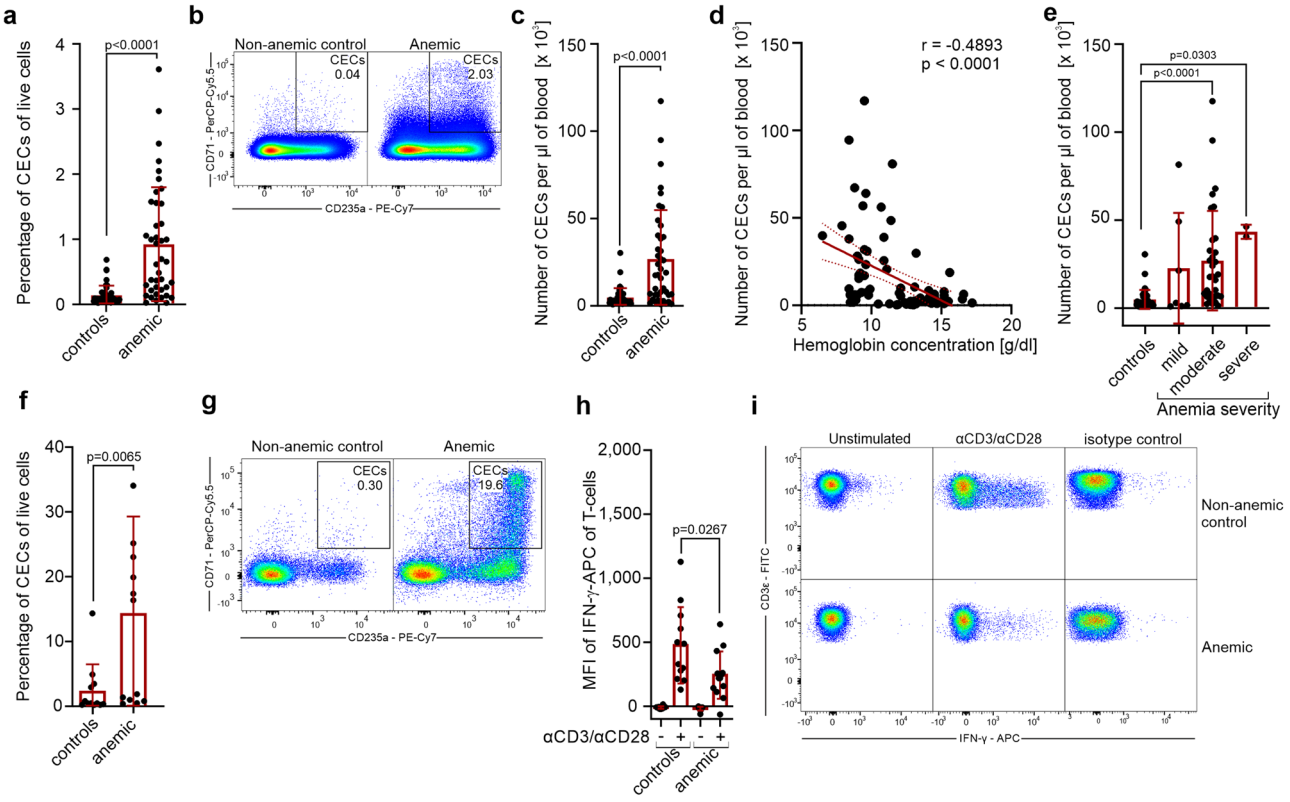


Fig. 7 CECs expand in the blood of anemic patients and suppress T cells response. a Percentages of live CD71^{+}CD235a^{+} CECs in the whole blood of non-anemic (controls, $n = 42$) and anemic patients ($n = 41$). *P* value was calculated with the Mann–Whitney test. **b** Representative dot plots of CECs in the blood of non-anemic and anemic patients. **c** CECs count per µl of blood in controls ($n = 42$) and anemic patients ($n = 41$). *P* value was calculated with the Mann–Whitney test. **d** Correlation of the number of CECs per µl of blood and hemoglobin concentration ($n = 82$). The correlation was calculated with Spearman *r*. **e** CECs count per µl of blood in non-anemic controls ($n = 42$) and patients with mild ($n = 7$), moderate ($n = 32$), and severe ($n = 2$) anemia. *P* values were calculated with Kruskal–Wallis test with Dunn's post hoc test. **f**, **g** Percentages of CECs in the fraction of peripheral blood mononuclear cells (PBMC) in controls ($n = 12$) and anemic patients ($n = 13$) (**f**) and representative dot plots of CECs (**g**). *P* value was calculated with the Mann–Whitney test. **h** PBMCs of controls ($n = 12$) and anemic patients ($n = 13$) were stimulated with αCD3/αCD28 for 12 h in the presence of a protein transport inhibitor. IFN-γ levels were determined by intracellular staining. *P* values were calculated with an unpaired *t*-test. **i** Representative plots of IFN-γ levels in unstimulated or αCD3/αCD28-stimulated CD3ε^{+} T cells from PBMCs of anemic patients or non-anemic controls. Isotype control-stained cells are shown as a negative control. Data show means ± SD. Each point in **a**, **c**–**f**, **h** represents data from individual patients. *n* values are the numbers of patients used to obtain the data or the number of biological replicates in in vitro experiments. The source data underlying **a**, **c**–**f**, **h** are provided as a Supplementary Data 2 file.

Fig. 19a, b). Similar to their bone marrow counterparts, PBMCs-derived CECs had high levels of both ARG1 and ARG2 (Supplementary Fig. 20a, b). Moreover, isolated PBMC-derived CECs (Fig. 10a) potently suppressed both CD4^{+} and CD8^{+} human T cell proliferation (Fig. 10b, c).

Next, we aimed to study the possible changes in immunoregulatory properties of erythroid cells during differentiation into RBC. First, we investigated whether hematopoietic stem and progenitor cells (HSPCs) exert immunosuppressive effects. Mobilized hematopoietic stem cells obtained from peripheral blood (peripheral blood stem cells, PBSCs, Supplementary Fig. 21a) had high ARG1 and ARG2 levels (Supplementary Fig. 21b) and included only a small percentage of CECs (Supplementary Fig. 21c). Despite high arginase expression, PBSCs had no impact on T cell proliferation (Supplementary Fig. 21d, e).

Then, using continuous CECs culture, we demonstrated that CECs differentiated from PBMCs (Fig. 10d) exert robust, but transient suppressive properties, that disappear during erythroid differentiation (Fig. 10e–g). We found that of all CECs developmental stages, cells at the stage of CD71^{high} CD235a^{mid} most strongly inhibited T cell proliferation (Fig. 10f, g). Moreover, these cells potently suppressed T cell activation based on the CD25 and CD69 levels (Supplementary Fig. 22a, b) as well as inhibited IFN-γ production by T cells (Supplementary Fig. 22c). The suppression depended on both ARG and ROS since only the combination of ARGi and ROSi significantly diminished suppressed T cell activation, IFN-γ production (Supplementary Fig. 22c), and proliferation (Supplementary Fig. 23a, b). Loss of the suppressive properties corresponded with a decrease in CD71 (Fig. 10h), an increase in CD235a (Fig. 10i) as well as a decrease in CD49d (Fig. 10k) levels, the latter being a marker of the transition to the reticulocyte stage[27,28]. Subsequent CEC differentiation resulted in a complete loss of suppressive effects on T cells.

Further, we observed that induction of erythroid differentiation of K562 cells by sodium butyrate[29] (Supplementary Fig. 24a) was associated with a decrease of immunosuppressive effects on T cells (Supplementary Fig. 24b). These differentiated cells had decreased ARG2, but not ARG1 levels (Supplementary Fig. 24c), and decreased total arginase activity as compared with non-differentiated K562 cells (Supplementary Fig. 24d). Downregulation of ARG2 was most probably caused by mitophagy, a crucial process during erythroid differentiation[30], as evidenced by the decreased signal from a mitochondrial probe in differentiated K562-erythroid cells (Supplementary Fig. 24e). Similar changes in

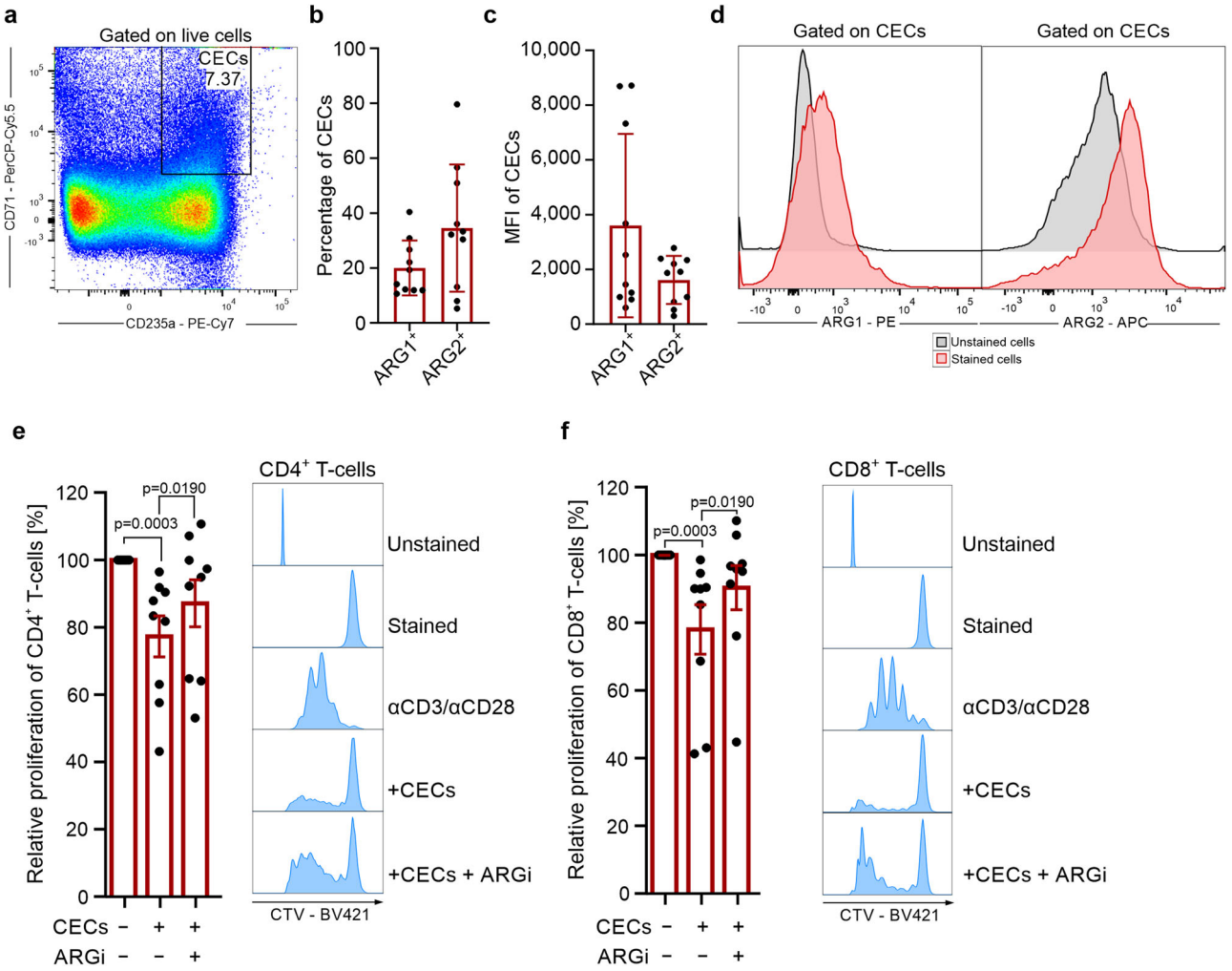


Fig. 8 CECs from human bone marrow express ARG1 and ARG2 and suppress T cells proliferation. a Representative plots of CD71$^+$ and CD235$^+$ CECs in the aspirate of human bone marrow of total live cells. **b** Percentages of ARG1$^+$ and ARG2$^+$ CECs in the human bone marrow based on intracellular staining. **c** Mean fluorescence intensity (MFI) of ARG1-PE and ARG2-APC in CECs. **d** Representative histograms of ARG1 and ARG2 levels in CECs from human bone marrow. Fluorescence-minus-one (FMO) is shown as unstained controls. **e**, **f** Proliferation triggered by αCD3/αCD28 of CTV-labeled CD4$^+$ (**e**) and CD8$^+$ (**f**) T cells co-cultured with CECs isolated from the human bone marrow. T cell to CEC ratio was 1:2 ($n = 9$). The proliferation of T cells in co-culture with CECs was calculated as a percentage of maximum proliferation (100%) of T cells that was triggered by αCD3/αCD28 antibodies and cultured without CECs. Representative histograms shows CTV-BV421 fluorescence of CD4$^+$ (**e**) or CD8$^+$ (**f**) T cells co-cultured with CECs at a 1:2 ratio in the presence of ARGi (OAT-1746, 500 nM). *P* values were calculated with Friedman's test with Dunn's post hoc test. Data show means ± SD (**b**, **c**) or means ± SEM (**e**, **f**). *n* values are the numbers of individual patients used to obtain the data or the number of biological replicates in in vitro experiments. The source data underlying **b**, **c**, **e**, **f** are provided as a Supplementary Data 2 file.

ARG expression were also detected in primary murine (Supplementary Fig. 6b–e) and human CECs (Supplementary Fig. 20b). Finally, we demonstrated that mature RBCs obtained from healthy donors had no impact on T cell proliferation (Supplementary Fig. 25a–c). Altogether, we show that human CECs possess robust, but transient suppressive properties that are most potent in the earliest developmental stages and disappear during erythroid cell maturation.

Discussion

In this study, we demonstrate that suppression of T cells is a general feature of murine and human CECs. Anemic CECs via arginases and ROS suppress proliferation and production of IFN-γ by T cells. Using continuous human erythroid cell culture, we show that the immunoregulatory properties of CECs are transient and disappear during maturation.

Recent studies have broadened our understanding of the many roles played by CECs expanded by different triggers[3]. Immunoregulatory functions of CECs were reported for the first time in neonates that are characterized by a physiological abundance of CECs[4]. Neonatal CECs suppress anti-bacterial immunity via ARG2 by decreasing the production of proinflammatory cytokines by myeloid cells[4] and by suppressing antibody production in response to *B. pertussis*[6]. Moreover, they have been described to be involved in the expansion of inducible regulatory T cells[31]. We found that in adult mice anemia induced the expansion of early-stage CECs that had the highest expression of ARG2. Neither ARG2-expressing CECs nor recombinant ARG1 suppressed the production of TNF-α from myeloid cells. However, arginases seem to primarily impair T cells by decreasing their activation and proliferation[32]. Accordingly, we observed decreased proliferation of adoptively transferred OT-I cells in the spleen of anemic mice, which was reflected ex vivo in the co-culture of

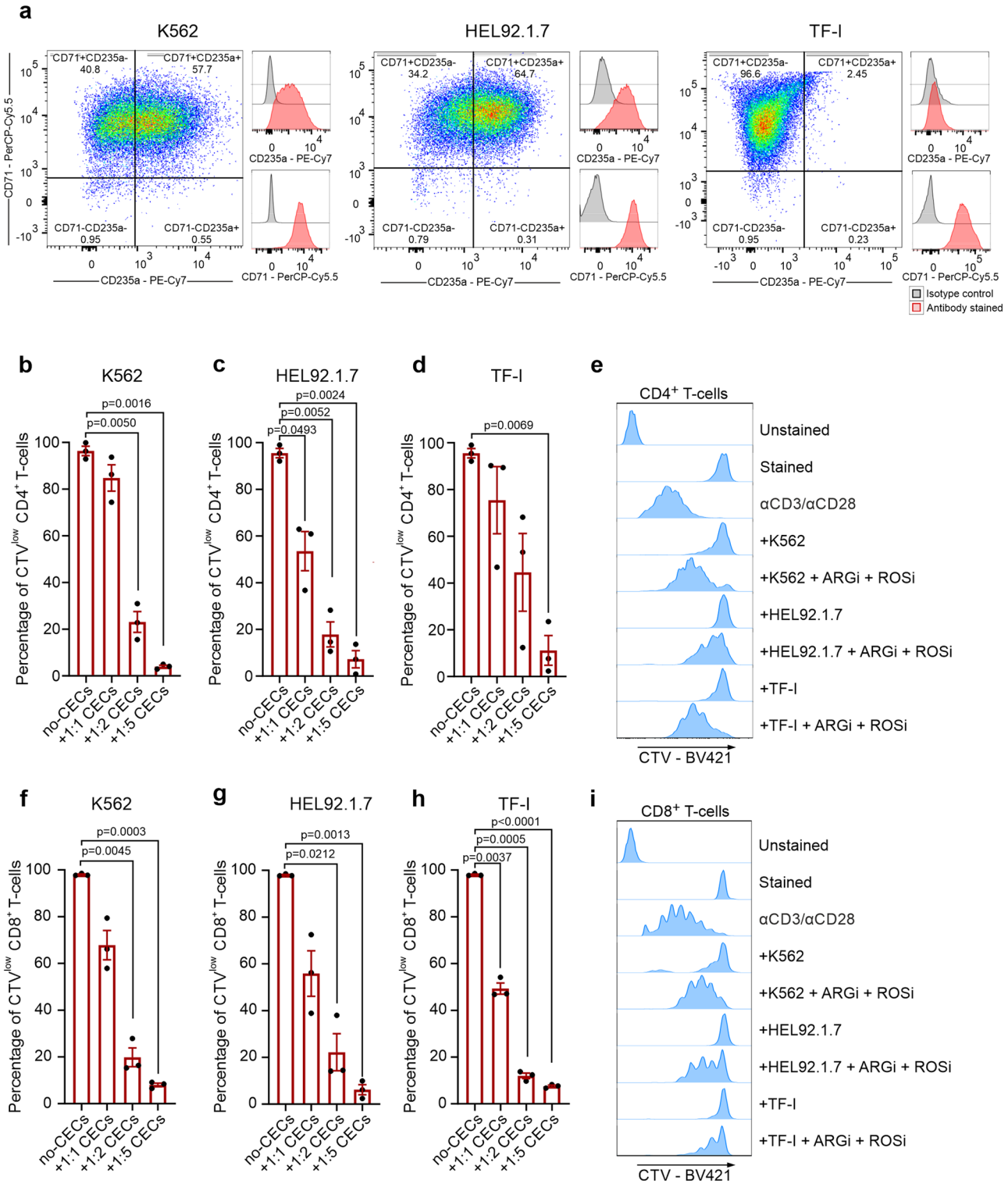


Fig. 9 Erythroleukemia-derived erythroid cell lines suppress T cells in an ARG- and ROS-dependent mechanism. a The levels of CD71 and CD235a in erythroleukemia-derived erythroid cell lines. **b**–**d** Proliferation of CTV-labeled CD4$^+$ T cells triggered by αCD3/αCD28 co-cultured with K562 (**b**), HEL92.1.7 (**c**), and TF-I (**d**) erythroid cell lines at different ratios (T cells:CECs) ($n = 3$). *P* values were calculated with repeated measures ANOVA with Dunnett's post hoc test. **e** Representative histograms of CTV-BV421 fluorescence of CD4$^+$ T cells co-cultured with erythroid cells at a 1:2 ratio in the presence of ARGi (OAT-1746, 1.5 μM) and ROSi (NAC, 200 μM) ($n = 2$). Statistical analyses are provided in Supplementary Fig. 18a. **f**–**h** Proliferation of CTV-labeled CD8$^+$ T cells triggered by αCD3/αCD28 co-cultured with K562 (**f**), HEL92.1.7 (**g**), or TF-I (**h**) erythroid cells at different ratios ($n = 3$). *P* values were calculated with repeated measures ANOVA with Dunnett's post hoc test. **i** Representative histograms of CTV-BV421 fluorescence of CD8$^+$ T cells co-cultured with erythroid cell lines at a 1:2 ratio in the presence of ARGi (OAT-1746, 1.5 μM) and ROSi (NAC, 200 μM) ($n = 2$). Statistical analyses are provided in Supplementary Fig. 18b. Data show means ± SEM. *n* values are the numbers of biological replicates in in vitro experiments. The source data underlying **b**–**d**, **f**–**h** are provided as a Supplementary Data 2 file.

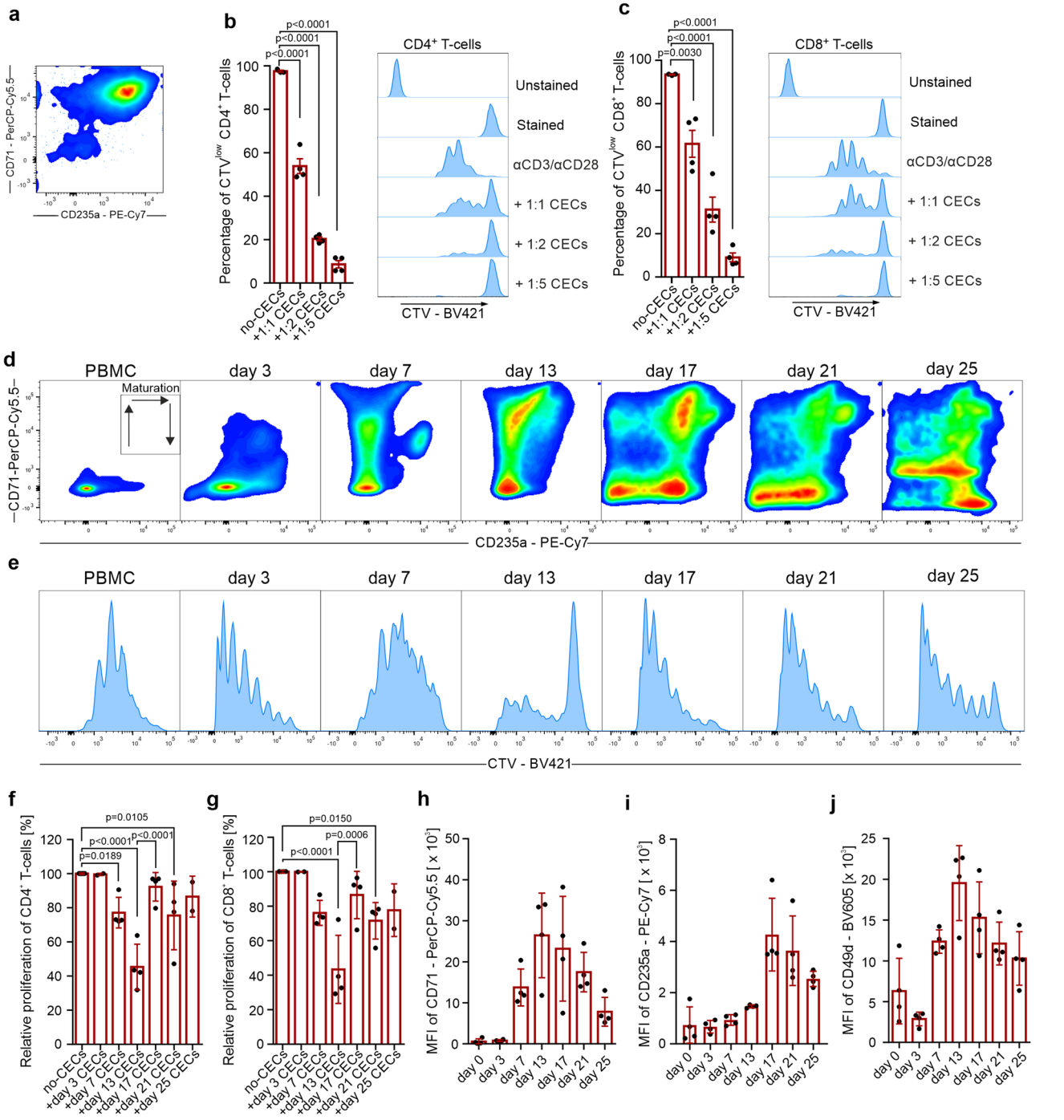


Fig. 10 Suppression of T cells is a general feature of erythroid cells that diminishes with CECs maturation. a Representative plot of isolated CECs differentiated from PBMCs. **b**, **c** Proliferation of CTV-labeled CD4$^+$ (**b**) and CD8$^+$ (**c**) T cells triggered by αCD3/αCD28 co-cultured with CECs differentiated from PBMCs ($n = 4$). *P* values were calculated with one-way ANOVA with Dunnett's post hoc test. **d** Representative density plots of CEC differentiation from PBMCs based on CD71 and CD235a expression. **e** Proliferation of CTV-labeled CD4$^+$ triggered by αCD3/αCD28 co-cultured with CEC-PBMCs at different developmental stages at 1:4 ratio. **f**, **g** Relative proliferation of CD4$^+$ (**f**) and CD8$^+$ (**g**) T cells co-cultured with CEC-PBMCs at different time points at a 1:4 ratio. *P* values were calculated with one-way ANOVA with Bonferroni's post hoc test. **h**–**j** Levels of CD71 (**h**), CD235a (**i**), and CD49d (**j**) during erythroid differentiation from PBMCs. Data show means ± SD. Each point in **b**, **c**, **f**–**j** represents data from individual patients. *n* values are the numbers of individual patients used to obtain the data or the number of biological replicates in in vitro experiments. The source data underlying **b**, **c**, **f**–**j** are provided as a Supplementary Data 2 file.

murine T cells with CECs. Expansion of CECs in the spleen of anemic mice resulted in the increased ARG activity in the spleen leading to the L-arginine starvation of T cells, decreased levels of CD3ζ, and suppressed proliferation. Moreover, human CECs expressed both ARG1 and ARG2 and suppressed T cell proliferation in an ARG-dependent manner. Thus, expansion of ARG-expressing CECs in anemia may induce immune suppression, similar to the expansion of ARG-expressing myeloid cells in cancer[33] and in females over males[34], and during pregnancy[35].

CECs were also reported to modulate immune response via ROS in tumor-bearing mice and cancer patients[10]. We found that ROSi restored T cell proliferation in co-culture with CECs from anemic mice to a similar extend as ARGi. Importantly, ROS also may decrease CD3ζ in T cells[36]. However, ROSi restored CD3ζ decreased by CECs only in combination with ARGi, which confirms that ARG cooperates with ROS in CECs to induce T cells hyporesponsiveness to proliferative triggers.

Importantly, we demonstrated that previously described lack of immunosuppressive capacities of CECs in anemic mice[10] resulted from the interaction between PHZ used to induced anemia and ARGs. PHZ-induced HA is one of the most commonly used models of anemia. PHZ leads to the formation of ferrihemoglobin from oxyhemoglobin and production of free radicals that disrupt the interactions between hem and globin chains leading to the formation of Heinz bodies and hemolysis[21]. However, PHZ-induced CECs are less effective in suppressing T cell proliferation as compared with CECs isolated from neonatal or other anemic mice. We show that PHZ targets ARGs, critical immunomodulating enzymes. It needs to be considered in future studies that the interaction between PHZ and ARGs may have considerable effects on the obtained results.

Anemia correlates with worse outcomes in many diseases, including pneumonia[37] or cancer[38]. Moreover, preoperative anemia is associated with an increased risk of infection and mortality in patients undergoing surgery[39,40]. We demonstrated that CECs expand in anemic patients and may suppress the production of cytokines by T cells. A small fraction of $CD45^+$ CECs in the peripheral blood of our cohort subject is consistent with a recent report on CECs in systemic juvenile idiopathic arthritis patients[41]. In line with our results, a recent study showed that anemia status influences the blood transcriptome with enrichment of erythrocyte differentiation genes as well as *ARG1* in anemic children, but decreased signatures of $CD4^+$ T cell activation and differentiation[42]. It remains unknown to which extent CECs are responsible for immune suppression and whether in these conditions supplementation of iron, vitamin B_{12}, or administration of erythropoiesis-stimulating agents including EPO may restore immune response.

Erythropoiesis is a continuum of developmental states that gives mature red blood cells from a hematopoietic stem cell (HSC) and is strictly regulated by multiple factors[43]. Recent studies demonstrated that immunomodulatory properties are strong in early-stage $CD45^+$ CECs in contrast to more mature $CD45^-$ CECs that lack these capacities[9,10,17,44]. However, other studies also reported the immunomodulatory role of $CD45^-$ CECs[41,45], which suggests that CD45 alone may not be a reliable marker of immunosuppressive CECs. Here, we showed that human CECs acquire immunomodulatory properties during erythroid differentiation and are the most potent in an early stage characterized by the $CD71^{high}$ $CD235a^{mid}$ phenotype. Further erythroid maturation is associated with the disappearance of the suppressive properties. These early-stage CECs are relatively rare in the peripheral blood of anemic individuals, which may be a reason for the lack of T cell proliferation suppression. In contrast, human bone marrow is enriched in early-stage CECs that suppress T cells proliferation. Nonetheless, the suppression of T cells by bone marrow CECs was substantially weaker than that of $CD71^{high}$ $CD235a^{mid}$ CECs from ex vivo culture. This may be explained by the fact that in human bone marrow CECs at the earliest stages of differentiation are still much less abundant than late-stage CECs[27,46].

The exact role of transient immunomodulatory properties of CECs remains elusive. It was suggested that expansion of CECs in neonates provides tolerance to harmless antigens, including the commensal microbiota[4], and minimalize damage caused by inflammation in the intestines[4], liver[7], and lungs[47] during the first days of postnatal life. In adults, the role of CECs seems to be similar. Recent studies demonstrated that stress erythropoiesis is a key inflammatory response[48]; therefore, expansion of CECs may prevent progression to chronic inflammation. Indeed, transfer of CECs suppressed inflammatory response and attenuated the wasting syndrome in murine models of colitis[11]. In cancer, which is characterized by a chronic inflammation[49], CECs substantially expand and suppress immune response facilitating tumor growth and increasing the susceptibility to pathogens[10]. On the other hand, impaired immunoregulatory properties of CECs may exacerbate the damage caused by inflammation[50]. Moreover, CECs by suppressing production of IFN-γ, a crucial inflammatory cytokine and a potent inhibitor of erythropoiesis[51,52], may allow maintenance of erythropoiesis and play a role in preventing systemic inflammation.

Importantly, most of the knowledge on CECs has been built based on murine models[5]. However, several crucial divergences between mice and humans may limit the translational character of CECs studies in mice, which include differences in stress erythropoiesis[53]. In mice, stress erythropoiesis primarily takes place in the spleen and relies on the expansion of early-stage CECs[54]. Thus, suppressive CECs may interact with immune cells in the spleen, which is also an active immune organ[55]. We observed that T cells in anemic mice are rather locally suppressed in the spleen, while not affected in the lymph nodes, similar to the conditions described for neonates[4]. In contrast, in humans stress erythropoiesis primarily involves the bone marrow, and expansion of CECs in extramedullary sites is rather occasional[5,53]. Moreover, CECs at the earliest stages of differentiation are relatively rare in healthy individuals[46]. Thus, it seems that CECs may have the most significant immunoregulatory role under conditions that are characterized by the impaired erythroid differentiation and robust enrichment of early-stage CECs, which include cancer[56].

Our findings might be of relevance in better understanding the mechanisms underlying suppressed cell-mediated immunity and anti-bactericidal capacity of leukocytes[57] and the impaired of T cell mediated immunity in anemic children[58]. Moreover, our study suggests that CECs may be a crucial regulator of immune response in different disease conditions.

Methods

Reagents. Recombinant human ARG1 was obtained from Biolegend (San Diego, CA, USA), recombinant murine Arg1 was obtained from Cloud-Clone Corp., arginase inhibitor OAT-1746 was synthesized at OncoArendi Therapeutics, Warsaw, Poland. All other reagents, if not otherwise stated, were obtained from Sigma-Aldrich.

Cell lines. K562, HEL92.1.7, and TF-I cell lines were purchased from American Type Culture Collection (ATCC). Cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone), 2 mM L-glutamine (Sigma-Aldrich) 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich) at 37 °C in a humidified atmosphere of 5% CO_2 in the air. Additionally, TF-I cells medium contained 2 ng/ml of recombinant human GM-CSF (R&D Systems). Cells have been cultured for no longer than 4 weeks after thawing and were regularly tested for *Mycoplasma* contamination using PCR technique and were confirmed to be negative.

Human samples. Human PBMCs used as a source of $CD4^+$ and $CD8^+$ T cells and used for the expansion and differentiation of PBMC-derived CECs were isolated from buffy coats obtained commercially from the Regional Blood Centre in Warsaw, Poland. PBMCs were isolated by density-gradient centrifugation method using Histopaque®-1077 (Sigma-Aldrich) or Lymphoprep™ (STEMCELL Technologies), according to the manufacturer's protocols. All donors were males between the ages of 18 and 45 years old. Donors were screened for general health and qualified by the physician for blood donation. All donors had negative clinical laboratory tests for HIV-1, HIV2, hepatitis B, and hepatitis C and hematology values within normal ranges.

Peripheral blood samples were obtained from patients hospitalized in the Central Teaching Clinical Hospital, Medical University of Warsaw or treated in the Outpatient Clinic of Central Teaching Clinical Hospital, Medical University of Warsaw, Warsaw. The study was conducted in accordance with the Declaration of Helsinki. The study was approved by the Bioethical Committee of the Medical University of Warsaw (KB/8/2021). Patients with or without anemia based on WHO diagnostic criteria[59] were enrolled in the study. Patients with proliferative diseases, including cancer, were excluded. The blood samples were obtained by venipuncture and subjected to complete blood count evaluation. The remaining blood was used for further examination. Flow cytometry was performed as described below. CountBright™ Absolute Counting Beads (Thermo Fisher Scientific) were used for CECs counting. PBMCs were purified from whole blood of anemic and non-anemic patients by density separation using Lymphoprep (STEMCELL Technologies).

Human bone marrow samples were commercially obtained from Lonza Walkersville, Inc. Aspirates were withdrawn from bilateral punctures of the posterior iliac crests. Every 100 ml of bone marrow was collected into syringes containing 10 ml of heparin (Porcine Intestinal Mucosa) Sodium Injection (~100 units heparin per ml bone marrow). Bone marrow samples were obtained from both healthy males ($n = 6$) and healthy non-pregnant females ($n = 3$) US-based donors between the ages of 23 and 45 years old. Samples were collected after obtaining permission for their use in research applications by informed consent. All donors were screened for general health and negative medical history for heart disease, kidney disease, liver disease, cancer, epilepsy, and blood or bleeding disorders. All donors had negative clinical laboratory tests for HIV-1, HIV2, hepatitis B, and hepatitis C.

Mobilized PBSCs were obtained from familial donors from the material remaining after allogeneic stem cell transplantation. PBSCs were mobilized with the granulocyte colony-stimulating factor (G-CSF) and isolated from the donor peripheral blood using apheresis according to the standard clinical protocol. Informed consent was obtained from PBSC donors. Collected PBSCs were subjected to density-gradient centrifugation using Lymphoprep (STEMCELL Technologies) to remove dead cells and debris, washed three times with RPMI medium, and used for the analysis.

Mice. Wild-type C57BL/6 mice, both males and females, 8- to 14-week-old were obtained from the Animal House of the Polish Academy of Sciences, Medical Research Institute (Warsaw, Poland). Transgenic mice of C57BL/6 genetic background, B6.129S4-Arg1^{tm1Lky}/J (YARG mice co-expressing Arg1 and eYFP[60], stock #015857), C57BL/6-Tg(TcraTcrb) 1100Mjb/J (OT-I, TCR transgenic mice producing OVA peptide-specific CD8$^+$ T cells[61], stock #003831) and Arg2^{tm1Weo}/J (Arg2$^{-/-}$, Arg2 functional knockout[26]), were purchased from the Jackson Laboratories and bred at the animal facility of the Department of Immunology, Medical University of Warsaw. Mice were housed in controlled environmental conditions in specific-pathogen-free (SPF) conditions (breeding cages, OT-I mice) or conventional (others) animal facility of the Department of Immunology, Medical University of Warsaw, with water and food provided ad libitum. For mice genotyping, DNA was isolated with DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. The concentration and purity of DNA were determined using the NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific). PCR reaction was performed using OneTaq® 2× Master Mix with Standard Buffer (New England Biolabs). Primers sequences, PCR, and agarose electrophoresis conditions were set according to genotyping protocols available on The Jackson Laboratory website (https://www.jax.org). The experiments were performed in accordance with the guidelines approved by the II Local Ethics Committee in Warsaw (approval No. WAW2/117/2019 and WAW2/143/2020) and in accordance with the requirements of the EU (Directive 2010/63/EU) and Polish (Dz. U. poz. 266/15.01.2015) legislation.

Animal anemia models. To induce NHA mice were phlebotomized 4 and 2 days before harvest. At least 100 µl of blood was collected each time. To induce HA, mice were injected intraperitoneally (i.p.) three days before harvest with 50 mg per kg body weight of PHZ hydrochloride solution (HA-PHZ) or mice were injected intravenously (i.v.) 6 days before harvest with 45 µg of anti-TER119 monoclonal antibody (TER-119, BioXCell) into the caudal vein. For the analysis of stress erythropoiesis, mice were injected i.v. with 30 µg of anti-TER119 monoclonal antibody (TER-119, BioXCell) into caudal vein followed by the monitoring of RBC count in the peripheral blood. For complete blood count, blood was collected into EDTA-coated tubes from inferior palpebral veins and examined using a Sysmex XN-2000 Hematology Analyzer. The parameters of complete blood counts and reference intervals[62] are presented in Supplementary Fig. 1. For the analysis of amino acid concentration, blood was collected into IMPROMINI® Gel & Clot Activator Tubes. Tubes were gently inverted five times to mix the clot activator and incubated for 30 min at room temperature (RT) followed by centrifugation for 10 min at 1000 × g at 4 °C. Serum was collected and stored at −20 °C until analysis. L-Arg concentration in the serum was determined with ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method on Waters Xevo TQ-S mass spectrometer equipped with Waters Acquity UPLC chromatograph (Waters) in the Mass Spectrometry Lab at the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland.

Antibodies. Fluorophore- or biotin-conjugated antibodies specific for mouse cell-surface antigens and cytokines were as follows: anti-CD71 (8D3, NovusBio; R17217, eBioscience, dilution 1:100), anti-TER119 (TER-119, BioLegend, 1:100), anti-CD45.2 (104, BD Biosciences, 1:50), anti-CD45 (30-F11, BioLegend, 1:100), anti-CD44 (IM7, BioLegend, 1:100), anti-CD3e (145-2C11, eBioscience, 1:100), anti-CD4 (GK1.5, eBioscience; RM4-5, eBioscience; 1:100), anti-CD8a (53-6.7, eBioscience, 1:100), anti-CD69 (H1.2F4, eBioscience, 1:100), anti-CD25 (PC61.5, eBioscience, 1:100), anti-CD62L (MEL-14, Invitrogen, 1:100), anti-CD11b (M1/70, BioLegend, 1:100), anti-CD11c (HL3, BD Bioscience, 1:100), anti-CD3 zeta (H146-968, Abcam, 1:500), anti-IFN-γ (XMG1.2, eBioscience, 2.5 µl per test in 50 µl volume), anti-TNF-α (MP6-XT22, eBioscience, 2.5 µl per test in 50 µl volume), anti-Arg1 (polyclonal, IC5868P/F, R&D Systems, 5 µl per test in 100 µl volume), anti-Arg2 (ab81505, Abcam, 1 µl per test in 50 µl volume), goat anti-rabbit IgG (Invitrogen, 1 µl per test in 100 µl volume).

Fluorophore- or biotin-conjugated antibodies specific for human cell-surface antigens and cytokines were as follows: anti-CD71 (CY1G4, BioLegend, DF1513, NovusBio, 1:50), anti-CD235a (HI264, BioLegend, 1:50), anti-CD44 (IM7, BioLegend, 1:100), anti-CD25 (BC96, eBioscience, 1:50), anti-CD69 (FN50, eBioscience, 1:50), anti-CD45 (HI30, BD Bioscience, 1:50), anti-CD49d (9F10, eBioscience, 1:50), anti-CD36 (NL07, eBioscience, 1:50), anti-CD34 (561, 25 BioLegend, 1:50), anti-CD3 (OKT3, eBioscience, 1:50), anti-CD4 (RPA-T4, eBioscience, 1:50), anti-CD8a (RPA-T8, eBioscience, 1:50), anti-IFN-γ (4S.B3, BioLegend, 2.5 µl per test in 50 µl volume), anti-TNF-α (MAb11, BD Bioscience, 2.5 µl per test in 50 µl volume), anti-Arg1 (polyclonal, IC5868P/F, R&D Systems, 5 µl per test in 100 µl volume), anti-Arg2 (ab137069, Abcam, 1 µl per test in 50 µl volume), goat anti-rabbit IgG (Invitrogen, 1 µl per test in 100 µl volume).

Flow cytometry analysis. Flow cytometry was performed on FACSCanto II (BD Biosciences) or Fortessa X20 (BD Biosciences) operated by FACSDiva software. For data analysis FlowJo v10.6.1 software (Tree Star) or BD FACSDiva software (BD Biosciences) were used. Murine whole blood was collected from the inferior palpebral vein to EDTA-coated tubes. Spleens were isolated from mice and mechanically dispersed by pressing gently through a 70-µm nylon cell strainer using a rubberized 1 ml syringe piston. Murine bone marrow was isolated from the femur by the centrifugation method. Briefly, femurs were dissected, followed by the removal of any muscle or connective tissue. The condyles and epiphysis were removed and a cleared bone was placed in microcentrifuge tubes followed by centrifugation at 2500 × g for 30 s. Bone marrow cells were filtered through a 70-µm nylon strainer and used for further analysis. No erythrocyte lysis was performed in flow cytometry analysis or experiments that involved analysis or isolation of CECs or RBCs. If only other types of cells were analyzed using flow cytometry, erythrocytes were lysed using ACK (ammonium-chloride-potassium) Lysing Buffer (Thermo Fisher Scientific), according to the manufacturer's protocol.

For cell surface staining, cells were stained with Zombie NIR™, Zombie UV™ or Zombie Aqua™ Fixable Viability Kit (BioLegend), blocked on ice with 5% normal rat serum in FACS buffer (PBS; 1% BSA, 0.01% sodium azide), and then incubated for 30 min on ice with fluorochrome-labeled antibodies. Fluorochrome-conjugated antibodies used for the staining are listed above. After washing in FACS buffer, cells were immediately analyzed.

For nucleus staining, cells were incubated before cell surface staining with Hoechst 33342 Fluorescent Stain (Invitrogen) at a final concentration of 1 µg/ml in Dulbecco's phosphate-buffered saline (DPBS) for 30 min, followed by a wash in DPBS. For mitochondrial staining, cells were incubated before cell surface staining with MitoSpy™ Red CMXRos (BioLegend) at a final concentration of 50 nM in RPMI medium at 37 °C for 30 min, followed by a wash in FACS buffer.

For intracellular staining, membrane-stained cells were fixed using Fixation Buffer for 30 min at RT, followed by a wash with permeabilization buffer, and staining with an antibody diluted in permeabilization buffer for 30 min at RT (Intracellular Fixation & Permeabilization Buffer Set, eBioscience). For anti-ARG2 indirect intracellular staining, cells were fixed using Fixation Buffer for 30 min at RT, followed by a wash with permeabilization buffer, and staining with anti-ARG2 antibody for 1 h at RT, followed by a wash with permeabilization buffer and staining with fluorochrome-conjugated goat anti-rabbit IgG for 30 min at RT. Gating strategies used to analyze the flow cytometry data are presented in Supplementary Figs. 26–44.

IFN-γ and TNF-α production assay of murine cells. Murine splenocytes were isolated from anemic or healthy mice. Cells were plated in round-bottomed 96-well plates (1 × 10^6 cell per well) in L-arginine-free RPMI medium (SILAC RPMI medium, Thermo Fisher Scientific) supplemented with 10% dialyzed FBS (Thermo Fisher Scientific), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 40 mg/l L-lysine, and 150 µM L-arginine (all from Sigma-Aldrich). Splenocytes were stimulated with heat-killed *E. coli 0111:B4* (HKEc, InvivoGen) at the concentration 1 × 10^6 cells per ml or Dynabeads Mouse T-Activator CD3/CD28 (ratio 1:2, Thermo Fisher Scientific) in the presence of protein transport inhibitor (BD GolgiStop™) for 6 h. Then, cells were stained with cell surface antigen-binding antibodies, followed by fixation, permeabilization, and intracellular staining for IFN-γ and TNF-α. Flow cytometry was performed on Fortessa X20 (BD Biosciences). Cell viability after culture in the presence of protein transport inhibitor was >80%.

IFN-γ and TNF-α production assay of human cells. Human PBMCs were isolated from the peripheral blood of anemic or non-anemic patients. Cells were plated in round-bottomed 96-well plates (1×10^6 cell per well) in L-arginine-free RPMI medium (SILAC RPMI medium, Thermo Fisher Scientific) supplemented with 10% dialyzed FBS (Thermo Fisher Scientific), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 40 mg/l L-lysine, and 150 µM L-arginine (all from Sigma-Aldrich). PBMCs were stimulated with heat-killed *E. coli 0111:B4* (HKEc, InvivoGen) at the concentration 1×10^6 cells per ml for TNF-α assessment in myeloid cells or Dynabeads Human T-Activator CD3/CD28 (ratio 1:2, Thermo Fisher Scientific) for IFN-γ assessment in T cells in the presence of protein transport inhibitor (BD GolgiStop™) for 12 h. Then, cells were stained with cell surface antigen-binding antibodies, followed by fixation, permeabilization, and intracellular staining for IFN-γ and TNF-α. Flow cytometry was performed on Fortessa X20 (BD Biosciences). Cell viability after culture in the presence of protein transport inhibitor was >93%.

For the analysis of the effect of PBMC-derived CECs on T cell activation and IFN-γ production, PBMCs were isolated from the peripheral blood healthy blood donor. $CD4^+$ or $CD8^+$ T Cells were isolated from PBMC using EasySep™ Human $CD4^+$ or $CD8^+$ T-Cell Isolation Kit (STEMCELL Technologies) according to the manufacturer's protocols. $CD4^+$ or $CD8^+$ T cells were plated in round-bottomed 96-well plates (2×10^4 cell per well) in L-arginine-free RPMI medium (SILAC RPMI medium, Thermo Fisher Scientific) supplemented with 10% dialyzed FBS (Thermo Fisher Scientific), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 40 mg/l L-lysine, and 150 µM L-arginine (all from Sigma-Aldrich). T cells were stimulated with Dynabeads Human T-Activator CD3/CD28 (ratio 1:2, Thermo Fisher Scientific) in the presence of PBMC-derived CECs (ratio 1:2, 4×10^5 CECs per well). The arginase inhibitor OAT-1746 (1500 nM) or *N*-acetylcysteine (200 µM) was added as indicated in the figures. In these concentrations, ARGi and ROSi had no effects on T cells nor CECs viability. Human T cells were incubated for 72 h at 37 °C in 5% CO_2. Protein transport inhibitor (BD GolgiStop™) was added for the last 12 h. Then, cells were stained with cell surface antigen-binding antibodies, followed by fixation, permeabilization, and intracellular staining for IFN-γ and TNF-α. Flow cytometry was performed on Fortessa X20 (BD Biosciences). Cell viability after culture in the presence of protein transport inhibitor was >86%.

In vivo OVA immunization and analysis of the humoral response. Control and NHA mice were immunized with albumin from chicken egg white (OVA, Ovalbumin) from Sigma (Grade VII). Each mouse received 25 µg of OVA with Imject™ Alum Adjuvant (ALUM, Thermo Fisher Scientific) at a ratio of 1:1 in the final volume of 100 µl per mouse administered i.p. After 14 days, mice were challenged once again with the same dose of OVA-ALUM. NHA mice were divided into three groups. NHA before mice were phlebotomized before first immunization, NHA boost mice were phlebotomized before second OVA immunization, and NHA both were phlebotomized before first and second immunization (see Supplementary Fig. 3c). Untreated mice received Imject™ Alum Adjuvant without OVA. Blood was obtained from mice 14 days after the second immunization; plasma was isolated and stored at −80 °C. The concentration of anti-OVA IgG antibodies was determined using Anti-Ovalbumin IgG1 (mouse) ELISA Kit (Cayman Chemical).

In vivo proliferation assay. OVA (SIINFEKL)-specific $CD8^+$ T cells were isolated from the spleen and lymph nodes of healthy 6–8-week-old OT-I mice using EasySep™ Mouse $CD8^+$ T-Cell Isolation Kit (STEMCELL Technologies) according to the manufacturer's protocols. Isolated OT-I cells labeled with CTV for 20 min at 37 °C at a final concentration of 2.5 µM in PBS, washed, and transferred into the caudal tail vein of host C57BL/6 mice at a cell number of 7×10^6 in 150 µl of PBS. Twenty-four hours post OT-I T cells inoculation, host mice were challenged with 7.5 µg of full-length OVA protein (grade V, Sigma-Aldrich) injected into the caudal tail vein. Three mice from controls were injected only with PBS (negative control). On day 3 post OVA immunization, spleens were harvested, mashed through a 70-µm nylon strainer, stained with OVA-specific MHC tetramers (iTAg Tetramer/PE-H-2 K^b OVA (SIINFEKL), MBL Inc., WA, USA) to detect OT-I $CD8^+$ T cells, followed by anti-CD3 and anti-CD8 staining, and analyzed for proliferation by flow cytometry. The gate for proliferating cells (CTV^{low}) was set using unstimulated negative control. OT-I T cells with lower fluorescence of CTV than non-proliferating T cells were identified as proliferating cells.

Murine T cell proliferation assay. Murine T cells were isolated from spleens of healthy 6–8-week-old C57BL/6 mice using EasySep™ Mouse $CD4^+$ or $CD8^+$ T-Cell Isolation Kit (STEMCELL Technologies) according to the manufacturer's protocols. CECs were isolated from the spleens of anemic mice using the EasySep™ Release Mouse Biotin Positive Selection Kit (STEMCELL Technologies) according to the manufacturer's protocols. Biotin-conjugated anti-CD71 antibodies (anti-mouse clone 8D3, NovusBio,) were used at a final concentration of 1 µg/ml. CECs purity was >80%. For cell proliferation assay, T cells were labeled with Cell Trace Violet (CTV) dye (Thermo Fisher Scientific) for 20 min at 37 °C at a final concentration of 2.5 µM in PBS. Next, the labeled T cells were plated in round-bottomed 96-well plates (5×10^4 cell per well) in L-arginine-free RPMI medium (SILAC RPMI medium, Thermo Fisher Scientific) supplemented with 10% (v/v) dialyzed FBS (Thermo Fisher Scientific), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% (v/v) MEM non-essential amino acids solution (Thermo Fisher Scientific), 50 µM 2-mercaptoethanol (Thermo Fisher Scientific), and 150 µM L-arginine and 40 mg/l L-lysine (Sigma-Aldrich). Proliferation was triggered by the stimulation with Dynabeads Mouse T-Activator CD3/CD28 (ratio 1:2, Thermo Fisher Scientific). The arginase inhibitor OAT-1746 (ARGi, 500 nM), L-arginine (1000 µM), or *N*-acetylcysteine (ROSi, 100 µM) were added as indicated in the figures. In these concentrations, ARGi and ROSi had no effects on T cells nor CEC viability. Murine CECs were added to the wells in a 1:2 ratio (1×10^5 CECs per well). Murine T cells were incubated for 72 h at 37 °C in 5% CO_2. Then, cells were harvested, stained with live/dead Zombie dye (Biolegend), anti-CD3 and anti-CD4 or anti-CD8 antibody, and analyzed by flow cytometry. The gate for proliferating cells was set based on the unstimulated controls. Cell autofluorescence was determined using CTV-unstained controls. Percentages of proliferating cells were calculated using the FlowJo Software v10.6.1 (Tree Star).

Human T cell proliferation assay. Human T cells were isolated from PBMC isolated from buffy coats commercially obtained from the Regional Blood Centre in Warsaw, Poland using EasySep™ Human $CD4^+$ or $CD8^+$ T-Cell Isolation Kit (STEMCELL Technologies) according to the manufacturer's protocols. CECs were isolated from human bone marrow aspirates or PBMC-derived CECs culture using EasySep™ Release Mouse Biotin Positive Selection Kit (STEMCELL Technologies) according to the manufacturer's protocols. Biotin-conjugated anti-CD71 antibodies (anti-human clone DF1513, NovusBio) were used at a final concentration of 1 µg/ml. CECs purity was >80%. For cell proliferation assay, T cells were labeled with Cell Trace Violet (CTV) dye (Thermo Fisher Scientific) for 20 min at 37 °C at a final concentration of 2.5 µM in PBS. Next, the labeled T cells were plated in round-bottomed 96-well plates (2×10^4 cell per well) in L-arginine-free RPMI medium (SILAC RPMI medium, Thermo Fisher Scientific) supplemented with 10% (v/v) dialyzed FBS (Thermo Fisher Scientific), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% (v/v) MEM non-essential amino acids solution (Thermo Fisher Scientific), 50 µM 2-mercaptoethanol (Thermo Fisher Scientific), and 150 µM L-arginine and 40 mg/l L-lysine (Sigma-Aldrich). Proliferation was triggered by the stimulation with Dynabeads Human T-Activator CD3/CD28 (ratio 1:2, Thermo Fisher Scientific). The arginase inhibitor OAT-1746 (1500 nM) or *N*-acetylcysteine (200 µM) was added as indicated in the figures. In these concentrations, ARGi and ROSi had no effects on T cells nor CECs viability. Human CECs were added to the wells in a 1:2 ratio (4×10^5 CECs per well). T cells were incubated for 120 h at 37 °C in 5% CO_2. Then, cells were harvested, stained with live/dead Zombie dye (Biolegend), anti-CD3 and anti-CD4 or anti-CD8 antibody, and analyzed by flow cytometry. The gate for proliferating cells was set based on the unstimulated controls. Cell autofluorescence was determined using CTV-unstained controls. Percentages of proliferating cells were calculated using the FlowJo Software v10.6.1 (Tree Star).

CEC-conditioned medium (CM). Conditioned medium was obtained by culturing CECs in the arginine-free RPMI medium (SILAC RPMI medium, Thermo Fisher Scientific) supplemented with 150 µM L-arginine at the density 1×10^6 cells/ml for 24 h. Cells were centrifuged and the supernatant was collected and immediately frozen at −80 °C. After thawing, the supernatant was filtered through a 0.45 µm Syringe Filter (Wenk LabTec) and was used in experiments a 1:1 ratio with 150 µM L-arginine RPMI SILAC medium.

ROS detection. The level of ROS in cells was determined using CellROX Green Reagent (Thermo Fisher Scientific) or 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA). Cells were stained with CellROX at a final concentration of 5 µM or DCFDA at a final concentration of 10 µM in pre-warmed PBS for 30 min at 37 °C, followed by three washes with PBS. H_2O_2-treated cells served as positive controls. For some experiments, cells stained with CellROX or DCFDA were further stained with fluorochrome-labeled antibodies on ice. Stained cells were acquired on Fortessa X20 flow cytometer (BD Biosciences).

Arginase activity assay and Griess test. Recombinant enzymes (ARG1 and ARG2) to study ARGi were produced at OncoArendi Therapeutics in *E. coli* expression system and purified by the FPLC method. The proteins were purified by FPLC and stored at −80 °C in the storage buffer containing: 20 mM Tris pH 8.0, 100 mM NaCl, 10 mM DDT, and 10% glycerol. The basic assay buffer was composed of 100 mM sodium phosphate buffer, 130 mM sodium chloride, 1 mg/ml BSA, pH 7.4. The enzymatic reaction was carried out in the presence of 200 µM $MnCl_2$ (cofactor) and 10 or 20 mM L-arginine hydrochloride (for hARG1 or hARG2, respectively), mixed at the final volume of 25 µL. Basic developing buffer contained 50 mM boric acid, 1 M sulfuric acid, 0.03% (m/v) Brij® 35 detergent. PHZ or ABH was diluted in basic assay buffer at the volume of 50 µL. The recombinant enzyme was diluted in a basic assay buffer at the volume of 25 µL. The reaction was performed at the final volume of 100 µL. Developing mixture included freshly prepared equal volume mixture of developing solution A (4 mM *o*-phthaldialdehyde) and solution B (4 mM *N*-(1-naphthyl)ethylenediamine dihydrochloride) prepared in the basic developing buffer. The compound background wells contained each of the tested compounds and the substrate/cofactor mixture,

but not the recombinant enzyme (data were excluded from the analysis when the compound background exceeded 10% of the signal obtained in the wells with enzyme). The "0% activity" background wells contained only the substrate/cofactor mixture. Following 1 h incubation at 37 °C, the freshly prepared developing reagent was added (150 µl) and the colorimetric reaction was developed (12 min at RT, gentle shaking). The absorbance, proportional to the amount of the produced urea, was measured at 515 nm using Tecan's Spark™ microplate reader. Data were normalized by referring the absorbance values to the positive control wells (100% enzyme activity). The IC_{50} value was determined by the nonlinear regression method. Arginase activity in the CECs or splenocytes lysates and cell supernatant was determined using Arginase Activity Assay (Sigma) according to the manufacturer's protocol.

To evaluate nitric oxide (NO) production as a measure of NOS (nitric oxide synthase) activity, Griess Reagent System (Promega) was used according to the manufacturer's protocol. Splenocytes or CECs were isolated from murine spleens and were cultured in non-adherent six-well plate 1×10^6 or 5×10^5 cells per 2 ml, respectively, for 24 h followed by supernatants collection and measurement of NO concentration.

Bioinformatical analysis of arginase structure. The structure and predicted binding energies for the complexes of PHZ, L-arginine, and 2-amino-6-borono-2-(2-(piperidin-1-yl)ethyl)hexanoic acid with both human and mouse arginases were compared. The 3D models of mouse arginases were proposed using available structures of human arginases (pdb|4hww and pdb|4hze for ARG1 and ARG2, respectively) as templates. Both templates shared more than 87% sequence identity with their respective target. The sequence to structure alignments between mouse arginases and selected templates were calculated with the muscle program[63]. The 3D structure was proposed with MODELLER[64]. Models quality was assessed with the Molprobity webserver[65]. Next, both human and mouse proteins were prepared for docking using the Chimera dock prep module. Molecular docking was carried out with two programs—GOLD[66] and Surflex[67]. The active site was specified based on the position of the inhibitor present in the active site of the arginase 1 (pdb|4hww). The default parameters of both programs were used.

To assess if PHZ remains stably bound to the active site of both human arginases short molecular dynamics simulations were performed. The initial configurations of ligand–protein complexes were derived from docking results for PHZ. For the PHZ–arginase complexes the simulation included the following steps. First protein and ligand were put in a dodecahedron box with the distance between solute and a box equal to 1 nm. The 0.1 M NaCl was added to the system including neutralizing counterions. After energy minimization using the steepest descent algorithm, 100 ps NVT and NPT simulation were carried out. For this modified Berendsen thermostat was used to maintain the temperature at 310 K using and Berendsen barostat to keep the pressure at 1 atm. Positions of both protein and ligand heavy atoms remained constrained. During the following 300 ps of simulation time, the ligand's constraints were gradually removed. Finally, an unconstrained 100 ns simulation is performed in which Berendsen barostat was replaced by Parrinello-Rahman barostat. During simulation short-range nonbonded interactions were cut off at 1.4 nm, with long-range electrostatics calculated using the particle mesh Ewald (PME) algorithm. Bonds were constrained using the lincs algorithm. Simulations were carried out with Gromacs[68] using the gromos54a7 force field, modified to include parameters for Mn^{2+} ion adopted from[69]. Spc model was used for water molecules. Parameters for the ligand were obtained with Automated Topology Builder (ATB)[70].

Additional analyses were performed to assess if PHZ can migrate to the arginase active site when present in solute in high concentration. For this analysis protein was put in dodecahedron box with the distance between solute and a box equal to 1.5 nm in which 6 PHZ molecules were placed randomly. This corresponds to 0.02 M concentration of the compound. A similar simulation setup to one described above was used with exception that ligand molecules remained unconstrained throughout simulation.

Protein carbonylation assay. The carbonyl content of proteins was determined in a 2,4-DNPH reaction. Five micrograms of murine ARG1 (Cloud-System Corp) was resuspended in 400 µl of distilled water and incubated with PHZ (10 µM), PHZ (10 µM) with NAC (10 mM), H_2O_2 (10 mM), or water (negative control) as indicated in the Fig. 3d for 1 h at 37 °C. Proteins were precipitated with 10% TCA. The precipitates were treated with either 2 N HCl alone (control) or 2 N HCl containing 5 mg/ml 2,4-DNPH at RT for 30 min. The resulting hydrazones were precipitated in 10% TCA and then washed three times with ethanol–ethyl acetate (1:1). Final precipitates were dissolved in 8 M guanidine chloride. Equal amounts of proteins were separated on 4–12% SDS-polyacrylamide gel (Bio-Rad), transferred onto nitrocellulose membranes (Bio-Rad) blocked with TBST [Tris-buffered saline (pH 7.4) and 0.05% Tween 20] supplemented with 5% non-fat milk. Anti-DNP antibodies (Life Diagnostics, Inc.) at concentration 1 U/ml were used for overnight incubation at 4 °C. After washing with TBST, the membranes were incubated with horseradish peroxidase-coupled secondary antibodies (Jackson Immunores.). The reaction was developed using SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and imaged using ChemiDoc Touch Gel Imaging System (Bio-Rad). Densitometry was done using ImageJ software.

RNA isolation from CECs, reverse transcription, and quantitative polymerase chain reaction. Total RNA was isolated from CECs isolated from murine spleens using RNeasy Mini Kit (Qiagen). RNA was subjected to reverse transcription using the GoScript™ Reverse Transcriptase system (Promega). All qPCRs were performed in MicroAmp Fast Optical 96 WellReaction Plates (Thermo Fisher Scientific) using AppliedBiosystems 7500 Fast Real-Time PCR System with 7500Software V2.0.6 (Thermo Fisher Scientific). Samples were assayed in triplicates. Primers sequences used in the study: *ARG1* forward 5′- CTCCAAGCCAAAGTCCTTAGAG-3′, reverse 5′-AGGAGCTGTCATTAGGGACATC-3′; *ARG2* forward 5′-AGGAGTGGAATATGGTCCAGC-3′, reverse 5′-GGGATCATCTTGTGGGACATT-3′; and GAPDH forward 5′-GAAGGTGGTGAAGCAGGCATC-3′, reverse 5′-GCATCGAAGGTGGAAGAGTGG-3′ as an endogenous control. The mean Ct values of a target gene and endogenous control were used to calculate relative expression using the $2^{-\Delta Ct}$ method.

Western blot. Splenocytes lysates were prepared using Cell Lysis Buffer (#9803, Cell Signaling Technology) supplemented with protease inhibitors (Roche) according to the manufacturer's protocol. Total protein concentration was assessed using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Equal amounts of whole-cell protein lysates samples were boiled in Laemmli loading buffer, separated on 4–12% SDS-polyacrylamide gel (Bio-Rad), transferred onto nitrocellulose membranes (Bio-Rad) blocked with TBST [Tris-buffered saline (pH 7.4) and 0.05% Tween 20] supplemented with 5% non-fat milk. Anti-Arg1 antibodies (polyclonal, GTX109242, GeneTex) at dilution 1:2000 or anti-Arg2 antibodies (polyclonal, ab81505, Abcam) at dilution 1:1000 were used for overnight incubation at 4 °C. After washing with TBST, the membranes were incubated with horseradish peroxidase-coupled secondary antibodies (Jackson ImmunoResearch). The reaction was developed using SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and imaged using ChemiDoc Touch Gel Imaging System (Bio-Rad). After imaging, bound antibodies were removed from membranes using Restore™ PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific), followed by blocking with TBST supplemented with 5% non-fat milk. Next, the membranes were incubated with anti-β-Actin (A5060, Santa Cruz) conjugated with peroxidase. Densitometric quantifications were done using ImageJ software.

Erythroid cells differentiation. CECs were differentiated from human PBMCs according to the protocol by Heshusius et al.[71] with modifications. Human PBMC were purified from buffy coats from healthy donors by density separation using Lymphoprep (STEMCELL Technologies). PBMC were seeded at 1×10^6 cells/ml in erythroid differentiation-promoting medium based on StemSpan™ Serum-Free Expansion Medium (SFEM) supplemented with human recombinant EPO (2 U/ml, Roche), human recombinant stem cell factor (25 ng/ml, R&D Systems), dexamethasone (1 µM, Sigma-Aldrich), human recombinant insulin (10 ng/ml, Sigma-Aldrich), L-glutamine (2 mM, Sigma-Aldrich), iron-saturated holo-transferrin (20 µg/ml, Sigma-Aldrich), sodium pyruvate (1 mM, Gibco), MEM non-essential amino acids (1% v/v, Gibco), bovine serum albumin (0.1% m/v, Sigma-Aldrich), EmbryoMax Nucleosides (1% v/v, Merck), and 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich). The expansion and differentiation of CECs were assessed by flow cytometry.

Statistics and reproducibility. Data are shown as means ± SD or means ± SEM, as indicated in the figure legends. Graphpad Prism 8.4.3 (GraphPad Software) was used for statistical analyses. Data distribution was tested using the Shapiro–Wilk test, D'Agostino & Pearson test, and Kolmogorov–Smirnov test. Statistical analyses of three or more groups were compared using one-way analysis of variance (ANOVA) or Brown–Forsythe ANOVA followed by Tukey's, Dunnett's, or Bonferroni's multiple comparisons test or Kruskal–Wallis test followed by Dunn's multiple comparisons test. Repeated measures ANOVA with Sidak's or Holm–Sidak's post hoc tests were used to analyze the differences in paired samples. Statistical analyses of two groups were compared using unpaired *t*-test, paired *t*-test, or Mann–Whitney test. Methods of statistical analyses are defined in every figure legend. A *P* value of less than 0.05 was statistically significant. Each experiment was performed in technical duplicates or triplicates. The number of biological replicates for each experiment is mentioned in the figure legends.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Source data underlying the graphs and charts presented in the figures are available in the Supplementary Data 2. Uncropped western blots are provided in the Supplementary Data 2 file. Any remaining information can be obtained from corresponding authors upon reasonable request.

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Author contributions

T.M.G. designed and supervised the study, conducted the experiments, analyzed the data, and wrote the manuscript. A.S. participated in in vivo studies. Z.R. participated in in vitro experiments. M.L. and D.P. performed molecular docking and molecular dynamics simulations, K.K. performed real-time qPCR and participated in in vitro experiments, M.M. and O.C. collected and provided human blood samples, A.R.-L. performed analysis of murine blood, M.J. participated in in vitro experiments, P.P. and M.M.-G. carried out arginase activity assays, R.B. designed and synthesized OAT-1746, M.W. bred and provided *Arg2*$^{-/-}$ mice, A.T. and G.B. collected and provided HSPCs. J.G. conceived, designed and supervised the study, provided funding and wrote the manuscript. D.N. provided funding, performed in vivo studies, designed and supervised the study, and wrote the manuscript. All authors edited and approved the final manuscript.

Competing interests

P.P., M.M.-G. and R.B. are employees of OncoArendi Therapeutics, Warsaw, Poland. The remaining authors declare no competing interests.

Additional information

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Potent but transient immunosuppression of T-cells is a general feature of CD71+ erythroid cells

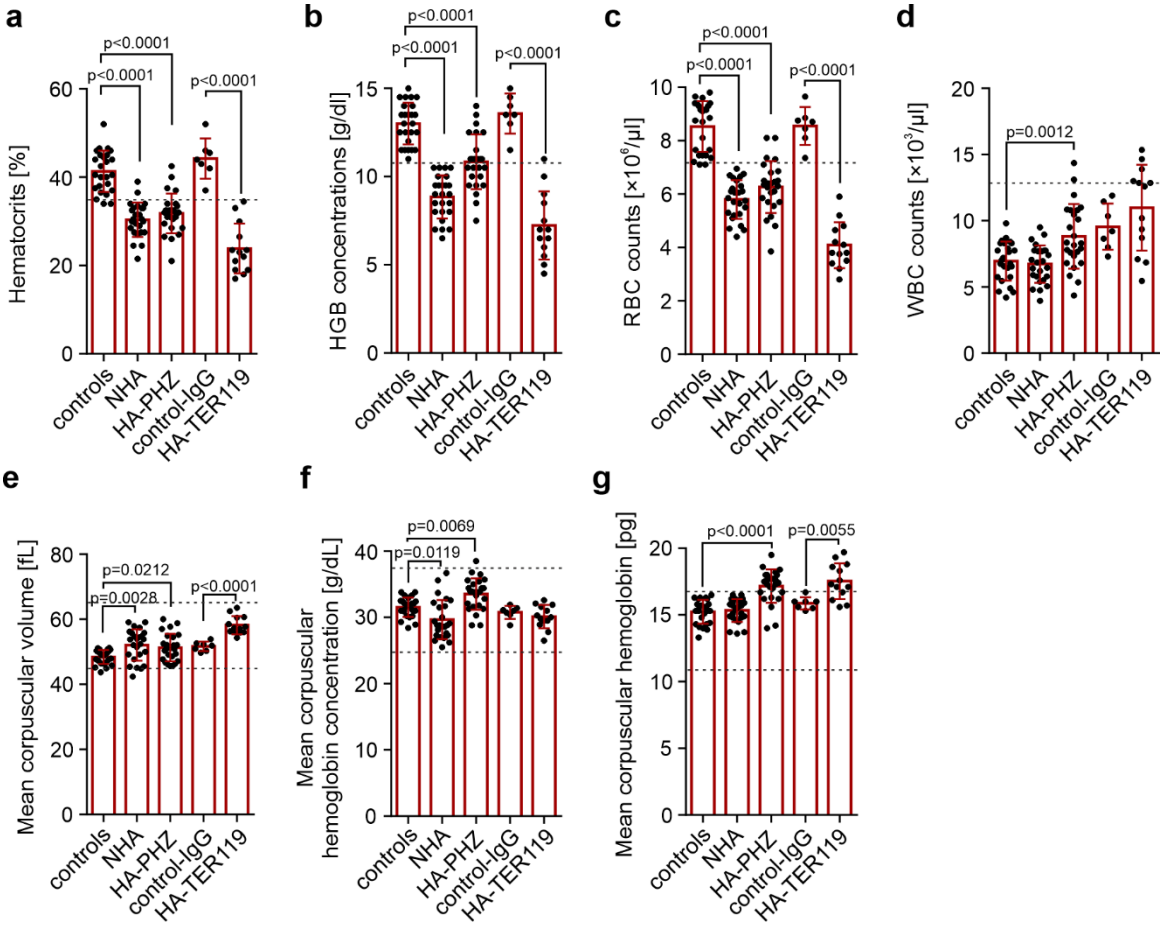
Tomasz M. Grzywa et al.

Supplementary Table 1. Clinical data of anemic and non-anemic patients

Parameter		Non-anemic	Anemic	*P*	Reference
Sex	**F**	23	19	*0.3888*	n/a
	M	19	23		
Age		53.71 ±18.72	63.66 ±19.50	*0.0209*	n/a
WBC		7.12 ±1.76	7.59 ±3.44	*0.9100*	4.00-11.00
RBC		4.73 ±0.47	3.68 ±0.83	*<0.0001*	3.8-5.2 (F) 4.2-5.7 (M)
HGB		14.13 ±1.29	9.62 ±1.32	*<0.0001*	12.0-16.0 (F) 14.0-18.0 (M)
HCT		40.99 ±3.87	29.73 ±4.10	*<0.0001*	37.0-47.0 (F) 40.0-54.0 (M)
MCV		86.88 ±3.09	83.21 ±14.15	*0.0371*	80.0-96.0
MCH		29.93 ±1.50	27.14 ±5.68	*0.0178*	27.0-31.2
RDW-CV		14.88 ±1.752	18.90 ±3.54	*<0.0001*	11.6-14.8
%CEC		0.124 ±0.15	0.907 ±0.87	*<0.0001*	n/a
CEC count		4.20 ±5.391	26.33 ±28.03	*<0.0001*	n/a

Supplementary Figures

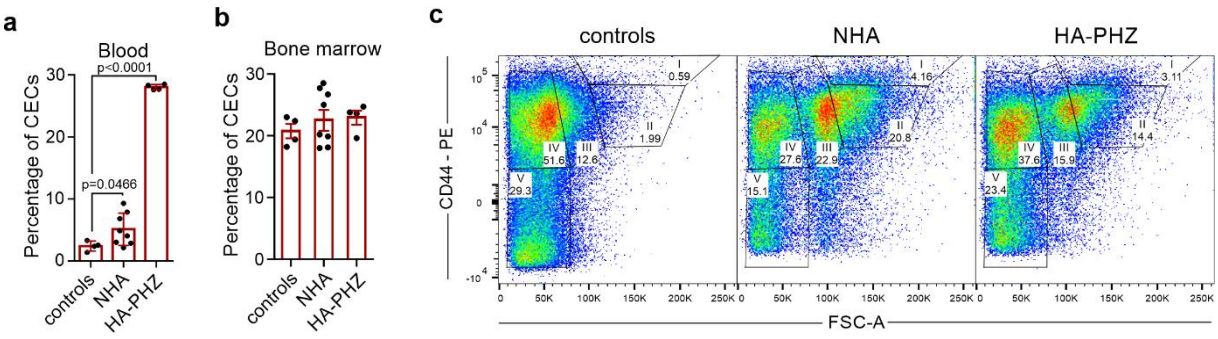
Supplementary Figure 1. Hematological parameters of anemia models



a-d, Complete blood count parameters including RBC count (**a**), hematocrit (**b**), hemoglobin concentration (**c**), and WBC count (**d**) in control healthy mice (n=25), control IgG mice (n=7), NHA mice (n=25), HA-PHZ mice (n=25), and HA-TER119 mice (n=13). **e-g,** RBC parameters including mean corpuscular volume (MCV, **e**), mean corpuscular hemoglobin concentration (MCHC, **f**), and mean corpuscular hemoglobin (MCH, **g**) in control healthy mice (n=25), control IgG mice (n=7), NHA mice (n=25), HA-PHZ mice (n=25), and HA-TER119 mice (n=13). Data show means ± SD. Each point in a-g represents data from individual mice. n values are the numbers of mice used to obtain the data. *P*-values were calculated with ordinary one-

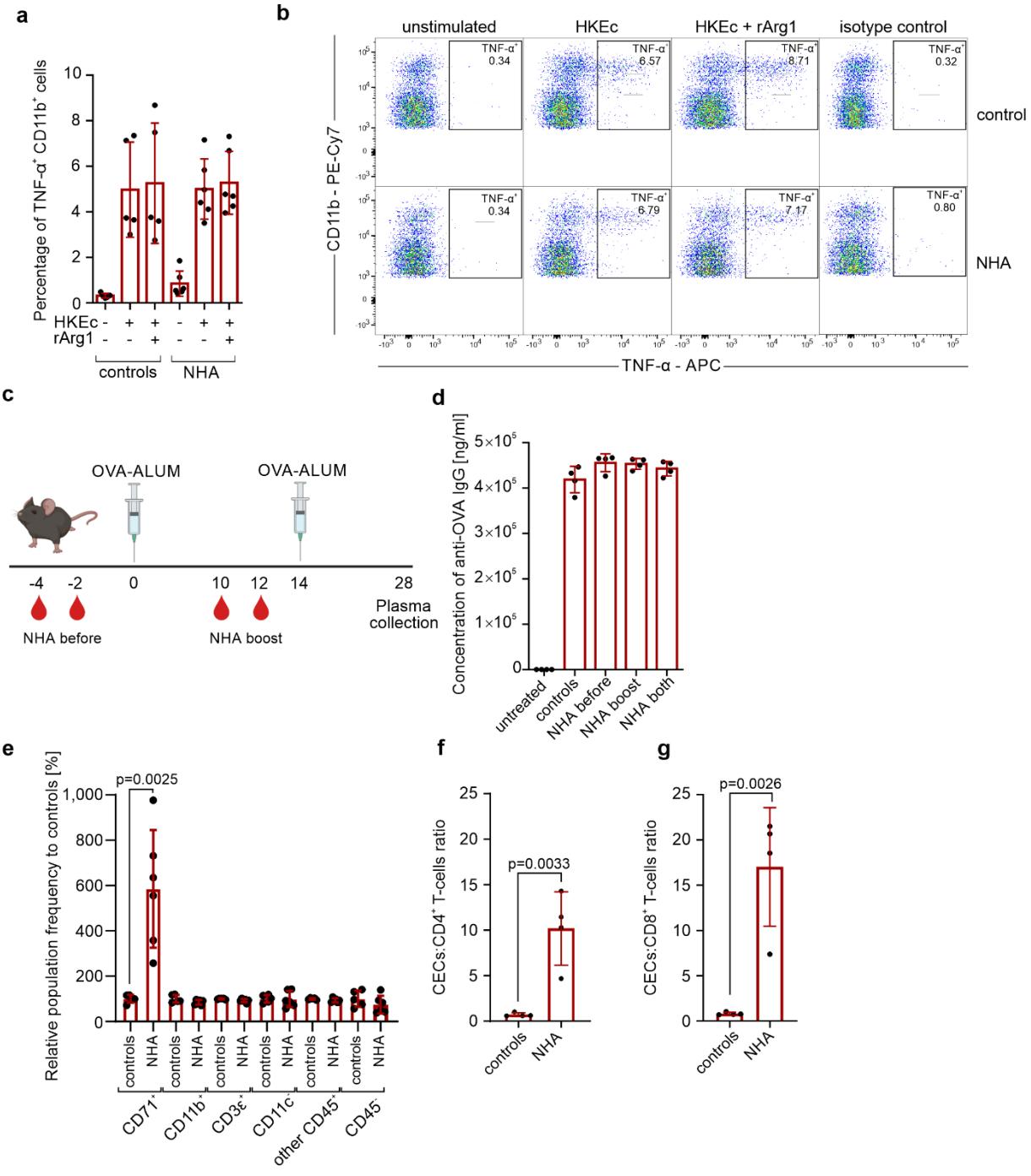
way ANOVA with Dunnett's post-hoc test (controls, NHA, HA-PHZ) or unpaired t-test (control-IgG and HA-TER119). The source data underlying Supplementary Fig. 1a-g, are provided as a Supplementary Data file.

Supplementary Figure 2. Expansion of CECs in anemic mice



a, Percentages of CECs in the blood in anemic (NHA n=8, HA-PHZ n=4) and control mice (n=4). **b,** Percentages of CECs in the bone marrow of anemic (NHA n=8, HA-PHZ n=4) and control mice (n=4). *P*-value was calculated with Brown-Forsythe ANOVA test with Dunnett's T3 post-hoc test. **c,** Representative plot of CD44 and cell size (FSC) in CECs in healthy control mice and anemic (NHA, HA-PHZ) mice. Data show means ± SD. Each point in a-b represents data from individual mice. n values are the numbers of mice used to obtain the data. The source data underlying Supplementary Fig. 2a-b are provided as a Supplementary Data file.

Supplementary Figure 3. Pro-inflammatory response by myeloid cells and induction of humoral immunity are not impaired in anemic mice

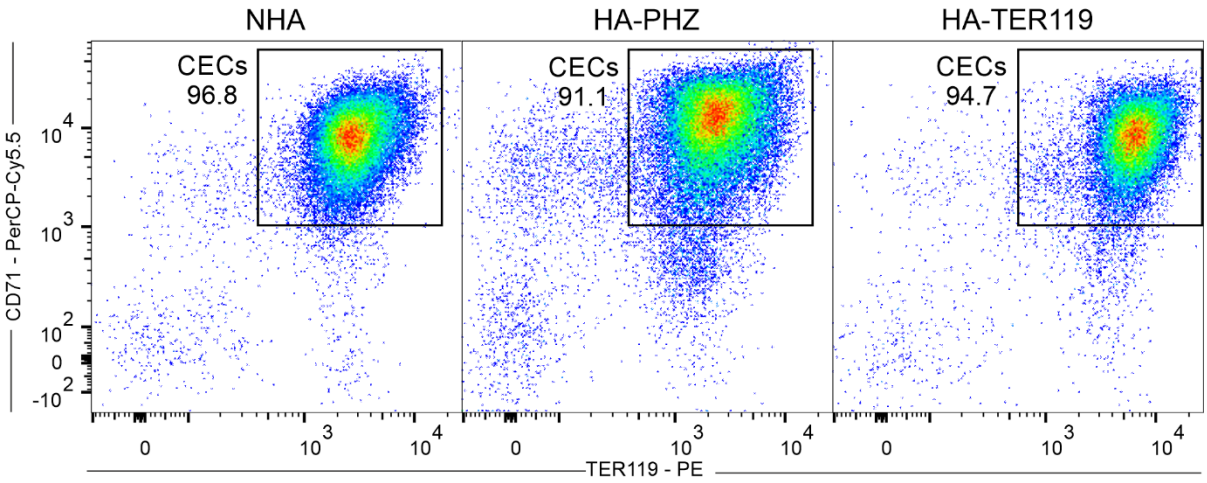


a, Splenocytes of control mice (n=5) and NHA mice (n=6) were stimulated with heat-killed *E. coli* (HKEc) for 6 hours in the presence of a protein transport inhibitor. TNF-α levels in CD11b$^+$ cells were determined by intracellular staining with fluorochrome-labeled antibodies. rARG1 – recombinant ARG1 (125 ng/ml). **b,** Representative plots

of TNF-α levels in $CD11b^+$ cells from controls and NHA mice based on intracellular staining. **c,** Schematic presentation of the experimental setting. Non-hemolytic anemia (NHA) was induced in mice by phlebotomy before immunization (NHA before) with ovalbumin (OVA) with Alum adjuvant, before second immunization (NHA boost) or each time (NHA both). Blood was collected 28 days after first immunization and plasma was isolated for anti-OVA antibodies measurement. Scheme generated using BioRender.com. **d,** Concentrations of anti-OVA IgG antibodies in the plasma of controls (n=4) and NHA mice (each group n=4) were determined using ELISA. Untreated mice were not immunized with OVA (n=4). **e,** Relative population frequency in the spleen of anemic mice (NHA, n=6) compared to healthy controls (n=5). **f,** Ratio of CECs to $CD4^+$ T-cells in the spleen of control (n=5) and anemic mice (n=5). **g** Ratio of CECs to $CD8^+$ T-cells in the spleen of control (n=5) and anemic mice (n=5). *P*-value was calculated with an unpaired *t*-test. Data show means ± SEM (a) or means ± SD (d-g). Each point in a, d, e, f, g represents data from individual mice. n values are the numbers of mice used to obtain the data. The source data underlying Supplementary Fig. 3a, Supplementary Figure 3d-g are provided as a Supplementary Data file.

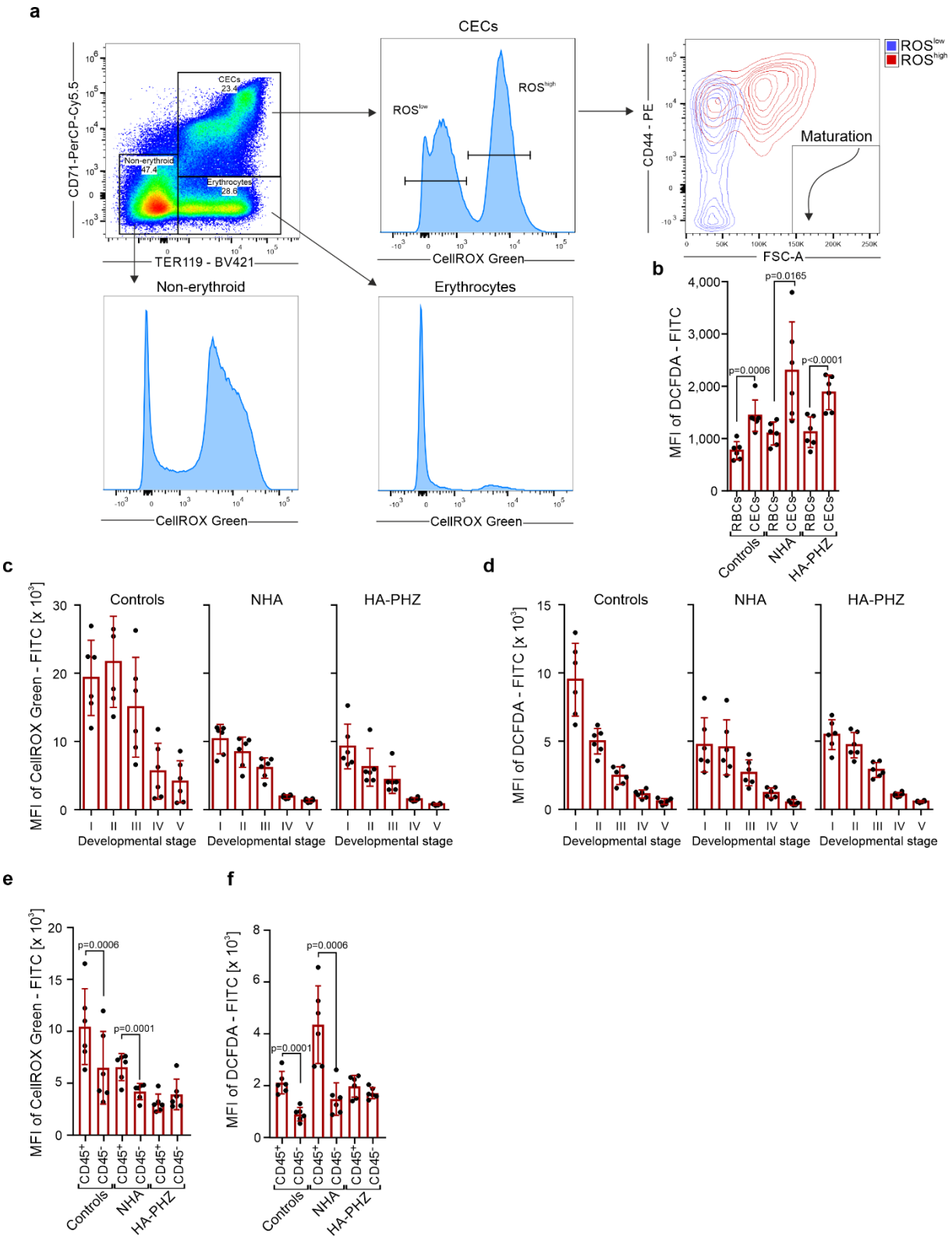
Supplementary Figure 4. The purity of isolated murine CECs

a



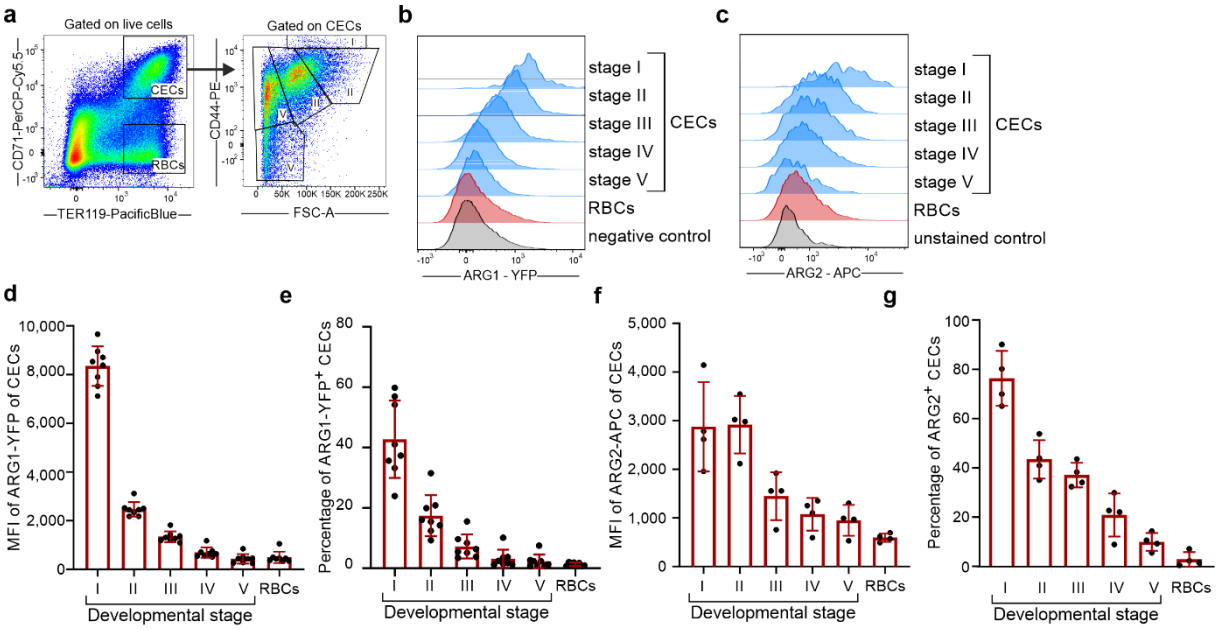
a, Representative plots of CD71 and TER119 in CECs isolated from the spleens of anemic mice (NHA, HA-PHZ, HA-TER119) using positive immunomagnetic selection with anti-CD71 antibody.

Supplementary Figure 5. The level of reactive oxygen species (ROS) is the highest in early-stage CECs



a, Representative plot of CD71 and TER119 levels in live cells and histograms of reactive oxygen species (ROS) levels based on CellROX Green – FITC in CECs ($CD71^+TER119^+$), erythrocytes (RBCs, $CD71^-TER119^+$), and non-erythroid cells ($CD71^-TER119^-$). **b,** ROS levels in CECs and RBCs in the spleen based on mean fluorescence intensity (MFI) 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) – FITC in anemic (NHA n=6, HA-PHZ n=6) and control mice (n=6). *P*-values were calculated with an unpaired *t*-test. **c,d,** ROS levels in CECs at different stages of differentiation based on mean fluorescence intensity (MFI) of CellRox Green – FITC (**c**) and 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) – FITC (**d**) in anemic (NHA n=6, HA-PHZ n=6) and control mice (n=6). **e,f,** ROS levels in $CD45^+$ CECs and $CD45^-$ CECs based on mean fluorescence intensity (MFI) of CellRox Green – FITC (**e**) and 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) – FITC (**f**) in anemic (NHA n=6, HA-PHZ n=6) and control mice (n=6). *P* values were calculated with paired *t*-test. Data show means ± SD. Each point in b-f represents data from individual mice. n values are the numbers of mice used to obtain the data. The source data underlying Supplementary Fig. 5b-f are provided as a Supplementary Data file.

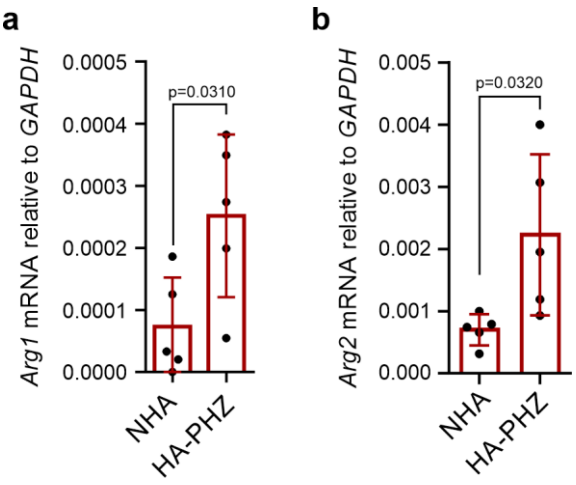
Supplementary Figure 6. ARG1 and ARG2 levels are the highest in the early-stages CECs



a, Representative plot of CD71 and TER119 levels in live cells in the spleens of NHA mice and gating strategy of CECs developmental stages based on CD44 levels and relative cell size. **b,** Representative histograms of ARG1 expression in different developmental stages of CECs and RBCs in NHA mouse based on YFP mean fluorescence intensity (MFI) in reporter B6.129S4-Arg1^{tm1Lky}/J mice. Negative control represents the background fluorescence of YFP in wild-type C57Bl/6 mouse. **c,** Representative histograms of ARG2 expression in different developmental stages of CECs and RBCs based on intracellular staining in NHA mouse. Unstained control represents sample unstained for ARG2. **d,e,** ARG1 expression (**d**) and percentage of ARG1^{+} cells (**e**) in different developmental stages of CECs in NHA mice based on YFP mean fluorescence intensity (MFI) in reporter B6.129S4-Arg1^{tm1Lky}/J mice (n=8). **f,g,** ARG2 levels (**f**) and percentage of ARG2^{+} cells (**g**) in different developmental stages of CECs in NHA mice based on intracellular staining (n=4). Data show means ± SD. Each point in d-g represents data from individual mice. n values are the

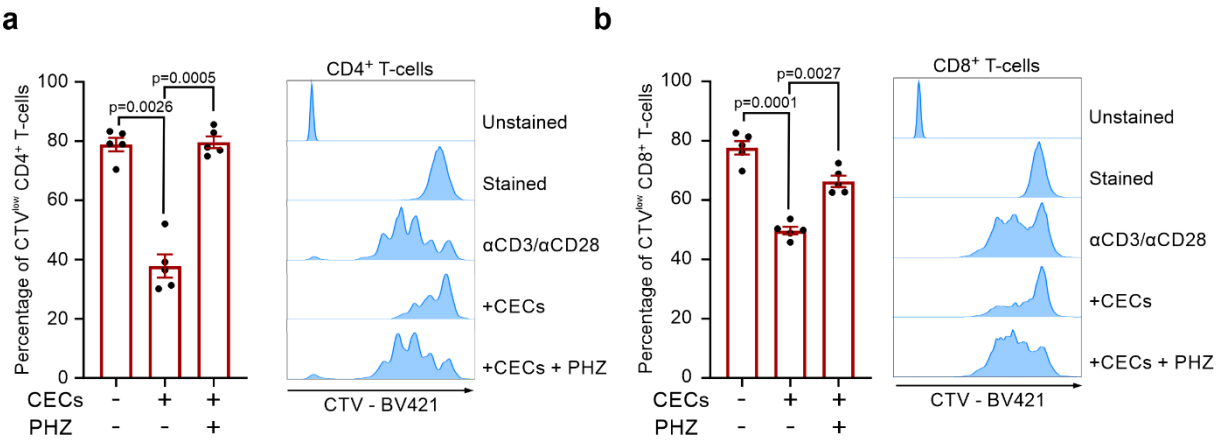
numbers of mice used to obtain the data. The source data underlying Supplementary Fig. 6d-g are provided as a Supplementary Data file.

Supplementary Figure 7. ARG1 and ARG2 expression in CECs of anemic mice



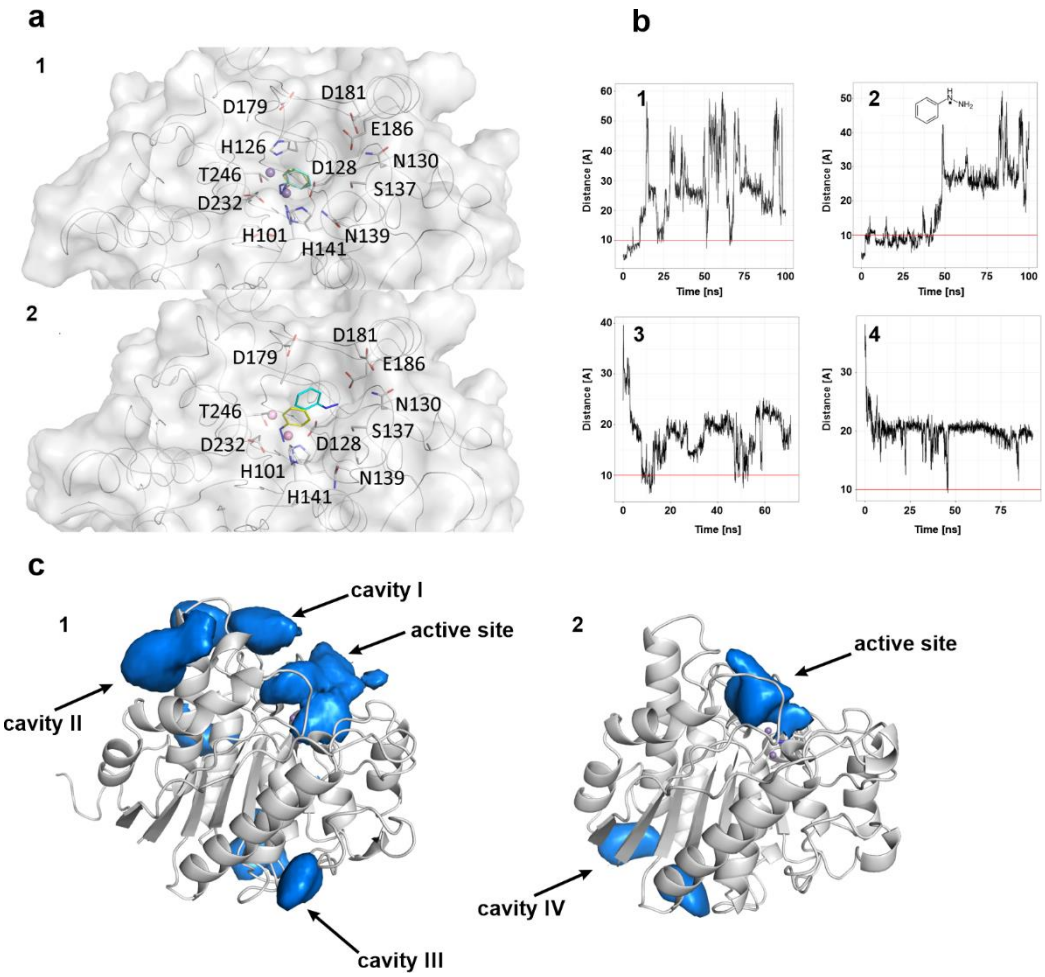
a,b, mRNA expression of ARG1 (**a**) and ARG2 (**b**) in CECs isolated from NHA (n=5) and HA-PHZ (n=5) mice was determined using real-time quantitative PCR method. *P*-values were calculated with an unpaired *t*-test. Data show means ± SD. Each point in a, b represents data from individual mice. n values are the numbers of mice used to obtain the data. The source data underlying Supplementary Fig. 7a,b are provided as a Supplementary Data file.

Supplementary Figure 8. PHZ diminishes immunoregulatory functions of murine CECs *ex vivo*



a,b, Effects of phenylhydrazine (PHZ, 100 μM) on the proliferation of $CD4^+$ (**a**) and $CD8^+$ (**b**) T-cells stimulated with αCD3/αCD28 and co-cultured with CECs at a 1:2 ratio (T-cells:CECs) isolated from the spleens of NHA mice (n=5). Representative proliferation histograms of αCD3/αCD28-stimulated $CD4^+$ (**a**) and $CD8^+$ (**b**) T-cells co-cultured with CECs in the presence of PHZ. Histograms show the fluorescence of CTV (CellTraceViolet) – BV421. *P*-values were calculated with repeated-measures ANOVA with Tukey's post-hoc test. Data show means ± SEM. Each point in a represents data from individual mice. n values are the numbers of mice used to obtain the data. The source data underlying Supplementary Fig. 8a,b are provided as a Supplementary Data file

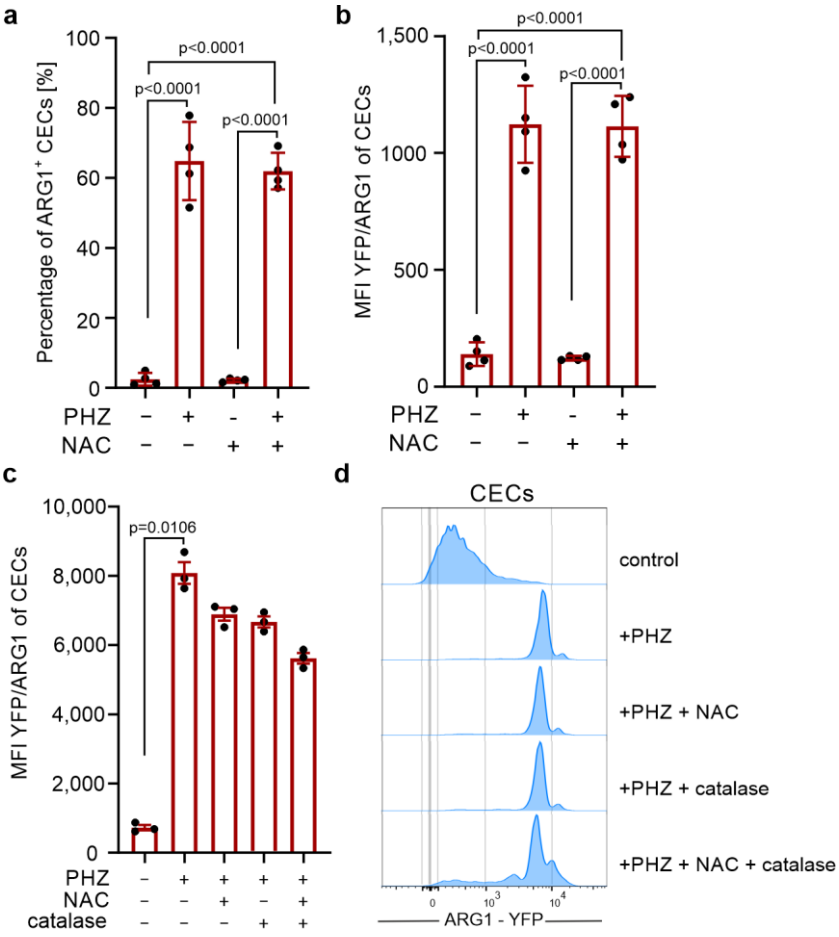
Supplementary Figure 9. Molecular analysis of arginases – PHZ complexes



a, The predicted conformation of PHZ in complex with human (panel 1) and mouse (panel 2) ARG1. The best-scored conformations of the ligand proposed independently by surflex and GOLD are colored cyan and yellow, respectively. Sidechains of key active site residues are also depicted. **b,** The distance between carboxyl group of D126 and benzene-substituted nitrogen atom of PHZ was observed during 100 ns MD simulations. (1) PHZ and human ARG1 (2) PHZ and human ARG2. In both cases the ligand was initially present in the active site. (3) The same distance but for MD simulations involving human ARG1 and six molecules of PHZ randomly placed around the enzyme. The distance for a molecule closest to the active site at a given moment in time is reported. (4) Same as 3 but for ARG2. The

vertical red line marks 10 Å value. If the distance is below that threshold we assume the ligand can effectively shield the active site from other molecules **c,** During MD simulation involving PHZ initially placed in the active site of ARG1 (1) and ARG2 (2) the ligand binds to several other regions on the surface of the protein.

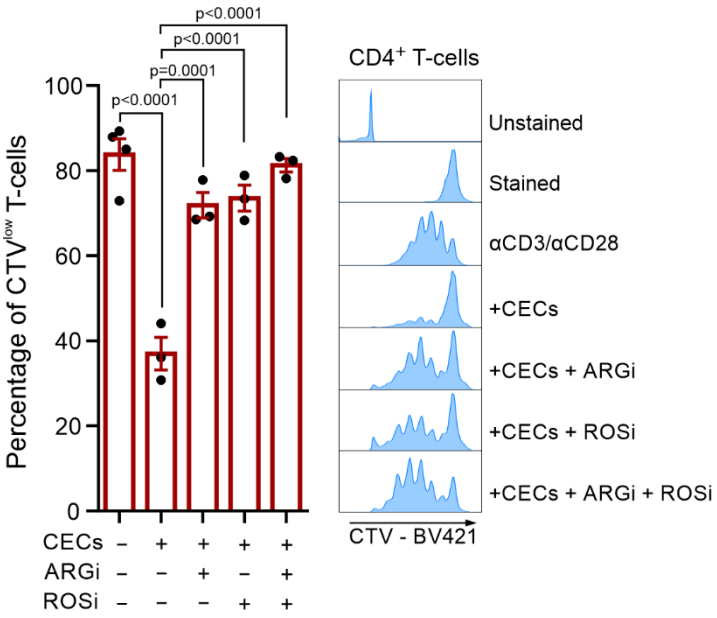
Supplementary Figure 10. Reactive oxygen species scavengers do not prevent ARG1 induction by PHZ.



a-b, YARG mice were injected intraperitoneally (i.p.) three days before harvest with 50 mg per kg body weight of phenylhydrazine (PHZ) and three times every 24h with 150 mg per kg body weight of N-acetylcysteine (NAC) (each group n=4). Percentages of ARG1$^+$ CECs (**a**) and mean fluorescence intensity (MFI) of YFP-ARG1 (**b**). **c,d**, CECs were isolated from the spleens of NHA YARG mice and incubated with 100 µM phenylhydrazine (PHZ), 100 µM NAC, and/or 100 µg/ml catalase. The fluorescence of YFP/ARG1 was assessed after 24h by flow cytometry (**c**). Histograms show representative fluorescence of ARG1 – YFP of CEC (**d**). *P*-values were calculated with repeated-measures ANOVA with Bonferroni's post-hoc test. Data show means ± SD (a-b) or means ± SEM (c). Each point in a-c represents

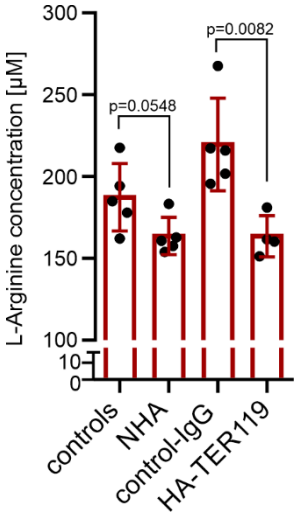
data from individual mice. n values are the numbers of mice used to obtain the data. The source data underlying Supplementary Fig. 10a-c are provided as a Supplementary Data file.

Supplementary Figure 11. Neonatal CECs suppress T-cells proliferation by arginase and ROS



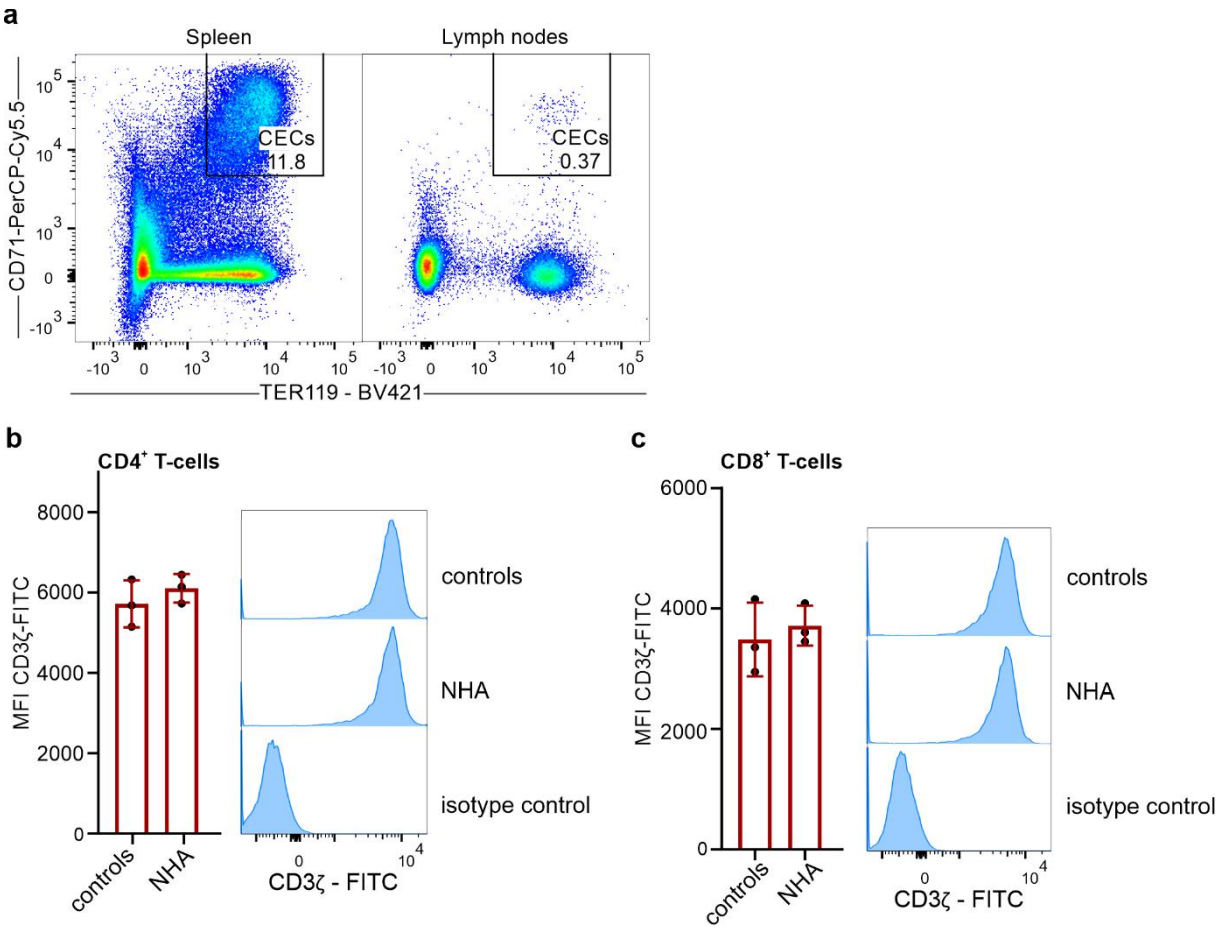
Effects of ARGi (OAT-1746, 500 nM) and ROSi (N-acetylcysteine, 100 μM) on the proliferation of αCD3/αCD28-stimulated $CD4^+$ T-cells co-cultured with CECs in a ratio of 1:2 isolated from the spleens of neonatal mice (n=3). Representative proliferation histograms of αCD3/αCD28-stimulated $CD4^+$ T-cells co-cultured with CECs in the presence of ARGi or ROSi. Histograms show the fluorescence of CTV (CellTraceViolet) – BV421. *P*-values were calculated with one-way ANOVA with Bonferroni's post-hoc test. Data show means ± SEM. Each point represents data from individual mice. n values are the numbers of mice used to obtain the data. The source data underlying Supplementary Fig. 11 are provided as a Supplementary Data file

Supplementary Figure 12. Anemic mice have slightly decreased L-arginine concentration in serum



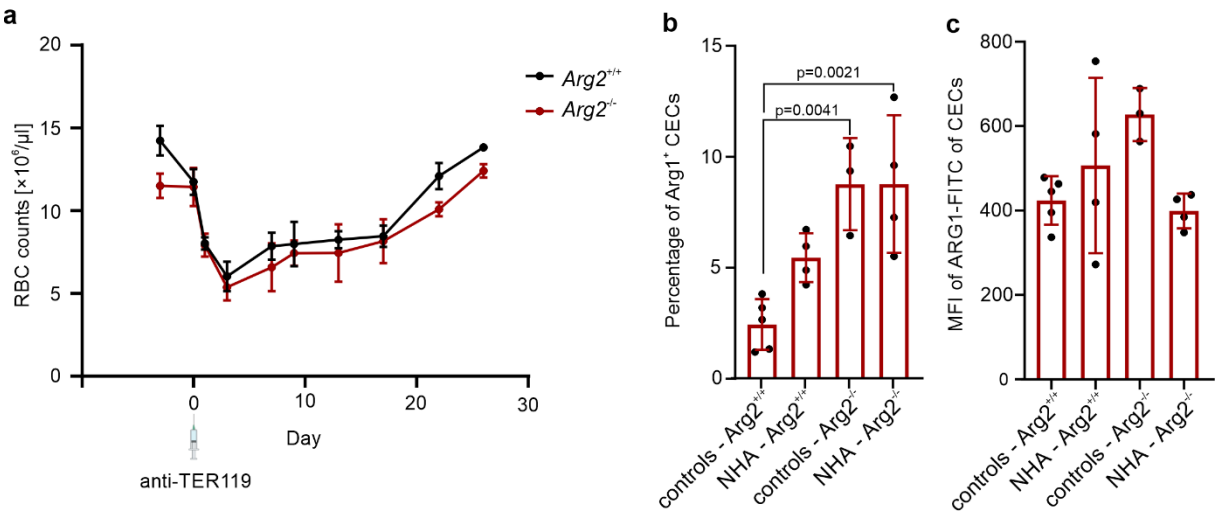
L-Arginine concentration in the serum of anemic and healthy mice determined by mass spectrometry (controls n=5, NHA n=5, control-IgG n=5, HA-TER119 n=4). *P*-values were calculated using unpaired *t*-tests. Data show means ± SD. Each point in a represents data from individual mice. n values are the numbers of mice used to obtain the data. The source data underlying Supplementary Fig. 12 are provided as a Supplementary Data file.

Supplementary Figure 13. Lymph node T-cells have normal CD3ζ levels in anemic mice



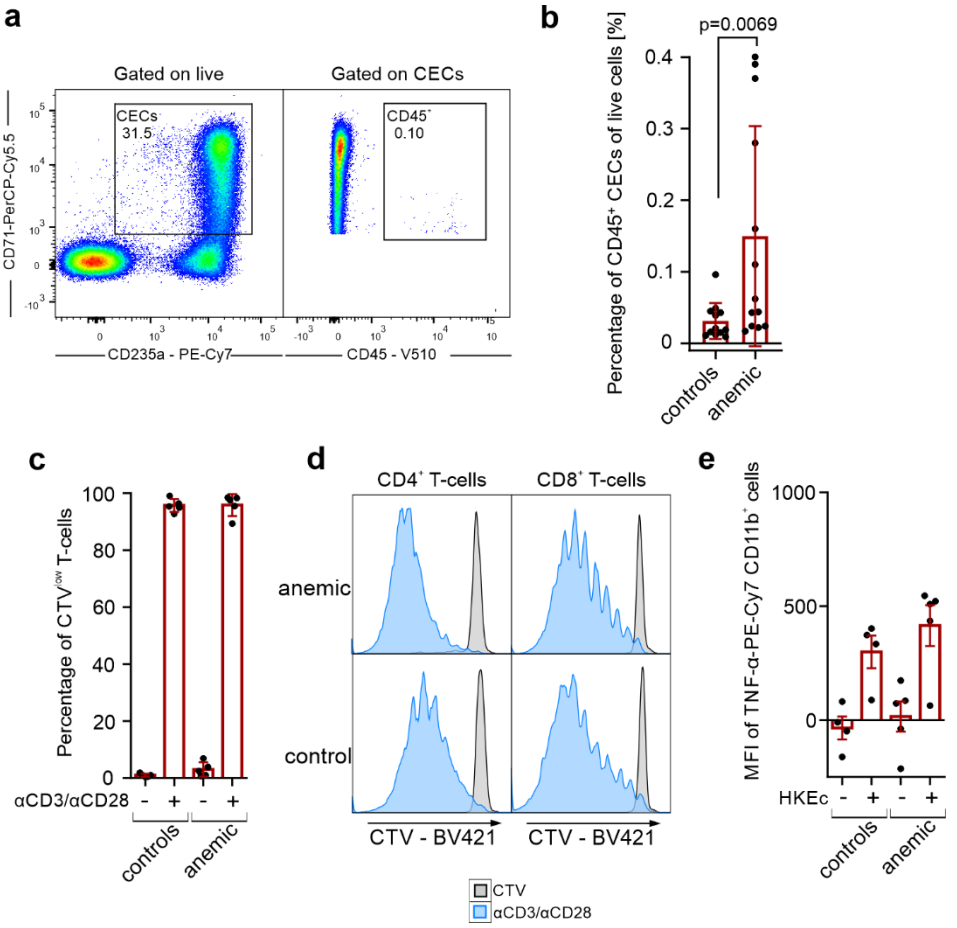
a, Representative plots of CD71 and TER119 in live cells from the spleen as well as inguinal and axillary lymph nodes isolated from NHA mice. **b,c,** The levels of CD3ζ in the lymph node $CD4^+$ (**b**) and $CD8^+$ T-cells (**c**) of controls (n=3) and anemic (n=3) mice. Histograms show the fluorescence of CD3ζ – FITC. *P*-values were >0.05. *P*-value was calculated with the Mann-Whitney test. Data show means ± SD. Each point in b, c represents data from individual mice. n values are the numbers of mice used to obtain the data. The source data underlying Supplementary Fig. 13b,c are provided as a Source Data file.

Supplementary Figure 14. Stress erythropoiesis and Arg1 expression in *Arg2*$^{-/-}$ mice



a, *Arg2*$^{-/-}$ knockout mice (n=3) and wild-type mice (n=3) were administered with 30 µg anti-TER119 antibody to induce stress erythropoiesis. Red blood cell (RBC) count was determined using an automated hematology analyzer. *P* > 0.05 for the analysis of the differences between *Arg2*$^{-/-}$ knockout mice and wild-type *Arg2*$^{+/+}$ mice in all time points. *P* values were calculated with the Mann-Whitney test. **b,** Percentages of ARG1^{+} CECs in the spleens of control (n=5), anemic wild-type (n=4), *Arg2*$^{-/-}$ knockout anemic (n=4), and control mice (n=3). **c,** ARG1 expressison in CECs based on intracellular staining in the spleen of control (n=5), anemic wild-type (n=4), *Arg2*$^{-/-}$ knockout anemic (n=4), and control mice (n=3). *P*-values were calculated with one-way ANOVA with Bonferroni's post-hoc test. Data show means ± SD. Each point in b, c represents data from individual mice. n values are the numbers of mice used to obtain the data. The source data underlying Supplementary Fig. 14a-c are provided as a Source Data file.

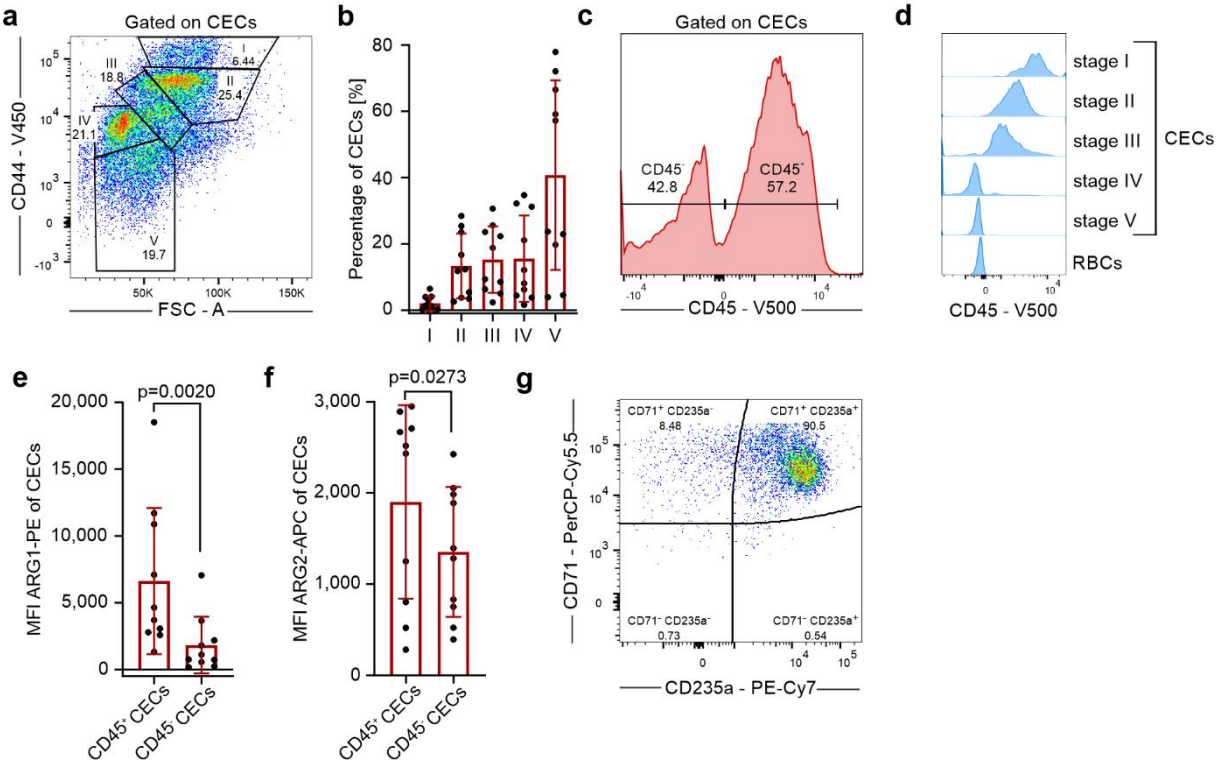
Supplementary Figure 15. T-cells proliferation and TNF-α production by myeloid cells is unimpaired in PBMCs of anemic patients



a, Representative plots of CD71 and CD235a levels in PBMCs and CD45 levels in CECs from an anemic individual. **b,** Percentages of live CD45$^+$ CECs cells in PBMCs of controls (n=12) and anemic patients (n=13). **c,** Proliferation of αCD3/αCD28-stimulated CD3ε$^+$ T-cells in PBMCs of controls (n=5) and anemic patients (n=5). **c,** Representative proliferation histograms of αCD3/αCD28-stimulated PBMC from anemic or control patients. **d,** Representative histograms of the fluorescence of CTV (CellTraceViolet) – BV421 in αCD3/αCD28-stimulated CD4$^+$ and CD8$^+$ T-cells. **e**, PBMCs of control patients (n=4) and anemic patients (n=5) were stimulated with Heat-killed *E. coli* (HKEc) for 12 hours in the presence of a protein transport inhibitor. TNF-α levels in CD11b$^+$ cells were determined by intracellular staining with

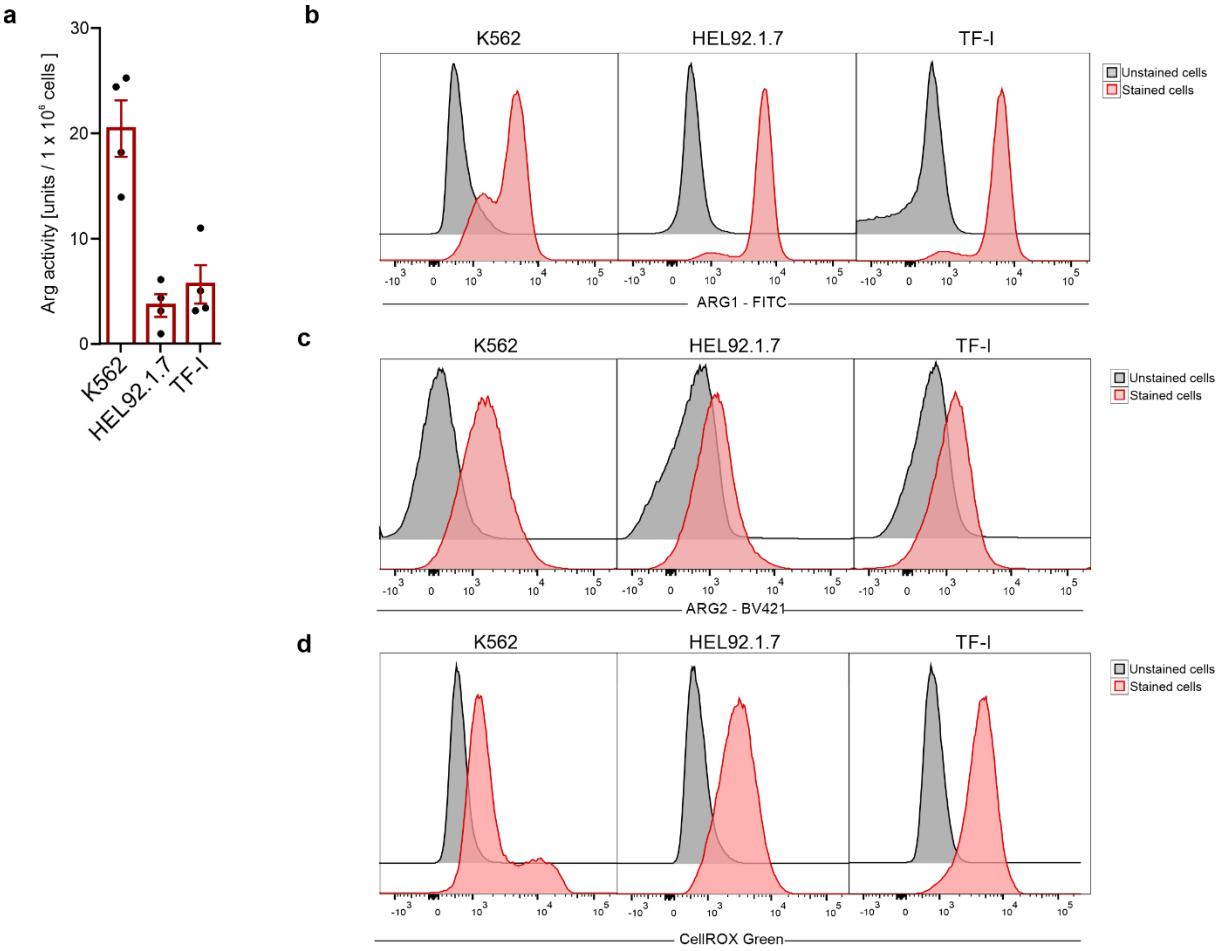
fluorochrome-labeled antibodies. *P* values were calculated using the Mann-Whitney test (**b,c,d**). Data show means ± SD (**b**) or means ± SEM (**c,e**). Each point in **b, c, e** represents data from individual patients. n values are the numbers of individual patients used to obtain the data. The source data underlying Supplementary Fig. 15b,c,e are provided as a Source Data file.

Supplementary Figure 16. CECs in the human bone marrow are enriched in early-stage progenitors and express ARG1 and ARG2



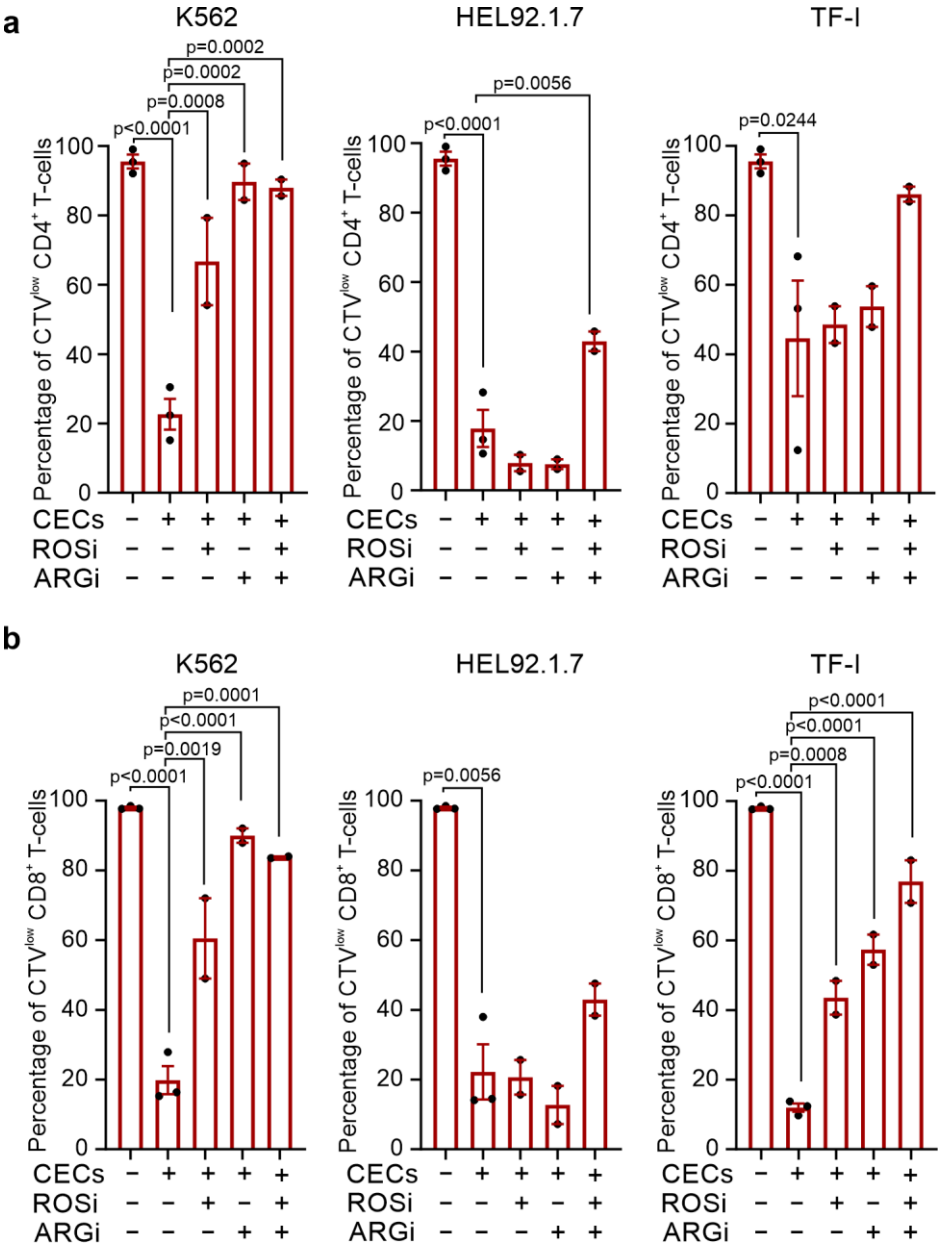
a, Representative dot plot of the gating of CECs developmental stages based on CD44 levels and relative cell size (FSC-A) in CECs from human bone marrow. **b,** Percentages of CECs in different developmental stages in human bone marrow (n=10). **c,** Representative histogram of CD45 levels in CECs from human bone marrow. **d,** Representative histogram of CD45 levels in CECs at different developmental stages. Red blood cells (RBCs) are shown as CD45-negative control. **e,f,** The levels of ARG1 (**e**) and ARG2 (**f**) in $CD45^+$ and $CD45^-$ CECs from human bone marrow. **g,** Representative plot of CD71 and CD235a in isolated CECs from human bone marrow. *P* values were calculated using the Mann-Whitney test (**d,e**). Data show means ± SD. Each point in **b, e, f** represents data from individual patients. n values are the numbers of individual patients used to obtain the data. The source data underlying Supplementary Fig. 16b,e,f are provided as a Source Data file.

Supplementary Figure 17. Erythroleukemia-derived erythroid cell lines have high arginase activity and ROS levels



a, Arginase activity in erythroid cells calculated per 1×10^6 cells (n=4). **b,c,** Representative histograms of ARG1 (**b**) and ARG2 (**c**) expression in erythroid cell lines. **d,** Representative histograms of ROS levels based on CellRox Green – FITC fluorescence in erythroid cell lines. Data show means ± SEM. Each point in **a** represents data from one biological replicate. n values are the numbers of biological replicates used to obtain the data. The source data underlying Supplementary Fig. 17a are provided as a Source Data file.

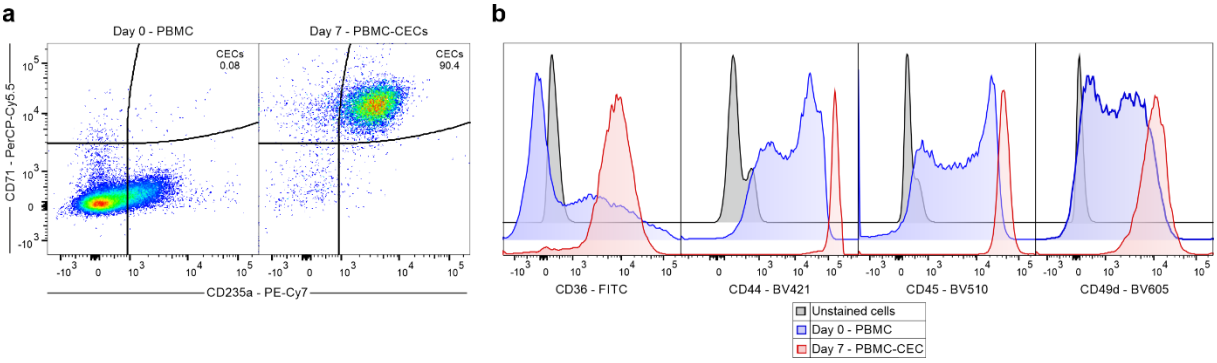
Supplementary Figure 18. Erythroid cells suppress T-cells in an ARG- and ROS-dependent mechanism



a,b, Effects of ARGi (OAT-1746, 1.5 μM) and ROSi (N-acetylcysteine, 200 μM) on proliferation of CTV-labelled $CD4^+$ (**a,** n=2) and $CD8^+$ (**b,** n=2) T-cells triggered by αCD3/αCD28 and co-cultured with erythroid cell lines at a 1:2 ratio. Data correspond to Fig. 9e (**a**) and 9i (**b**). Representative histograms are shown in Fig. 9e and Fig. 9i. *P* values were calculated with ordinary one-way ANOVA with Holm-Sidak's post-hoc test. Data show means ± SEM. Each point in a, b represents data from a mean of technical duplicates. n values are the numbers of biological repetitions of *in vitro*

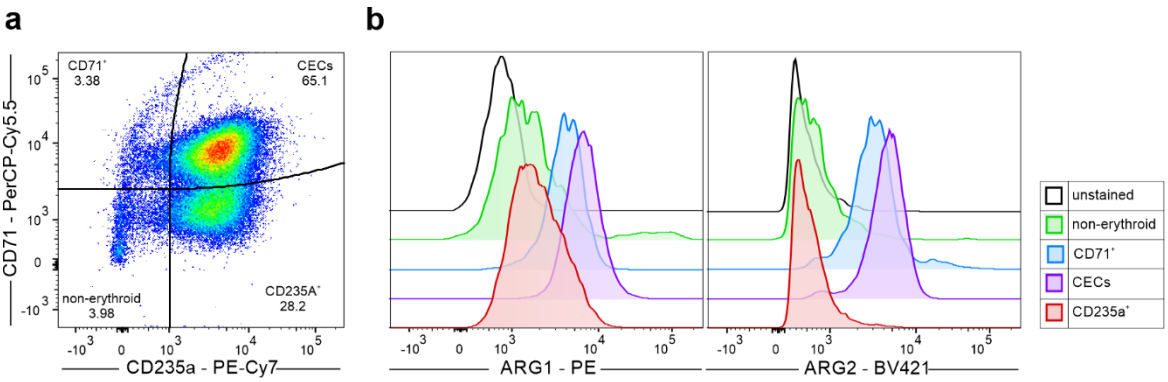
experiments. The source data underlying Supplementary Fig. 18a,b are provided as a Source Data file.

Supplementary Figure 19. CECs expansion and differentiation from PBMC



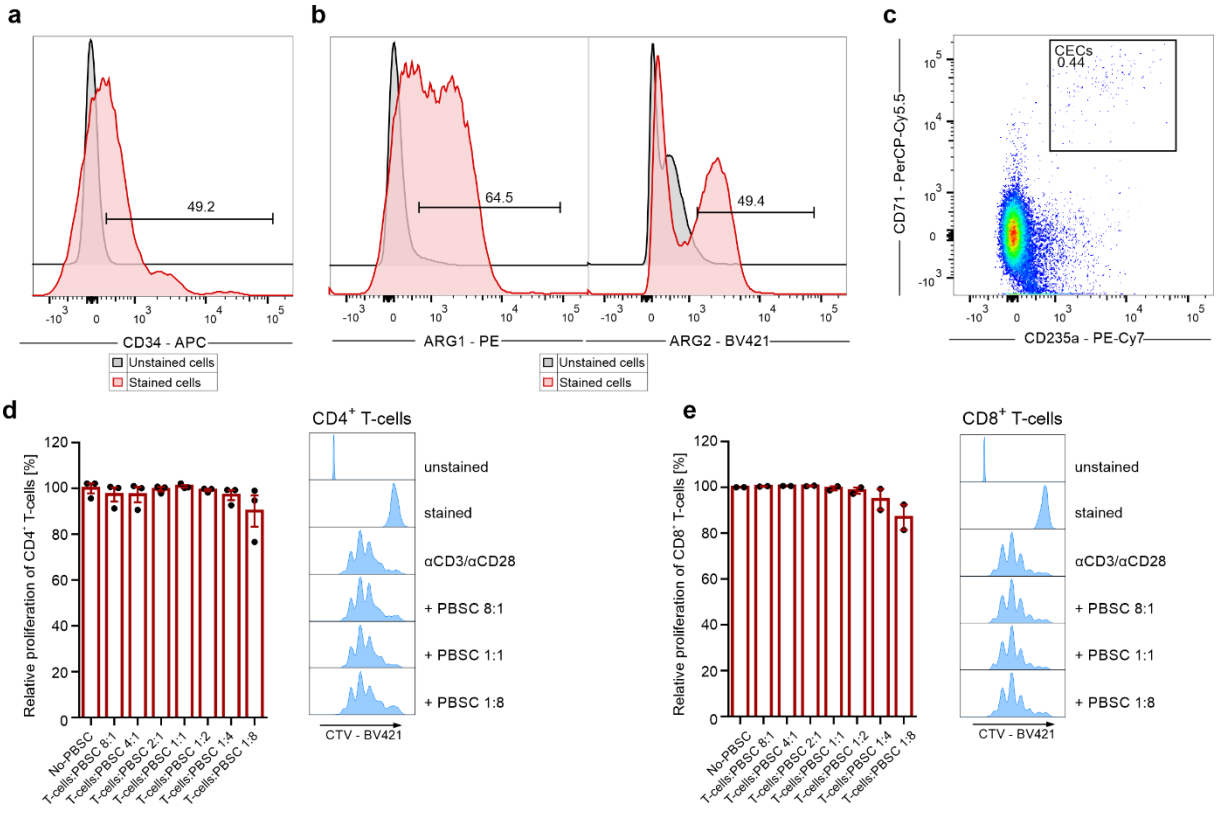
a, Representative plots of CD71 and CD235a expression in PBMCs on days 0 and 7 after erythroid differentiation. **b,** Representative histograms of CD36, CD44, CD45, and CD49d levels in PBMC and CECs differentiated from PBMCs (PBMC-CECs).

Supplementary Figure 20. PBMC-CECs had high ARG1 and ARG2 expression



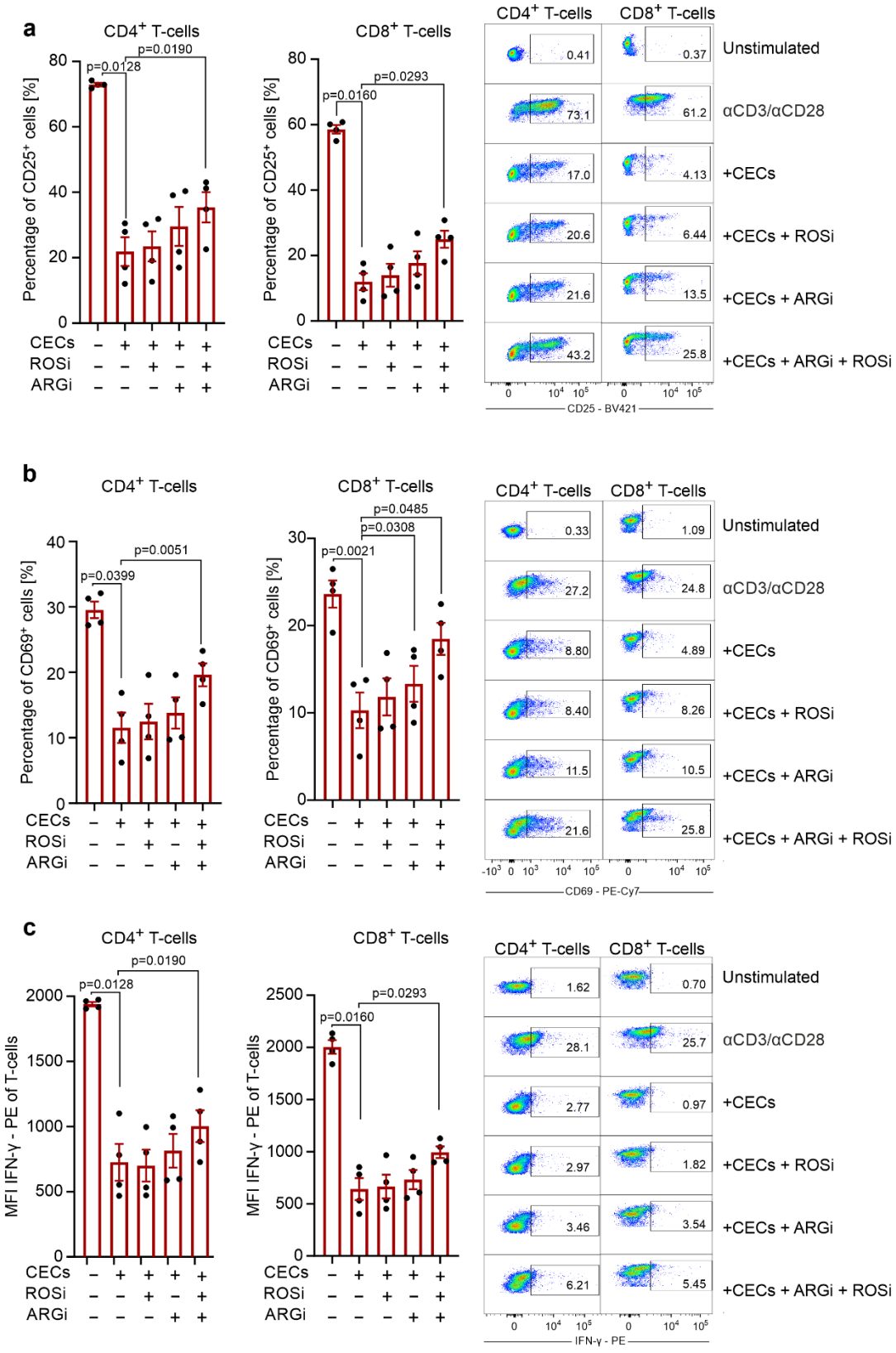
a, Representative plots of CD71 and CD235a expression in CECs differentiated from PBMC. **b,** Histograms of ARG1 and ARG2 expression in CECs ($CD71^+CD235a^+$), $CD71^+$ cells, RBCs ($CD71^-CD235a^+$) and non-erythroid cells ($CD71^-CD235a^-$).

Supplementary Figure 21. PBSCs have high ARG1 and ARG2 expression but do not suppress T-cells proliferation



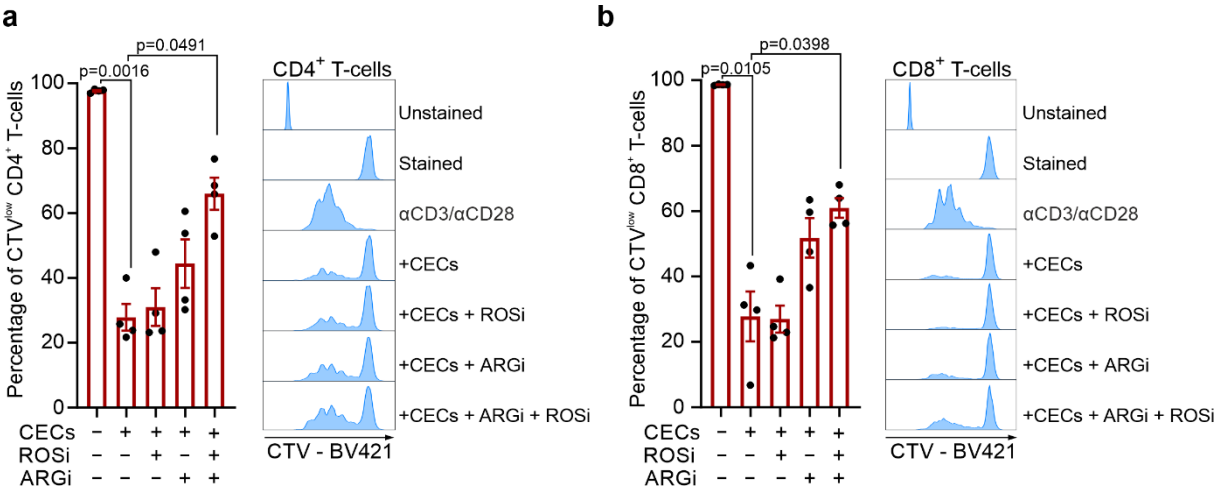
a, Representative histogram showing CD34 levels in peripheral blood stem cells (PBSC). **b,** Representative histograms of ARG1 and ARG2 expression in PBSCs. **c,** Representative plot of CD71 and CD235a expression in PBSCs. **d,e,** Proliferation of CTV-labelled $CD4^+$ (**d,** n=3) and $CD8^+$ (**e,** n=2) T-cells triggered by αCD3/αCD28 and co-cultured with PBSC. Relative proliferation was calculated with No-PBSC T-cells as a control. Representative histograms show the fluorescence of CTV (CellTraceViolet) – BV421 in $CD4^+$ (**d**) and $CD8^+$ T-cells (**e**) stimulated with αCD3/αCD28. Data show means ± SEM. Each point in **d**, **e** represents data from an individual patient. n values are the numbers of patients used to obtain the data. The source data underlying Supplementary Fig. 21d,e are provided as a Source Data file.

Supplementary Figure 22. PBMC-derived CECs suppress T-cells in ARG- and ROS-dependent mechanism



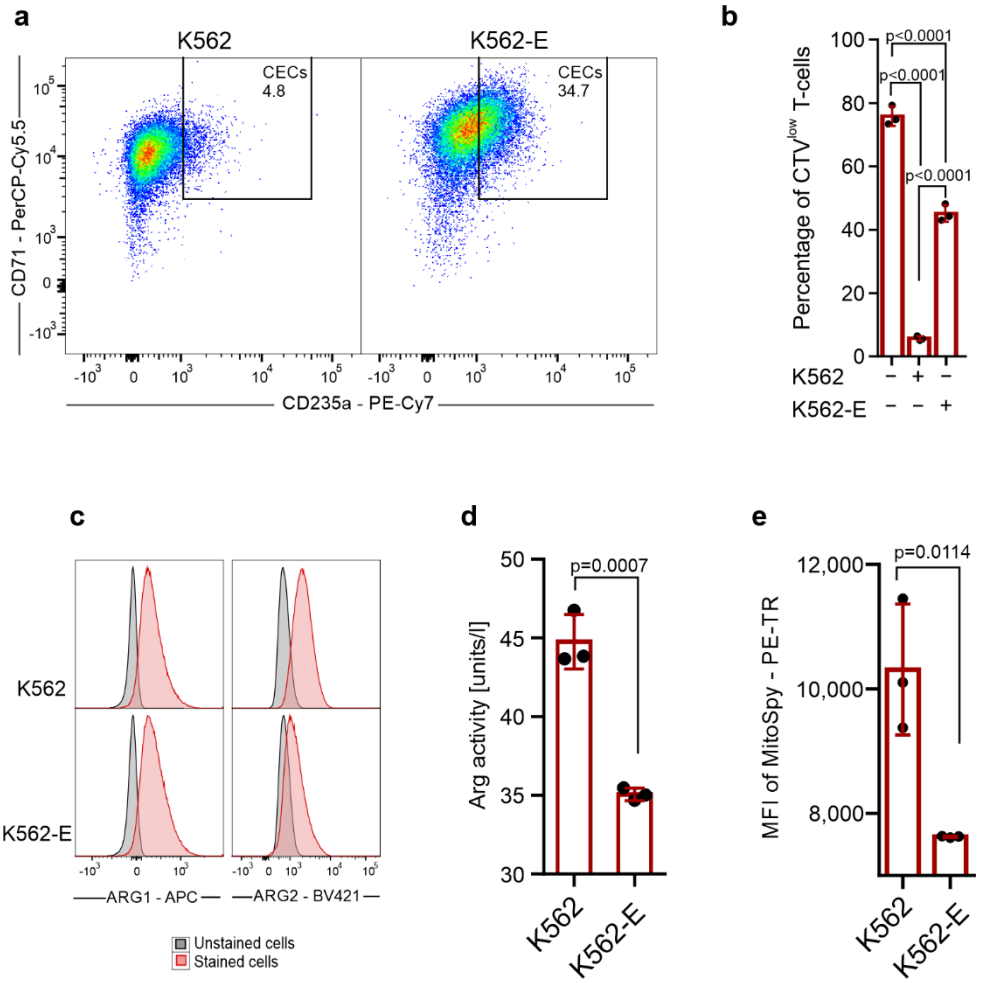
a,b, Effects of ARGi (OAT-1746, 1.5 μM) and ROSi (N-acetylcysteine, 200 μM) on $CD4^+$ or $CD8^+$ T-cell activation triggered by αCD3/αCD28 based on the percentage of $CD25^+$ T-cells (**a**) and $CD69^+$ T-cells (**b**). T-cells were co-cultured with PBMC-derived CECs at the stage of $CD71^{high}CD235a^{mid}$ at a ratio of 1:2 for 72h. Representative plots show the fluorescence of CD25 – BV421 (**a**) and CD69 – PE-Cy7 (**b**). **c,** Effects of ARGi (OAT-1746, 1.5 μM) and ROSi (N-acetylcysteine, 200 μM) on interferon γ (IFN-γ) production triggered by αCD3/αCD28 in $CD4^+$ or $CD8^+$ T-cells based on the level of intracellular staining with fluorochrome-labeled antibody. T-cells were co-cultured with PBMC-derived CECs at the stage of $CD71^{high}CD235a^{mid}$ at a 1:2 ratio for 72h. Protein transport inhibitor (BD GolgiStop™) was added for the last 12 hours. Representative plots show the fluorescence of IFN-γ – PE. *P* values were calculated using repeated-measures ANOVA with Sidak's post-hoc tests. Data show means ± SEM. Each point in a, b, c represents data from one individual patient. n values are the numbers of patients used to obtain the data. The source data underlying Supplementary Fig. 22a-c are provided as a Source Data file.

Supplementary Figure 23. PBMC-derived CECs suppress T-cells proliferation in ARG- and ROS-dependent mechanism



a,b, Proliferation triggered by αCD3/αCD28 in CTV-labelled $CD4^+$ (**a,** n=4) and $CD8^+$ (**b,** n=4) T-cells co-cultured with PBMC-derived CECs at ratio 1:2. CECs were at the stage of $CD71^{high}CD235a^{mid}$. *P* values were calculated using repeated-measures ANOVA with Sidak's post-hoc tests. Data show means ± SEM. Each point in a, b represents data from one individual patient. n values are the numbers of patients used to obtain the data. The source data underlying Supplementary Fig. 23a,b are provided as a Source Data file.

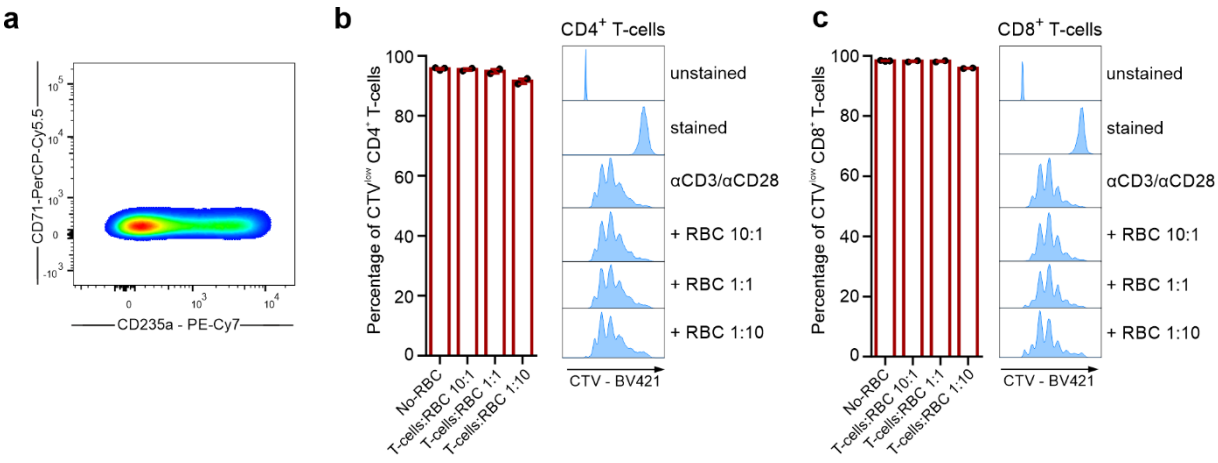
Supplementary Figure 24. Induction of differentiation of K562 cells decreases suppressive effects on T-cells and decreases ARG2 levels



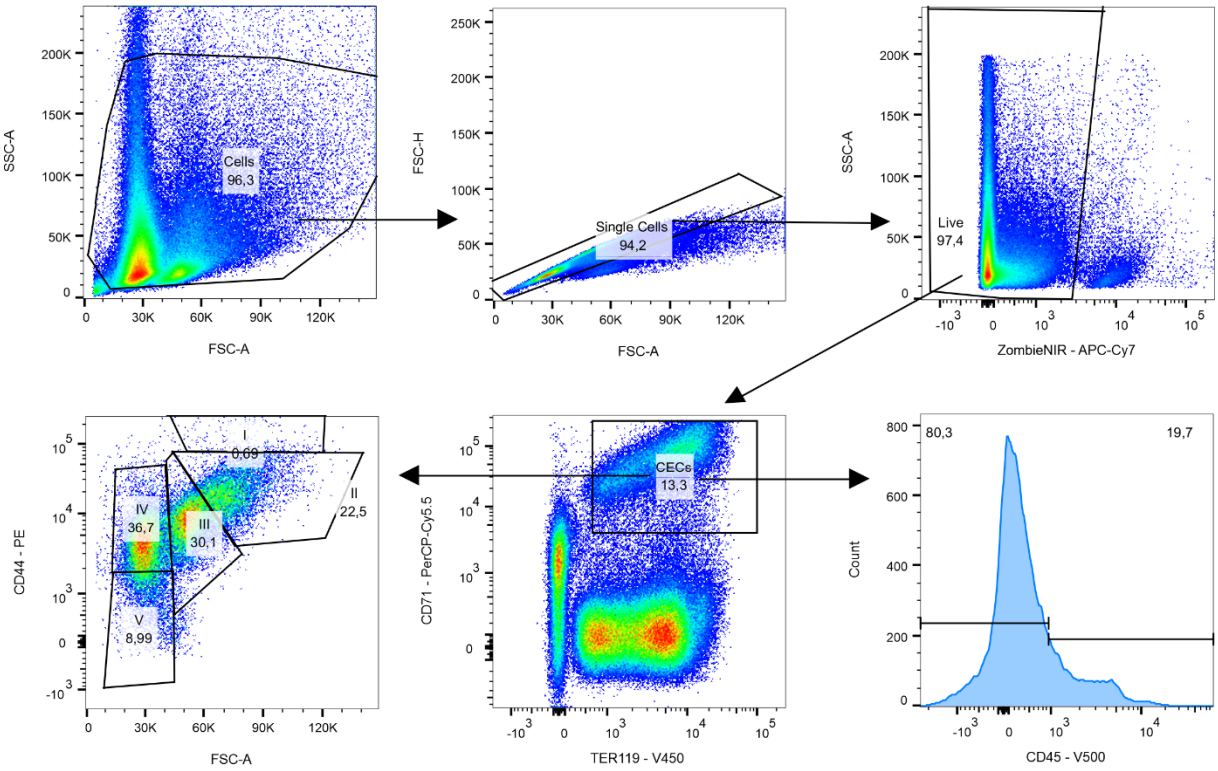
a, Representative plots of CD71 and CD235a levels in K562 and sodium butyrate-treated K562 cells (K562-Erythroid, K562-E). **b,** Proliferation of αCD3/αCD28-stimulated $CD4^+$ T-cells in co-culture with K562 and K562-E at a 1:2 ratio. Data show one representative experiment out of two. *P*-values were calculated with one-way ANOVA with Bonferroni's post-hoc test. **c,** Representative histograms of ARG1 and ARG2 expression in erythroid cell lines. **d,** Arginase activity of K562 and K562-E cells calculated per 1×10^6 cells (n=3). *P*-value was calculated with an unpaired *t*-test. **e,** Mean Fluorescence Intensity (MFI) of PE-TexasRed-MitoSpy, Mitochondrion probe of K562 and K562-E cells (n=3). *P*-value was calculated with an unpaired *t*-test. Data show means ± SD. Each point in d, e represents data from one biological replicate. n

values are the numbers of biological replicates used to obtain the data. The source data underlying Supplementary Fig. 24b, 24d-e are provided as a Source Data file

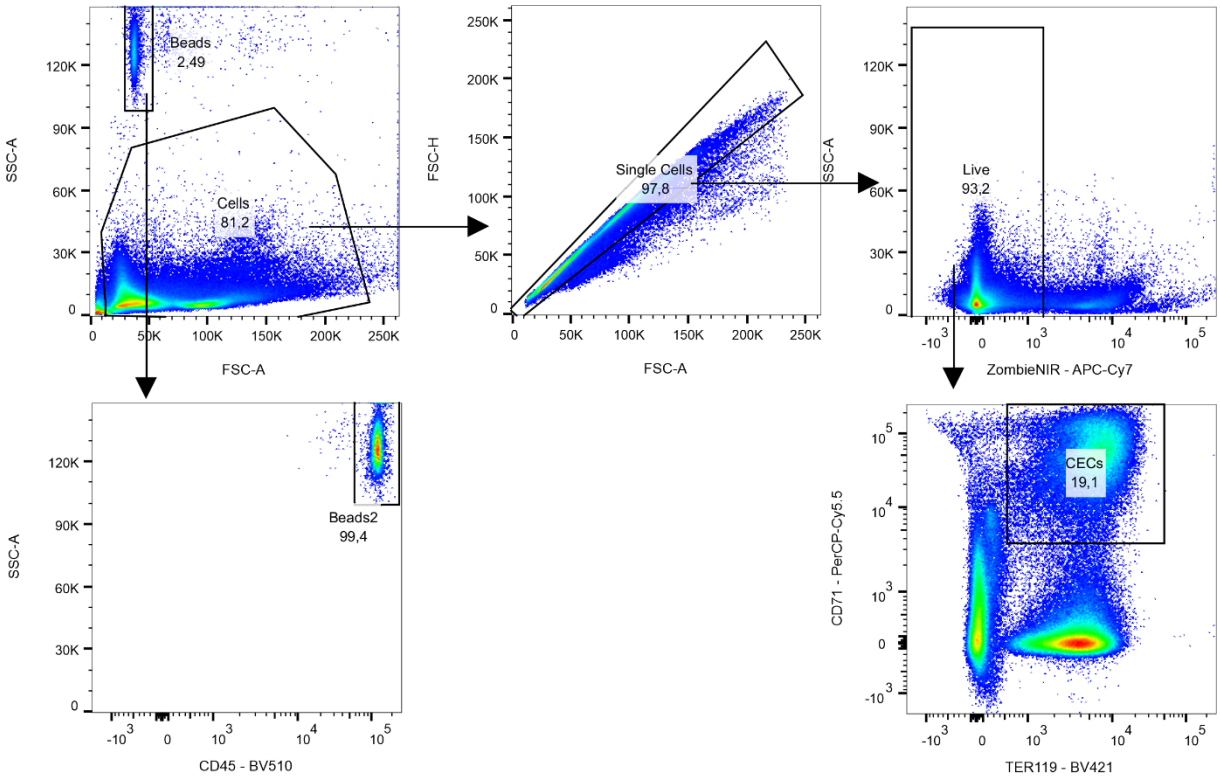
Supplementary Figure 25. Erythrocytes have no impact on T-cells proliferation



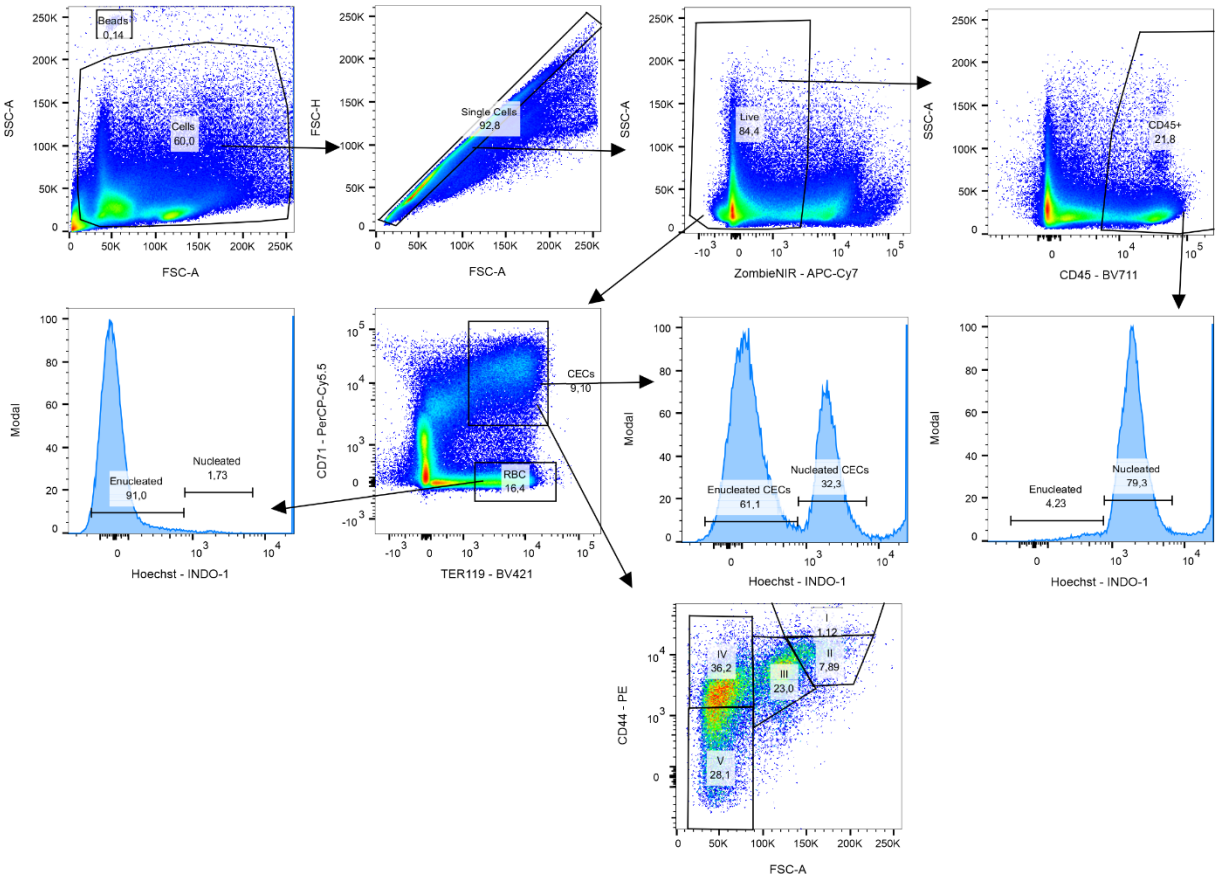
a, Representative plot of CD71 and CD235a expression in isolated erythrocytes. **B,c,** Proliferation triggered by αCD3/αCD28 in CTV-labelled $CD4^+$ (**b)** and $CD8^+$ (**c)** T-cells co-cultured with RBC (n=2). Data show means ± SEM. Each point in b,c represents data from an individual patient. n values are the numbers of patients used to obtain the data. The source data underlying Supplementary Fig. 25b,c are provided as a Source Data file.



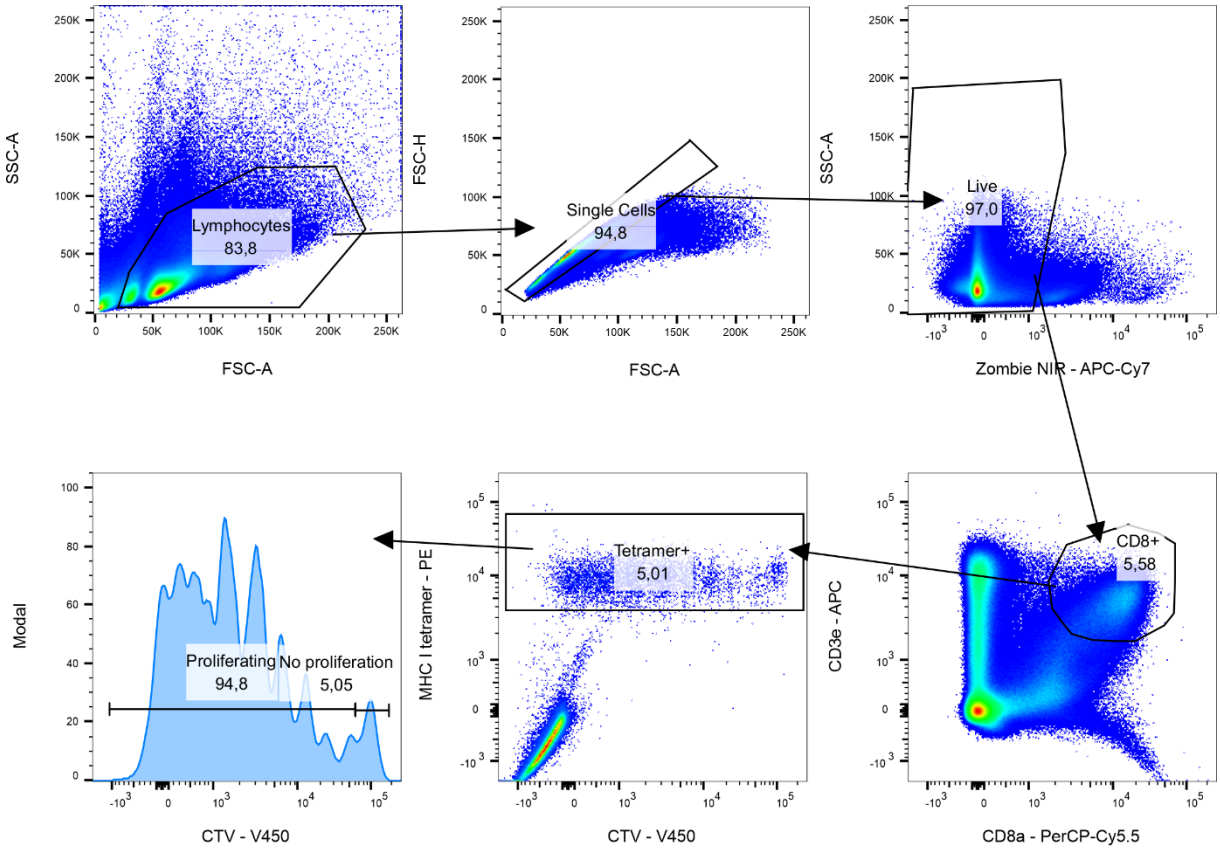
Supplementary Figure 26. Gating strategy used to analyze the data shown in Figure 1a-b, 1f-g, Supplementary Figure 2a-c, Supplementary Figure 6a-g, Supplementary Figure 13a. A minimum of 10 000 cells were acquired within the $CD45^{+}$ CECs gate.



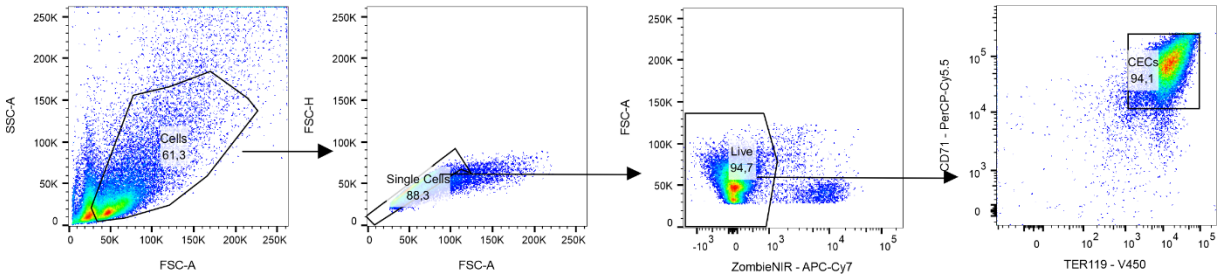
Supplementary Figure 27. Gating strategy used to analyze the data shown in Figure 1c. A minimum of 10 000 cells were acquired within the CECs gate.



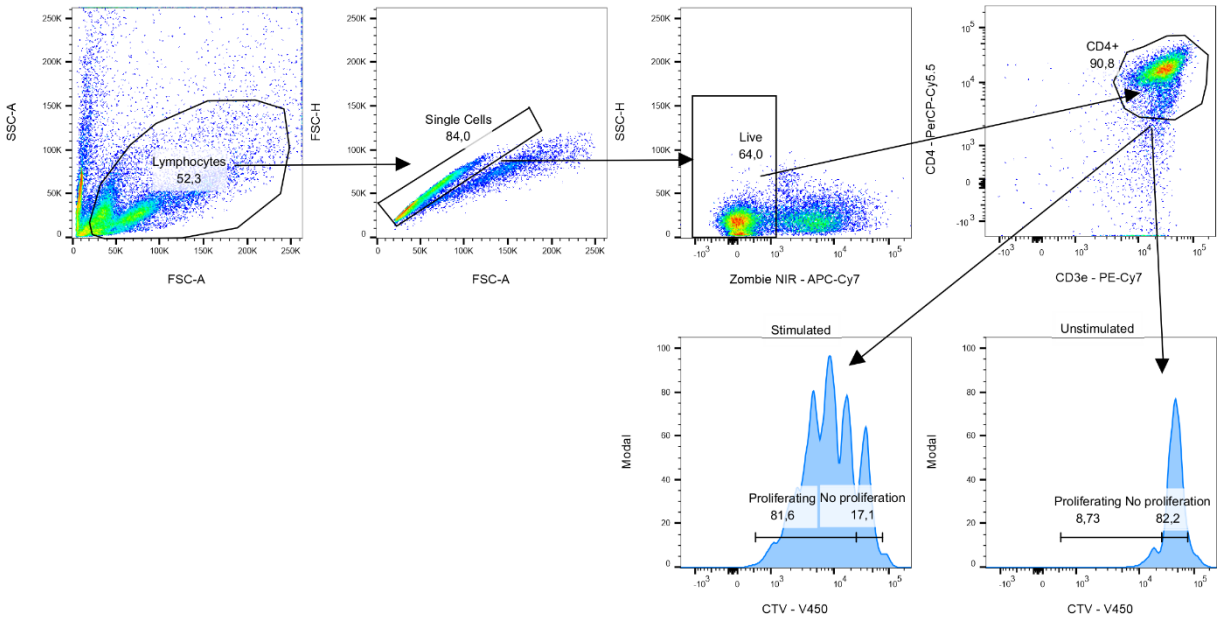
Supplementary Figure 28. Gating strategy used to analyze the data shown in Figure 1g-i. A minimum of 30 000 cells were acquired within the CECs gate.



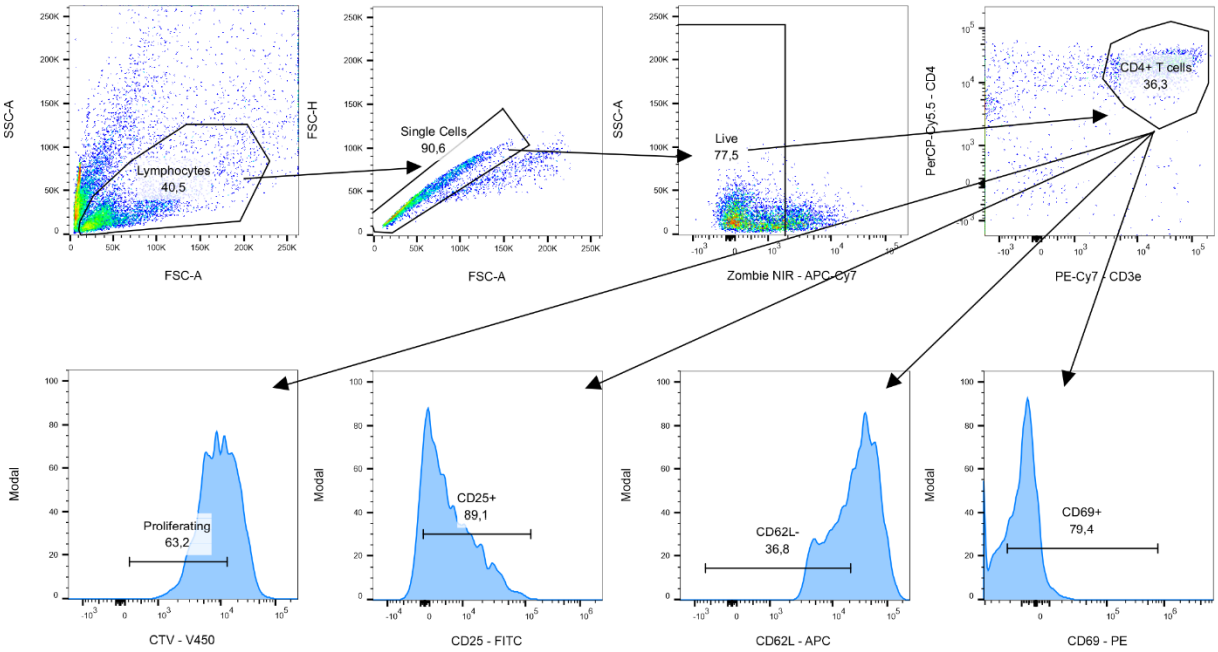
Supplementary Figure 29. Gating strategy used to analyze the data shown in Figure 2b. A minimum of 40 000 cells were acquired within the $CD8^+$ cells gate. The gate for proliferating (CTV^{low}) $Tetramer^+$ $CD8a^+$ T-cells was set based on the unstimulated control. Proliferation histograms were generated in FlowJo v10.6.2.



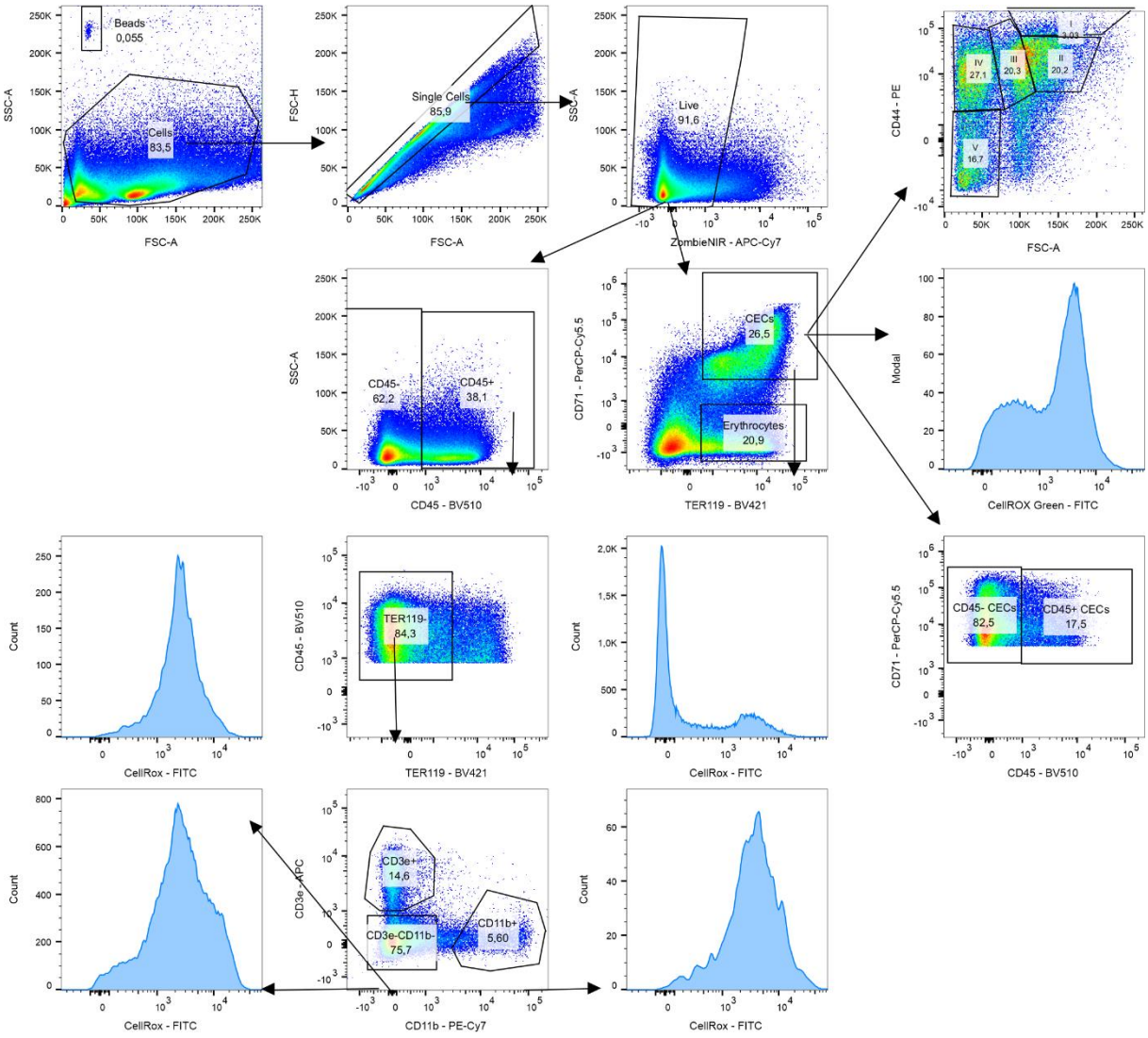
Supplementary Figure 30. Gating strategy used to analyze the data shown in Figure 2c, Supplementary Figure 4a. A minimum of 20 000 cells were acquired within the live cells gate.



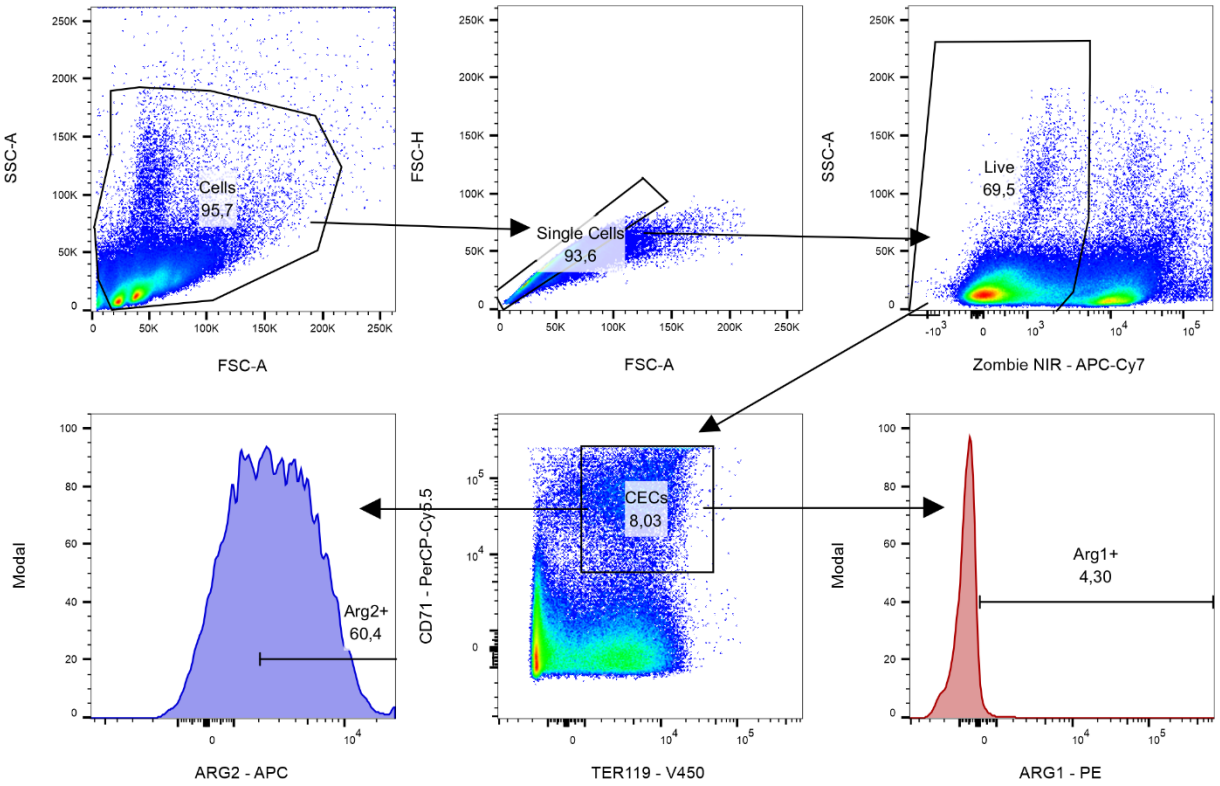
Supplementary Figure 31. Gating strategy used to analyze the data shown in Figure 2d, Figure 5b-f, Figure 6c, Figure 8e-f, Figure 9b-l, Figure 10b-c, Figure 10e-g, Supplementary Figure 8a-b, Supplementary Figure 11a, Supplementary Figure 15c-d, Supplementary Figure 18a-b, Supplementary Figure 21d-e, Supplementary Figure 23a-b, Supplementary Figure 24b, Supplementary Figure 25b-c. Exemplary gating strategy used for the murine $CD4^+$ T-cell population (analogous strategy was used for the human $CD4^+$ T-cell population and murine and human $CD8^+$ T-cell population). A minimum of 10 000 cells were acquired within the lymphocytes gate. The gate for proliferating (CTV^{low}) $CD4^+$ T-cells was set based on the unstimulated control. Proliferation histograms were generated in FlowJo v10.6.2.



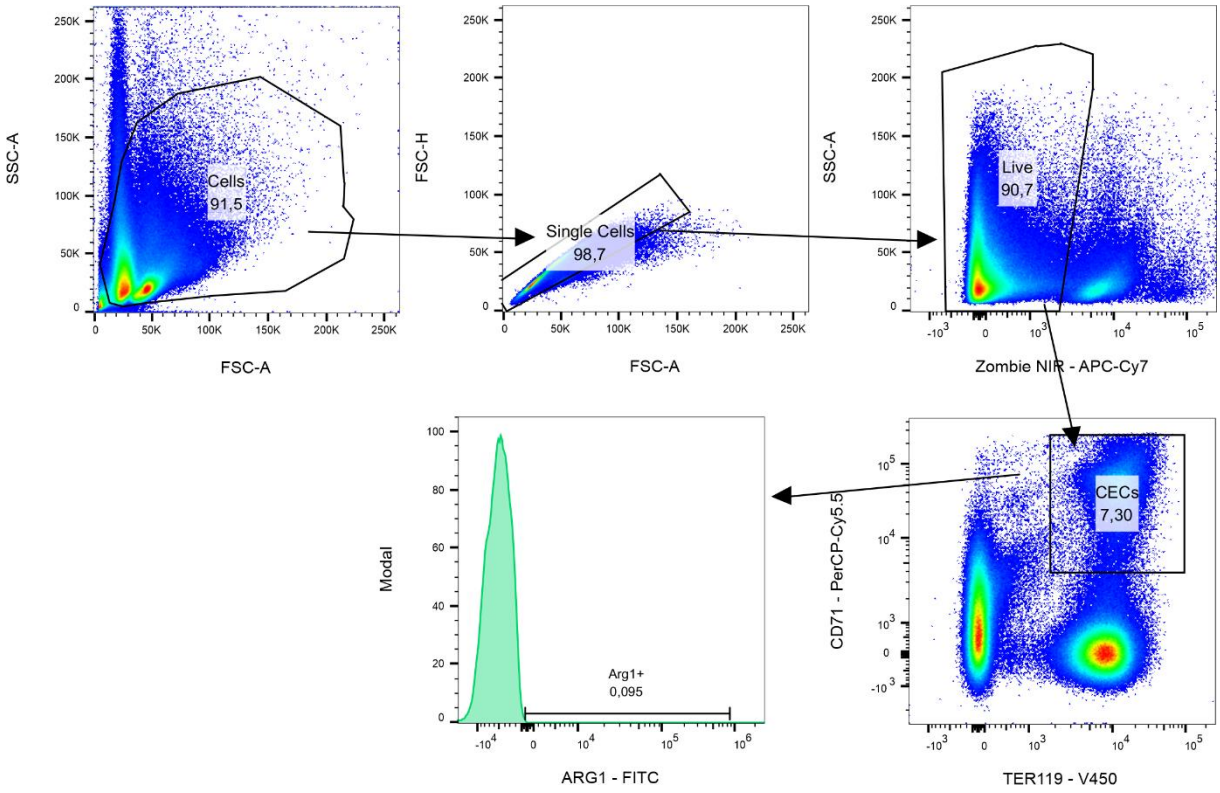
Supplementary Figure 32. Gating strategy used to analyze the data shown in Figure 5a. A minimum of 10 000 cells were acquired within the lymphocytes gate. The gate for proliferating (CTV^{low}) cells as well as $CD25^{+}$, $CD62L^{-}$ and $CD69^{+}$ cells was set based on the unstimulated control. Proliferation histograms were generated in FlowJo v10.6.2.



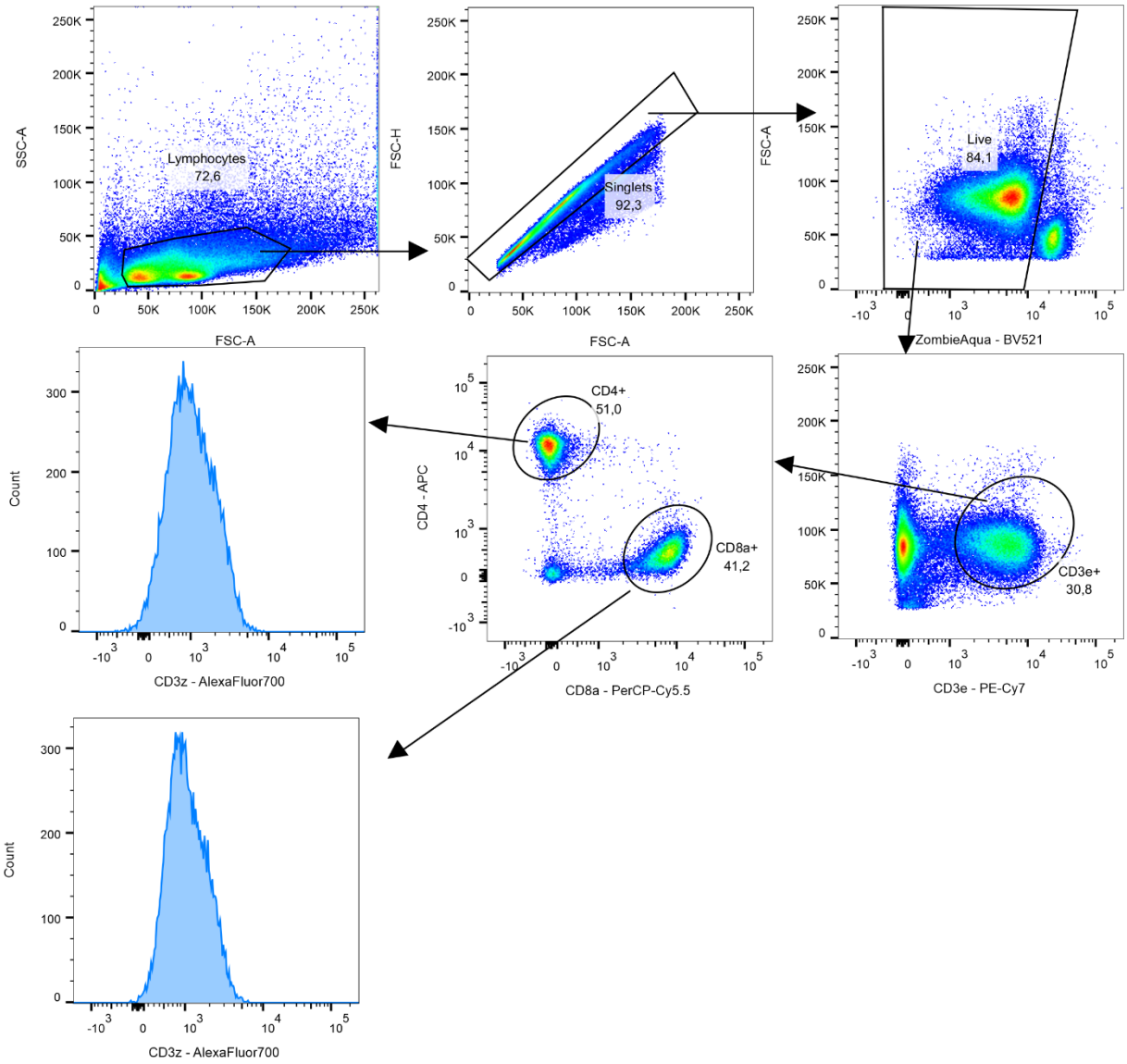
Supplementary Figure 33. Gating strategy used to analyze the data shown in Figure 3a, Figure 3b, Supplementary Figure 5a-f. A minimum of 100 000 cells were acquired within the live cell gate. Histograms were generated in FlowJo v10.6.2.



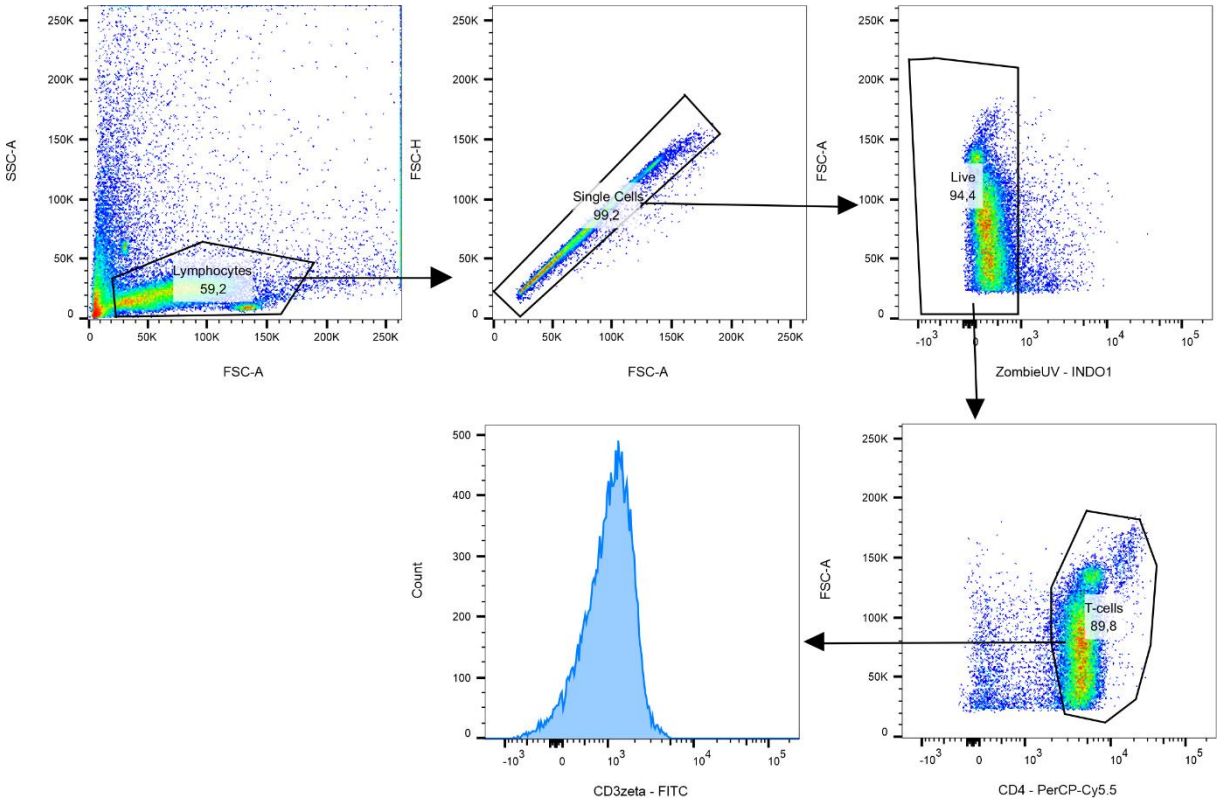
Supplementary Figure 34. Gating strategy used to analyze the data shown in Figure 3c-e, Supplementary Figure 14b-c. A minimum of 20 000 cells were acquired within the CECs cells gate. The gate for $ARG1^{+}$ cells and $ARG2^{+}$ was set based on the Fluorescence Minus One (FMO) control. Histograms were generated in FlowJo v10.6.2.



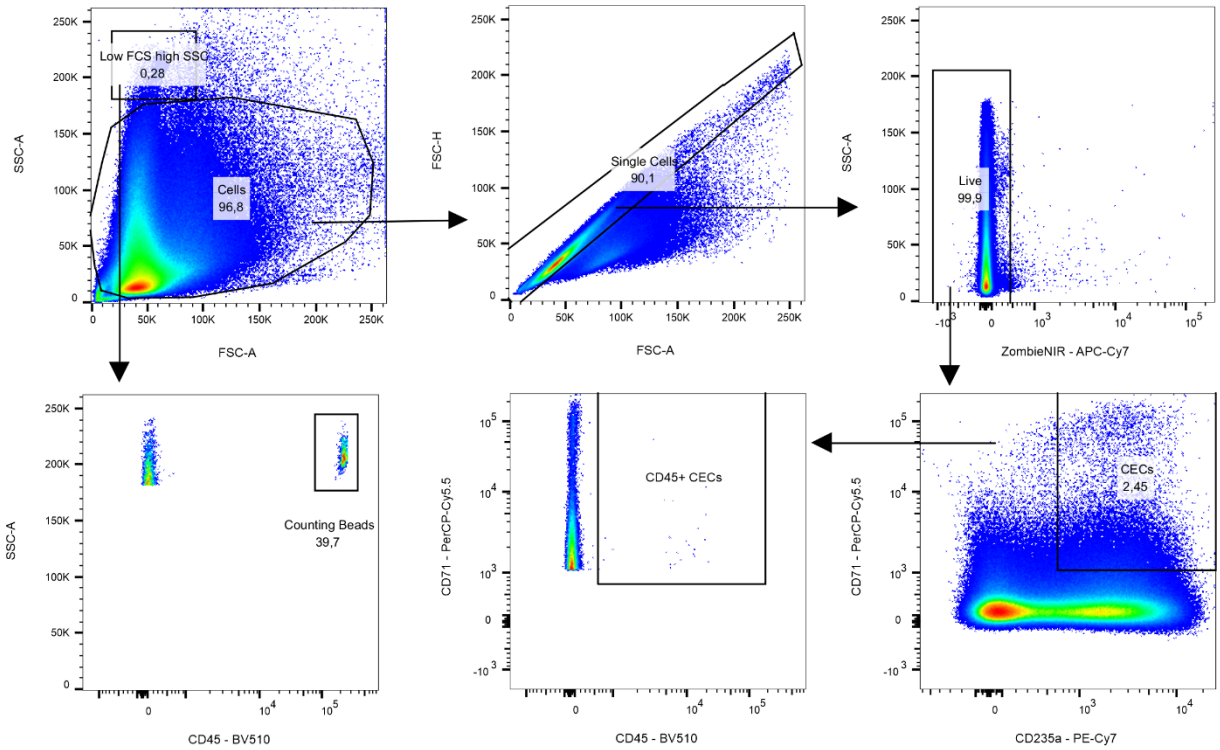
Supplementary Figure 35. Gating strategy used to analyze the data shown in Figure 3f-h, Figure 3k-l, Supplementary Fig. 10a-d. A minimum of 20 000 cells were acquired within the CECs cells gate. The gate for YFP/ARG1^{+} cells was set based YFP negative control from C57BL/6 mice. Histograms were generated in FlowJo v10.6.2.



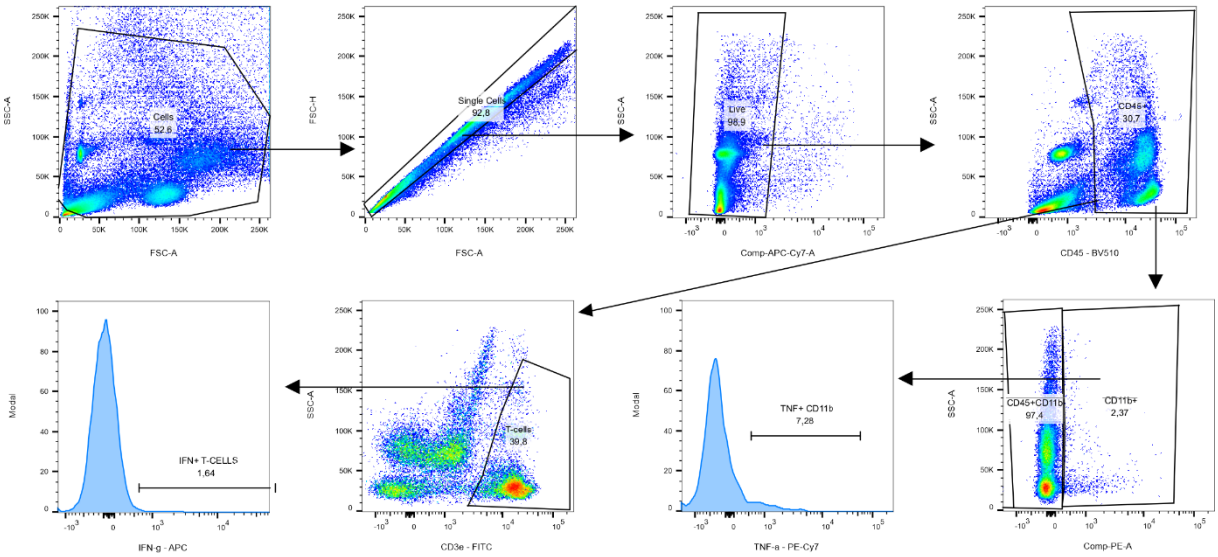
Supplementary Figure 36. Gating strategy used to analyze the data shown in Figure 5k-l, Supplementary Figure 13b-c. A minimum of 100 000 cells were acquired within the live cells gate. Histograms were generated in FlowJo v10.6.2.



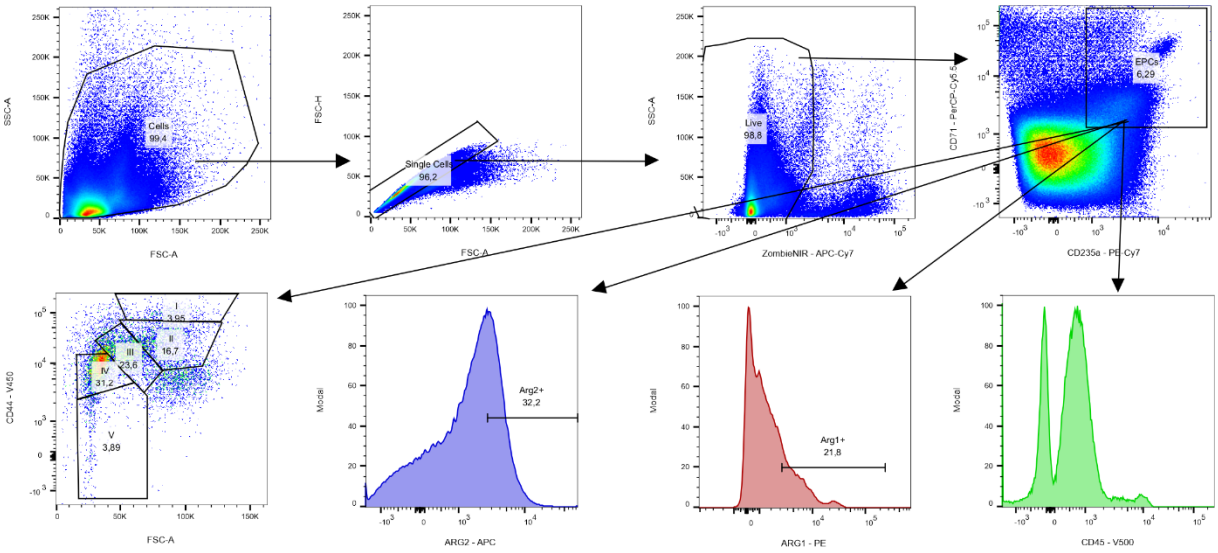
Supplementary Figure 37. Gating strategy used to analyze the data shown in Figure 5m-n, Figure 6a-b,. A minimum of 10 000 cells were acquired within the T-cells gate. Exemplary gating strategy used for the $CD4^+$ T-cell population (analogous strategy was used for the murine $CD8^+$ T-cell population). Histograms were generated in FlowJo v10.6.2.



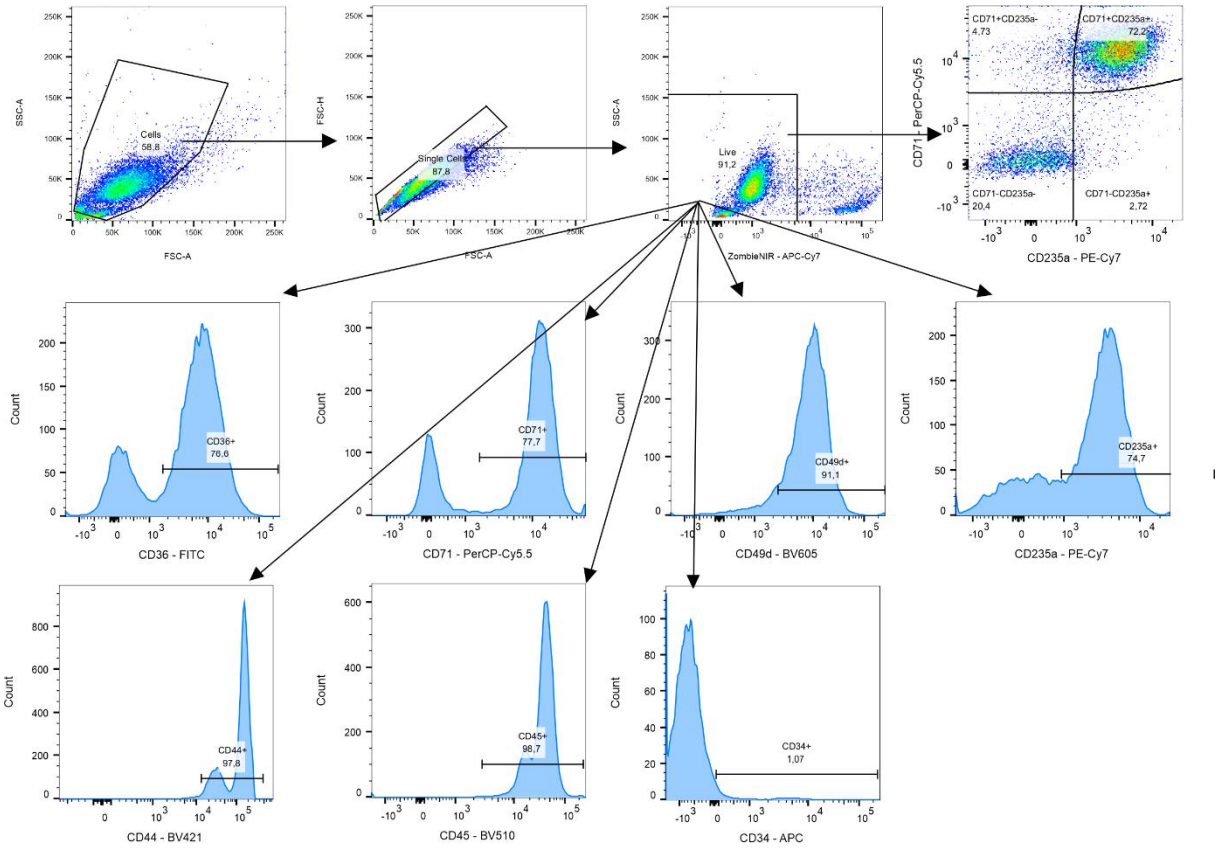
Supplementary Figure 38. Gating strategy used to analyze the data shown in Figure 7a-g, Figure 8a, Supplementary Figure 15a-b, Supplementary Figure 16g, Supplementary Figure 22c, Supplementary Figure 25a. A minimum of 500 000 cells were acquired within the live cells. For the data shown in Figure 7f-g, Figure 8a, Supplementary Figure 22a a minimum of 50 000 were acquired within the live cells.



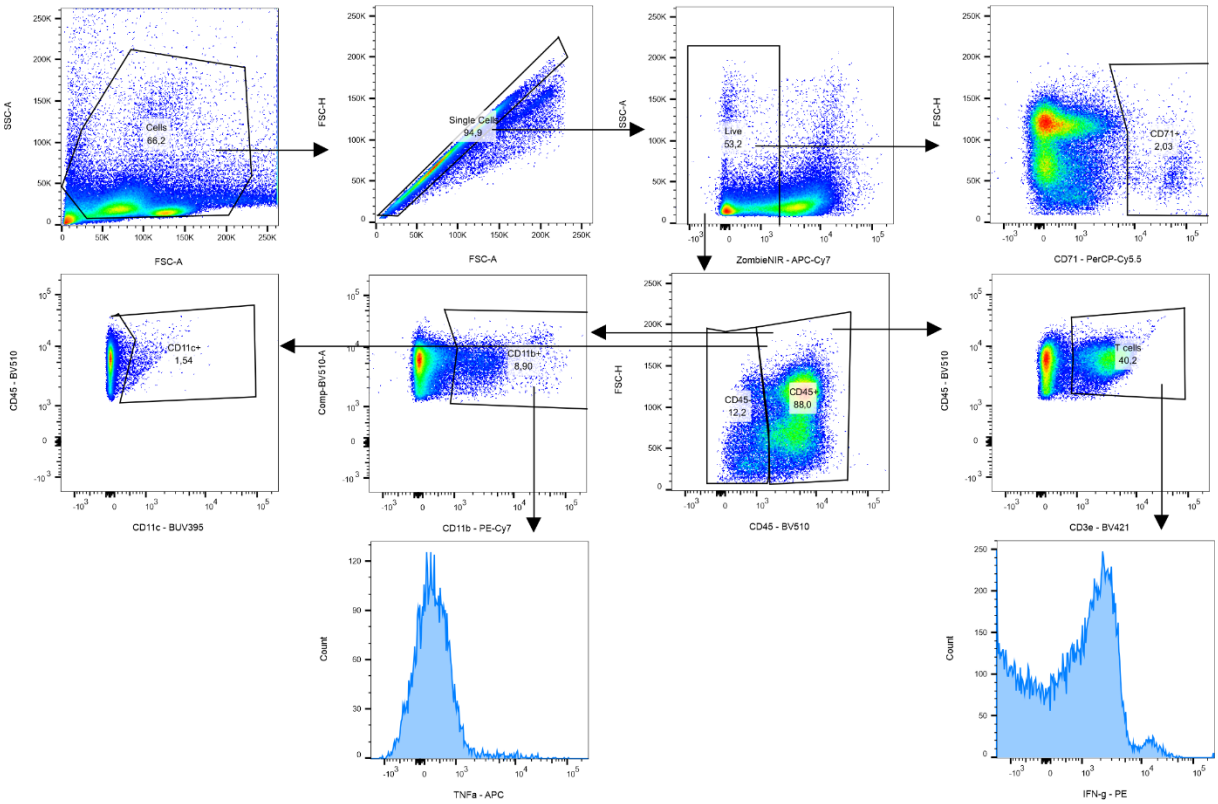
Supplementary Figure 39. Gating strategy used to analyze the data shown in Figure 7h-i, Supplementary Fig. 15e. A minimum of 100 000 cells were acquired within the live cells. The gate for IFN-γ^+ and TNF-α^+ cells were set based on unstimulated controls.



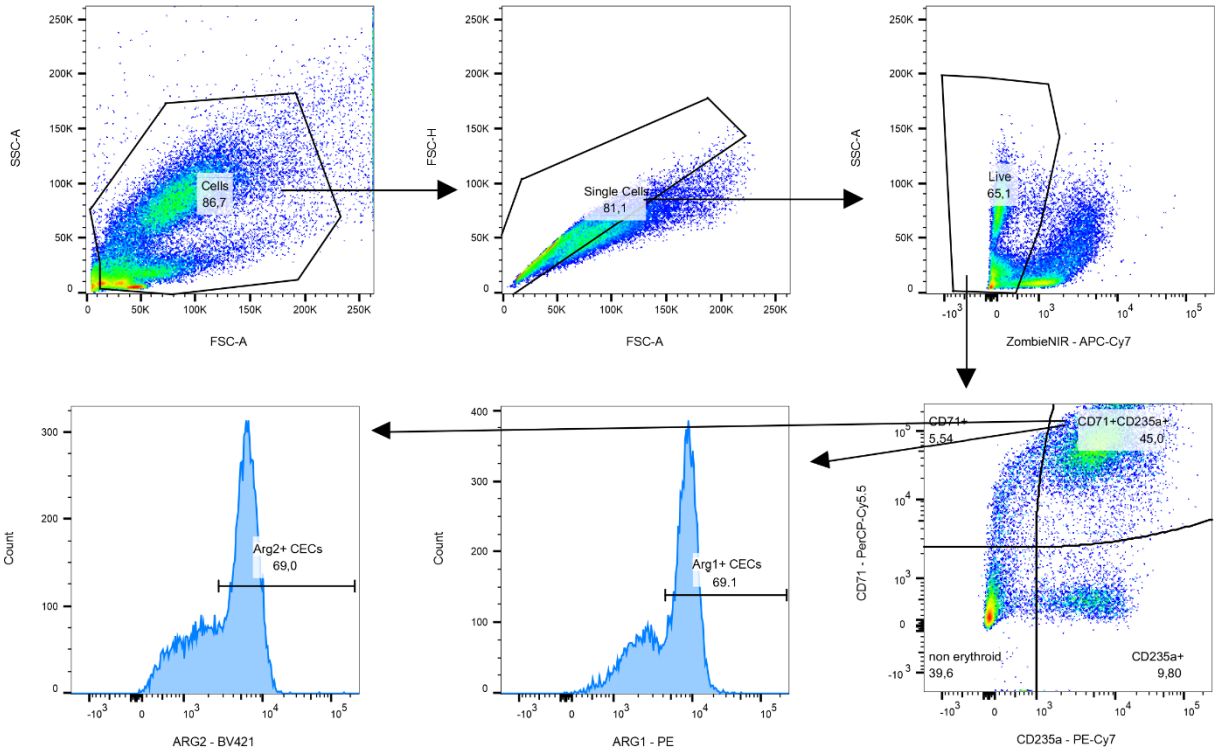
Supplementary Figure 40. Gating strategy used to analyze the data shown in Figure 8b-d, Supplementary Fig. 16a-f. A minimum of 100 000 cells were acquired within the live cells. The gate for ARG1^{+} and ARG2^{+} cells were set based on fluorescence minus one (FMO) controls.



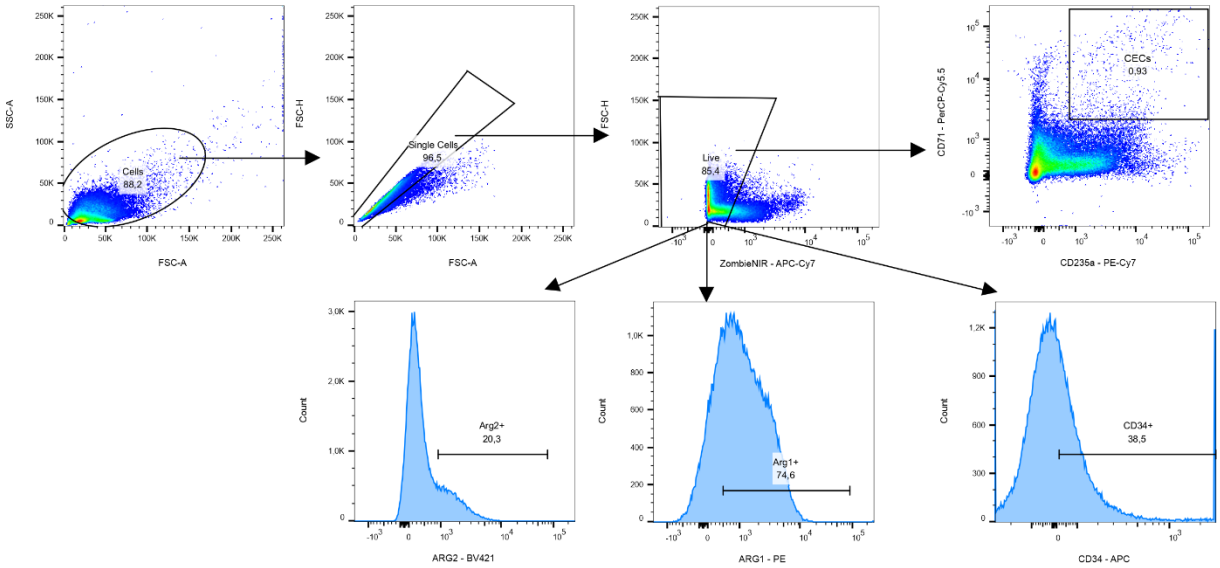
Supplementary Figure 41. Gating strategy used to analyze the data shown in Figure 9a, Figure 10a, Figure 10d, Figure 10h-j, Supplementary Fig. 19a,b,. A minimum of 50 000 cells were acquired within the live cells. The gate for $CD71^+$, $CD235a^+$, $CD36^+$, $CD49d^+$, $CD44^+$, $CD45^+$, $CD34^+$ cells were set based on fluorescence minus one (FMO) controls.



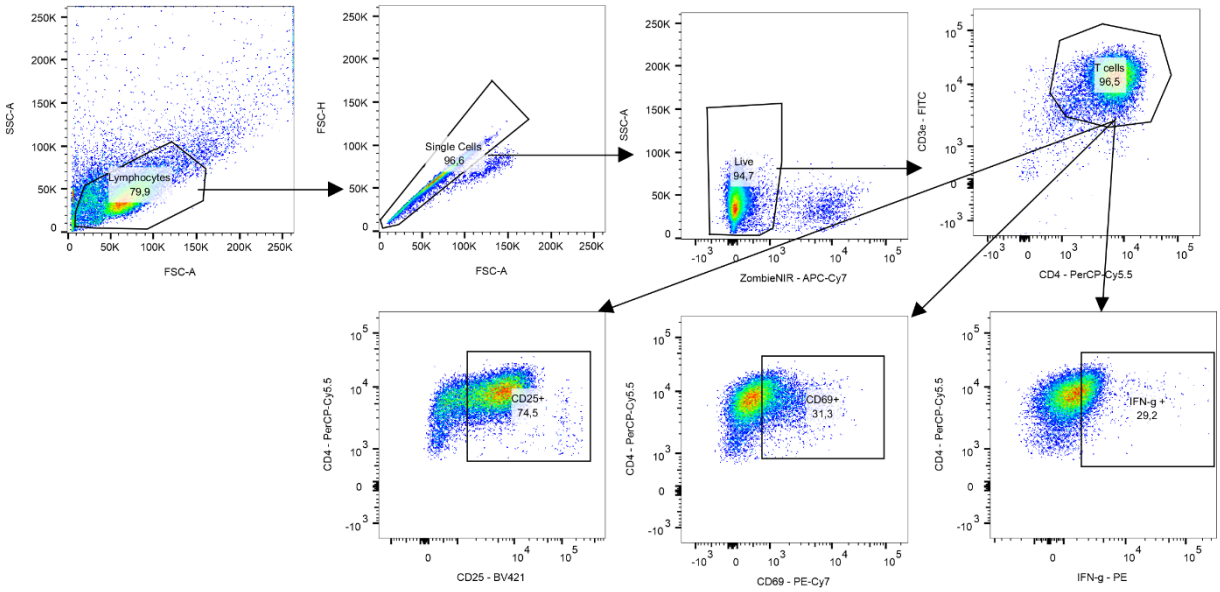
Supplementary Figure 42. Gating strategy used to analyze the data shown in Supplementary Figure 3a-b, Supplementary Figure 3d. A minimum of 50 000 cells were acquired within the live cells. The gate for TNF-α$^+$ and IFN-γ$^+$ cells were set based on isotype controls.



Supplementary Figure 43. Gating strategy used to analyze the data shown in Supplementary Figure 17b-d, Supplementary Figure 20a-b, Supplementary Figure 24a, Supplementary Figure 24c. A minimum of 20 000 cells were acquired within the live cells. The gate for ARG1^{+} and ARG2^{+} cells were set based on fluorescence minus one (FMO) controls.



Supplementary Figure 44. Gating strategy used to analyze the data shown in Supplementary Figure 21a-c. A minimum of 50 000 cells were acquired within the live cells. The gate for $CD34^{+}$, $ARG1^{+}$ and $ARG2^{+}$ cells were set based on fluorescence minus one (FMO) controls.



Supplementary Figure 45. Gating strategy used to analyze the data shown in Supplementary Figure 22a-c. A minimum of 20 000 cells were acquired within the live cells. The gate for $CD69^+$ and $CD25^+$ were set based on the unstimulated control. The gate for IFN-γ^+ cells were set based on the isotype control.

7. Podsumowanie i wnioski

W niniejszej pracy doktorskiej przedstawiono dane potwierdzające, iż supresja limfocytów T jest cechą wspólną mysich oraz ludzkich komórek CEC. Wykazano, iż właściwości immunoregulatorowe komórek CEC ulegają zmianom w trakcie ich różnicowania i są najsilniejsze w komórkach CEC znajdujących się we wczesnych stadiach dojrzewania. W ten sposób znacząco pogłębiono i poszerzono wiedzę na temat funkcji komórek CEC udowadniając ich kluczową w regulacji odpowiedzi układu odpornościowego. Strategie modulujące powstawanie oraz dojrzewanie komórek CEC, jak również wpływające na ich mechanizmy immunoregulatorowe są obiecującymi strategiami terapeutycznymi.

Szczegółowe wnioski wynikające z pracy doktorskiej to:

- komórki CEC ulegają ekspansji w śledzionach myszy z niedokrwistością;
- ostra niedokrwistość niehemolityczna u myszy prowadzi do upośledzenia odpowiedzi T-komórkowej;
- mysie komórki CEC charakteryzują się wysokim poziomem ARG2 i RFT, a niskim ARG1;
- poziom ARG1, ARG2 oraz RFT jest najwyższy w komórkach CEC znajdujących się w najwcześniejszych etapach dojrzewania i ulega sukcesywnemu obniżeniu w trakcie ich różnicowania;
- mysie komórki CEC indukowane niedokrwistością hamują aktywację i proliferację limfocytów w mechanizmie zależnym od ARG i RFT;
- komórki CEC ulegają ekspansji u chorych z niedokrwistością prowadząc do upośledzenia produkcji IFN-γ przez limfocyty T;
- komórki CEC izolowane ze szpiku kostnego zdrowych dawców hamują proliferację limfocytów T;
- ludzkie modelowe linie erytroidalne hamują proliferację limfocytów T w mechanizmie zależnym od ARG i RFT;
- ludzkie komórki CEC różnicowane z PBMCs hamują aktywację, produkcję IFN-γ oraz proliferację limfocytów T w mechanizmie zależnym od ARG i RFT;
- ekspresja ARG oraz właściwości immunoregulatorowe są najsilniejsze w komórkach CEC znajdujących się na wczesnych stadiach dojrzewania.

Opinie Komisji Etycznej i Komisji Bioetycznej

UCHWAŁA NR WAW2/117/2019

z dnia 12 lipca 2019 r.

II Lokalnej Komisji Etycznej do spraw doświadczeń na zwierzętach w Warszawie

§ 1

Na podstawie art. 48 ust. 1 pkt. 1[1] ustawy z dnia 15 stycznia 2015r. o ochronie zwierząt wykorzystywanych do celów naukowych lub edukacyjnych (Dz. U. poz. 266), zwanej dalej „ustawą" po rozpatrzeniu wniosku pt.: „Charakterystyka i rola erytroidalnych komórek progenitorowych (EPCs) w patogenezie szpiczaka plazmocytowego" z dnia 03 lipca 2019 roku, złożonego przez I Wydział Lekarski, Warszawski Uniwersytet Medyczny, adres: ul. Żwirki i Wigury 61, 02-091, Warszawa, zaplanowanego przez Jakuba Gołąba[2]

przy udziale[3] -

Lokalna Komisja Etyczna:

WYRAŻA ZGODĘ

na przeprowadzenie doświadczeń na zwierzętach w zakresie wniosku.

§ 2

W wyniku rozpatrzenia wniosku o którym mowa w § , Lokalna Komisja Etyczna ustaliła, że:

1. Wniosek należy przypisać do kategorii: [PB1] badania podstawowe, onkologia.
2. Najwyższy stopień dotkliwości proponowanych procedur to: umiarkowana.
3. Doświadczenia będą przeprowadzane na gatunkach lub grupach gatunków[4]:

Gatunek	Wiek/stadium	Liczba
Mysz domowa C57BL/6	6-7 tygodni	218
Mysz domowa B6.129S4-Arg1^{tm1Lky} (YARG)	6-7 tygodni	40

4. Doświadczenia będą przeprowadzane przez: Jakub Gołąb, Dominika Nowis, Zuzanna Rydzyńska, Anna Sosnowska.
5. Doświadczenie będzie przeprowadzane w terminie[5] od 01.08.2019 do 31.03.2022 r.
6. Doświadczenie będzie przeprowadzone w ośrodku[6]: Pracownia Hodowli Zwierząt Laboratoryjnych Katedry i Zakładu Histologii i Embriologii WUM, ul. Pawińskiego 3c, 02-106 Warszawa.
7. Doświadczenie będzie przeprowadzone poza ośrodkiem, w: nie dotyczy.
8. Użyte do procedur zwierzęta dzikie zostaną odłowione przez: nie dotyczy.
9. Doświadczenie ~~zostanie/~~nie zostanie poddane ocenie retrospektywnej~~ w terminie do 6 miesięcy od dnia przekazania przez użytkownika dokumentacji, mającej stanowić podstawę~~

[1] Niewłaściwy zapis usunąć

[2] imię i nazwisko osoby, która zaplanowała i jest odpowiedzialna za przeprowadzenie doświadczenia
[3] Wypełnić w przypadku dopuszczenia do postępowania organizacji społecznej.
[4] Podać liczbę, szczep/stado, wiek/stadium rozwoju
[5] Nie dłużej niż 5 lat
[6] Podać jeśli jest to inny ośrodek niż użytkownik

~~dokonania oceny retrospektywnej. Użytkownik jest zobowiązany do przekazania ww. dokumentacji niezwłocznie, tj. w terminie, o którym mowa w art. 52 ust. 2 ustawy~~.

§ 3

Uzasadnienie:

Komisja oceniła wniosek zgodnie z kryteriami zawartymi w art. 47.1. ustawy z dnia 15 stycznia 2015 r. o ochronie zwierząt wykorzystywanych do celów naukowych lub edukacyjnych (Dz. U. poz. 266). Po zapoznaniu się z problematyką badawczą przedstawioną we wniosku komisja stwierdza, że przedstawiony projekt spełnia zasady dopuszczenia doświadczeń na zwierzętach. Na podstawie art. 107 § 4 ustawy z dnia 14 czerwca 1960 r. – Kodeks postępowania administracyjnego z późniejszymi zmianami (Dz. U. z 2017 poz. 1257) odstąpiono od sporządzania uzasadnienia decyzji, gdyż uwzględnia ona w całości żądanie strony.

§ 4

Integralną część niniejszej uchwały stanowi kopia wniosku, o którym mowa w § 1.

(Pieczęć lokalnej komisji etycznej)

(Podpis Przewodniczącej komisji)

Pouczenie:

Zgodnie z art. 33 ust. 3 i art. 40 ustawy w zw. z art. 127 § 1 i 2 oraz 129 § 2 ustawy z dnia z dnia 14 czerwca 1960 r. Kodeks postępowania administracyjnego (Dz. U. 2017, poz. 1257 – tj.; dalej KPA) od uchwały Lokalnej Komisji Etycznej strona może wnieść, za jej pośrednictwem, odwołanie do Krajowej Komisji Etycznej do Spraw Doświadczeń na Zwierzętach w terminie 14 od dnia doręczenia uchwały.

Na podstawie art. 127a KPA w trakcie biegu terminu do wniesienia odwołania strona może zrzec się prawa do jego wniesienia, co należy uczynić wobec Lokalnej Komisji Etycznej, która wydała uchwałę. Z dniem doręczenia Lokalnej Komisji Etycznej oświadczenia o zrzeczeniu się prawa do wniesienia odwołania przez ostatnią ze stron postępowania, decyzja staje się ostateczna i prawomocna.

Otrzymuje:
1) Użytkownik,
2) Organizacja społeczna dopuszczona do udziału w postępowaniu (jeśli dotyczy)
3) a/a

Użytkownik kopie przekazuje:
- Osoba planująca doświadczenie
- Zespół ds. dobrostanu

UCHWAŁA NR WAW2/143/2020

z dnia 2 grudnia 2020 r.

II Lokalnej Komisji Etycznej do Spraw Doświadczeń na Zwierzętach w Warszawie

§ 1

Na podstawie art. 48 ust. 1 pkt. 1[1] ustawy z dnia 15 stycznia 2015r. o ochronie zwierząt wykorzystywanych do celów naukowych lub edukacyjnych (Dz. U. z 2019 r. poz. 1392), zwanej dalej „ustawą" po rozpatrzeniu wniosku pt.: **„Rola arginazy 1 (Arg1) oraz arginazy 2 (Arg2) w regulacji funkcji erytroidalnych komórek progenitorowych (EPCs)"** z dnia 12 listopada 2020 roku, złożonego przez: Zakład Immunologii, Wydziału Lekarskiego, Warszawskiego Uniwersytetu Medycznego, adres: 02-097 Warszawa, ul. Nielubowicza 5, zaplanowanego przez Dominikę Nowis[2] przy udziale[3] -

Lokalna Komisja Etyczna:

WYRAŻA ZGODĘ

na przeprowadzenie doświadczeń na zwierzętach w zakresie wniosku.

§ 2

W wyniku rozpatrzenia wniosku o którym mowa w § 1, Lokalna Komisja Etyczna ustaliła, że:

1. Wniosek należy przypisać do kategorii: badania podstawowe - układ odpornościowy [PB7].
2. Najwyższy stopień dotkliwości proponowanych procedur to: umiarkowana.
3. Doświadczenia będą przeprowadzane na gatunkach lub grupach gatunków[4]:

Gatunek	Wiek/stadium rozwoju	Liczba
Mysz domowa (C57BL/6 WT)	6-7 tyg.	15
Mysz domowa (*Arg2* KO, Arg2^{tm1Weo}/J)	6-7 tyg.	15

4. Doświadczenia będą przeprowadzane przez: Dominika Nowis, Tomasz Grzywa.
5. Doświadczenie będzie przeprowadzane w terminie[5] od 03.12.2020 do 01.12.2021 r.
6. Doświadczenie będzie przeprowadzone w ośrodku[6]: nie dotyczy.
7. Doświadczenie będzie przeprowadzone poza ośrodkiem, w: Pracownia Hodowli Zwierząt Laboratoryjnych Katedry i Zakładu Histologii i Embriologii WUM, 02-106 Warszawa, ul. Pawińskiego 3C.
8. Użyte do procedur zwierzęta dzikie zostaną odłowione przez: nie dotyczy.
9. Doświadczenie ~~zostanie/~~nie zostanie poddane ocenie retrospektywnej ~~na podstawie art. 53 ust. 1 ustawy w terminie do 6 miesięcy od dnia przekazania przez użytkownika dokumentacji, mającej stanowić podstawę dokonania oceny retrospektywnej. Użytkownik jest zobowiązany do przekazania ww. dokumentacji niezwłocznie, tj. w terminie, o którym mowa w art. 52 ust. 2 ustawy.~~

[1] Niewłaściwy zapis usunąć

[2] imię i nazwisko osoby, która zaplanowała i jest odpowiedzialna za przeprowadzenie doświadczenia
[3] Wypełnić w przypadku dopuszczenia do postępowania organizacji społecznej.
[4] Podać liczbę, szczep/stado, wiek/stadium rozwoju
[5] Nie dłużej niż 5 lat
[6] Podać jeśli jest to inny ośrodek niż użytkownik

§ 3

Uzasadnienie:

Komisja oceniła wniosek zgodnie z art. 47 ust. 1 i 2 ustawy z dnia 15 stycznia 2015 r. o ochronie zwierząt wykorzystywanych do celów naukowych lub edukacyjnych (Dz. U. z 2019 r. poz. 1392). Po zapoznaniu się z problematyką badawczą przedstawioną we wniosku komisja stwierdza, że przedstawiony projekt spełnia zasady dopuszczenia doświadczeń na zwierzętach pod kątem oceny etycznej. Na podstawie art. 107 § 4 ustawy z dnia 14 czerwca 1960 r. – Kodeks postępowania administracyjnego z późniejszymi zmianami (Dz. U. 2020 r. poz. 256) odstąpiono od sporządzania uzasadnienia decyzji, gdyż decyzja jest zgodna z wnioskiem strony.

§ 4

Integralną część niniejszej uchwały stanowi kopia wniosku, o którym mowa w § 1.

Szkoła Główna Gospodarstwa Wiejskiego
w Warszawie
II Lokalna Komisja Etyczna
ds. Doświadczeń na Zwierzętach
02-786 Warszawa, ul. Ciszewskiego 8
tel. 22 59-35622
(Pieczęć lokalnej komisji etycznej)

PRZEWODNICZĄCA
II Lokalnej Komisji Etycznej
ds. Doświadczeń na Zwierzętach przy SGGW
/ Prof. dr hab. Joanna Gromadzka-Ostrowska /
(Podpis Przewodniczącej Komisji)

Pouczenie:

Zgodnie z art. 33 ust. 3 i art. 40 ustawy w zw. z art. 127 § 1 i 2 oraz 129 § 2 ustawy z dnia z dnia 14 czerwca 1960 r. Kodeks postępowania administracyjnego (Dz. U. 2017, poz. 1257 – tj.; dalej KPA) od uchwały Lokalnej Komisji Etycznej strona może wnieść, za jej pośrednictwem, odwołanie do Krajowej Komisji Etycznej do Spraw Doświadczeń na Zwierzętach w terminie 14 od dnia doręczenia uchwały.

Na podstawie art. 127a KPA w trakcie biegu terminu do wniesienia odwołania strona może zrzec się prawa do jego wniesienia, co należy uczynić wobec Lokalnej Komisji Etycznej, która wydała uchwałę. Z dniem doręczenia Lokalnej Komisji Etycznej oświadczenia o zrzeczeniu się prawa do wniesienia odwołania przez ostatnią ze stron postępowania, decyzja staje się ostateczna i prawomocna.

Otrzymuje:
1) Użytkownik,
2) Organizacja społeczna dopuszczona do udziału w postępowaniu (jeśli dotyczy)
3) a/a

Użytkownik kopie przekazuje:
- Osoba planująca doświadczenie
- Zespół ds. dobrostanu

Komisja Bioetyczna
przy Warszawskim Uniwersytecie Medycznym

Tel.: 022/ 57 - 20 -303
Fax: 022/ 57 - 20 -165

ul. Żwirki i Wigury nr 61
02-091 Warszawa

e-mail: komisja.bioetyczna@wum.edu.pl
www.komisja-bioetyczna.wum.edu.pl

Warszawa, dnia 13 grudnia 2021r.

AKBE/ 246 / 2021

Prof. dr hab. med. Dominika Nowis
Laboratorium Medycyny Doświadczalnej
ul. Jana Nielubowicza 5,
02 – 097 Warszawa

OŚWIADCZENIE

Niniejszym oświadczam, że Komisja Bioetyczna przy Warszawskim Uniwersytecie Medycznym w dniu 13 grudnia 2021r. przyjęła do wiadomości informację na temat badania pt. "Rola komórek erytroidalnych wykazujących ekspresję cząsteczki CD71 w regulacji odpowiedzi immunologicznej u pacjentów z niedokrwistością." Przedstawione badanie nie stanowi eksperymentu medycznego w rozumieniu art. 21 ust. 1 ustawy z dnia 5 grudnia 1996 r. o zawodach lekarza i lekarza dentysty(Dz.U. z 2018 r. poz. 617) i nie wymaga uzyskania opinii Komisji Bioetycznej przy Warszawskim Uniwersytecie Medycznym, o której mowa w art. 29 ust.1 ww. ustawy.

Przewodnicząca Komisji Bioetycznej

Prof. dr hab. n. med. Magdalena Kuźma –Kozakiewicz

Oświadczenia współautorów publikacji

Warszawa, 16.12.2021
(miejscowość, data)

Tomasz Maciej Grzywa

OŚWIADCZENIE

Jako współautor pracy pt. „The role of CD71+ erythroid cells in the regulation of the immune response" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

konceptualizacja pracy, przegląd literatury, napisanie manuskryptu, przygotowanie figur.

Mój udział procentowy w przygotowaniu publikacji określam jako 60%.

Tomasz Grzywa
.....

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 16.12.2021
(miejscowość, data)

Prof. dr hab. Dominika Nowis

OŚWIADCZENIE

Jako współautor pracy pt. „The role of CD71+ erythroid cells in the regulation of the immune response" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

Zapewnienie finansowania, udział w przeglądzie literatury, udział w napisaniu manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 15%.
Wkład Tomasza Macieja Grzywa w powstawanie publikacji określam jako 60 %,

obejmował on: konceptualizacja pracy, przegląd literatury, napisanie manuskryptu, przygotowanie figur.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej Tomasza Macieja Grzywa.

.....

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 16.12.2021
(miejscowość, data)

Prof. dr hab. Jakub Gołąb

OŚWIADCZENIE

Jako współautor pracy pt. „The role of CD71+ erythroid cells in the regulation of the immune response" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Zapewnienie finansowania, udział w przeglądzie literatury, udział w napisaniu manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 25%.
Wkład Tomasza Macieja Grzywa w powstawanie publikacji określam jako 60 %,

obejmował on: konceptualizacja pracy, przegląd literatury, napisanie manuskryptu, przygotowanie figur.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej Tomasza Macieja Grzywa.

.....

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 16.12.2021
(miejscowość, data)

Tomasz Maciej Grzywa

OŚWIADCZENIE

Jako współautor pracy pt. „Tumor Immune Evasion Induced by Dysregulation of Erythroid Progenitor Cells Development" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

konceptualizacja pracy, przegląd literatury, napisanie manuskryptu, przygotowanie figur.

Mój udział procentowy w przygotowaniu publikacji określam jako 60%

Tomasz Grzywa

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 16.12.2021
(miejscowość, data)

Magdalena Justyniarska

OŚWIADCZENIE

Jako współautor pracy pt. „Tumor Immune Evasion Induced by Dysregulation of Erythroid Progenitor Cells Development" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

Udział w przeglądzie literatury, udział w napisaniu manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 8 %.
Wkład Tomasza Macieja Grzywa w powstawanie publikacji określam jako 60 %,

obejmował on: konceptualizacja pracy, przegląd literatury, napisanie manuskryptu, przygotowanie figur.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej Tomasza Macieja Grzywa.

Magdalena Justyniarska

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 16.12.2021
(miejscowość, data)

Prof. dr hab. Dominika Nowis

OŚWIADCZENIE

Jako współautor pracy pt. „Tumor Immune Evasion Induced by Dysregulation of Erythroid Progenitor Cells Development" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

Zapewnienie finansowania, udział w przeglądzie literatury, udział w napisaniu manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 7%.
Wkład Tomasza Macieja Grzywa w powstawanie publikacji określam jako 60 %,

obejmował on: konceptualizacja pracy, przegląd literatury, napisanie manuskryptu, przygotowanie figur.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej Tomasza Macieja Grzywa.

.....

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 16.12.2021
(miejscowość, data)

Prof. dr hab. Jakub Gołąb

OŚWIADCZENIE

Jako współautor pracy pt. „Tumor Immune Evasion Induced by Dysregulation of Erythroid Progenitor Cells Development" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Zapewnienie finansowania, udział w przeglądzie literatury, udział w napisaniu manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 25%.
Wkład Tomasza Macieja Grzywa w powstawanie publikacji określam jako 60 %,

obejmował on: konceptualizacja pracy, przegląd literatury, napisanie manuskryptu, przygotowanie figur.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej Tomasza Macieja Grzywa.

.....

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 16.12.2021
(miejscowość, data)

Tomasz Maciej Grzywa

OŚWIADCZENIE

Jako współautor pracy pt. „Potent but transient immunosuppression of T-cells is a general feature of CD71+ erythroid cells" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

przygotowanie koncepcji badania, opracowanie metodyki badania, wykonanie eksperymentów, opracowanie i analiza wyników, przygotowanie figur, napisanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 50%.

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 16.12.2021

Mgr Anna Sosnowska

OŚWIADCZENIE

Jako współautor pracy pt. „Potent but transient immunosuppression of T-cells is a general feature of CD71+ erythroid cells" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Udział w wykonaniu doświadczeń *in vivo* w mysim modelu niedokrwistości.

Mój udział procentowy w przygotowaniu publikacji określam jako 1%.
Wkład Tomasza Macieja Grzywy w powstawanie publikacji określam jako 50 %,

obejmował on: przygotowanie koncepcji badania, opracowanie metodyki badania, wykonanie eksperymentów, opracowanie i analiza wyników, przygotowanie figur, napisanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej Tomasza Macieja Grzywy.

Anna Sosnowska

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 16.12.2021

Mgr Zuzanna Rydzyńska

OŚWIADCZENIE

Jako współautor pracy pt. „Potent but transient immunosuppression of T-cells is a general feature of CD71+ erythroid cells" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Udział w wykonaniu doświadczeń *in vitro*.

Mój udział procentowy w przygotowaniu publikacji określam jako 1%.
Wkład Tomasza Macieja Grzywy w powstawanie publikacji określam jako 50 %,

obejmował on: przygotowanie koncepcji badania, opracowanie metodyki badania, wykonanie eksperymentów, opracowanie i analiza wyników, przygotowanie figur, napisanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej Tomasza Macieja Grzywy.

17.12.21 Zuzanna Rydzyńska

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 16.12.2021

Dr n. farm. Michał Łaźniewski

OŚWIADCZENIE

Jako współautor pracy pt. „Potent but transient immunosuppression of T-cells is a general feature of CD71+ erythroid cells" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Przeprowadzenie eksperymentów *in silico* dokowania molekularnego cząsteczki fenylohydrazyny oraz molekularnych symulacji.

Mój udział procentowy w przygotowaniu publikacji określam jako 1%.
Wkład Tomasza Macieja Grzywy w powstawanie publikacji określam jako 50 %,

obejmował on: przygotowanie koncepcji badania, opracowanie metodyki badania, wykonanie eksperymentów, opracowanie i analiza wyników, przygotowanie figur, napisanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej Tomasza Macieja Grzywy.

Michał Łaźniewski

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 16.12.2021

Prof. dr hab. Dariusz Plewczyński

OŚWIADCZENIE

Jako współautor pracy pt. „Potent but transient immunosuppression of T-cells is a general feature of CD71+ erythroid cells" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Przeprowadzenie eksperymentów *in silico* dokowania molekularnego cząsteczki fenylohydrazyny oraz molekularnych symulacji.

Mój udział procentowy w przygotowaniu publikacji określam jako 1%.
Wkład Tomasza Macieja Grzywy w powstawanie publikacji określam jako 50 %,

obejmował on: przygotowanie koncepcji badania, opracowanie metodyki badania, wykonanie eksperymentów, opracowanie i analiza wyników, przygotowanie figur, napisanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej Tomasza Macieja Grzywy.

.....

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 16.12.2021

Lek. Klaudia Klicka

OŚWIADCZENIE

Jako współautor pracy pt. „Potent but transient immunosuppression of T-cells is a general feature of CD71+ erythroid cells" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Przeprowadzenie reakcji real-time qPCR, udział w eksperymentach *in vitro*.

Mój udział procentowy w przygotowaniu publikacji określam jako 1%.
Wkład Tomasza Macieja Grzywy w powstawanie publikacji określam jako 50 %,

obejmował on: przygotowanie koncepcji badania, opracowanie metodyki badania, wykonanie eksperymentów, opracowanie i analiza wyników, przygotowanie figur, napisanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej Tomasza Macieja Grzywy.

Klaudia Klicka

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 16.12.2021

Mgr Milena Małecka-Giełdowska

OŚWIADCZENIE

Jako współautor pracy pt. „Potent but transient immunosuppression of T-cells is a general feature of CD71+ erythroid cells" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Zebranie, zapewnienie oraz analiza ludzkich próbek krwi.

Mój udział procentowy w przygotowaniu publikacji określam jako 0.5%.
Wkład Tomasza Macieja Grzywy w powstawanie publikacji określam jako 50 %,

obejmował on: przygotowanie koncepcji badania, opracowanie metodyki badania, wykonanie eksperymentów, opracowanie i analiza wyników, przygotowanie figur, napisanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej Tomasza Macieja Grzywy.

Milena Małecka-Giełdowska

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 16.12.2021

mgr Anna Rodziewicz-Lurzyńska

OŚWIADCZENIE

Jako współautor pracy pt. „Potent but transient immunosuppression of T-cells is a general feature of CD71+ erythroid cells" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

Analiza próbek mysiej krwi- morfologia krwi.

Mój udział procentowy w przygotowaniu publikacji określam jako 0.5%.
Wkład Tomasza Macieja Grzywy w powstawanie publikacji określam jako 50 %,

obejmował on: przygotowanie koncepcji badania, opracowanie metodyki badania, wykonanie eksperymentów, opracowanie i analiza wyników, przygotowanie figur, napisanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej Tomasza Macieja Grzywy.

09776 mgr Anna Rodziewicz-Lurzyńska
DIAGNOSTA LABORATORYJNY
specjalista diagnostyki laboratoryjnej

.....

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 16.12.2021

Dr hab. n. med. Olga Ciepiela

OŚWIADCZENIE

Jako współautor pracy pt. „Potent but transient immunosuppression of T-cells is a general feature of CD71+ erythroid cells" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
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Mój udział procentowy w przygotowaniu publikacji określam jako 0.5%.
Wkład Tomasza Macieja Grzywy w powstawanie publikacji określam jako 50 %,

obejmował on: przygotowanie koncepcji badania, opracowanie metodyki badania, wykonanie eksperymentów, opracowanie i analiza wyników, przygotowanie figur, napisanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej Tomasza Macieja Grzywy.

.....

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 16.12.2021

Magdalena Justyniarska

OŚWIADCZENIE

Jako współautor pracy pt. „Potent but transient immunosuppression of T-cells is a general feature of CD71+ erythroid cells" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Udział w wykonaniu doświadczeń *in vitro.*

Mój udział procentowy w przygotowaniu publikacji określam jako 0.5%.
Wkład Tomasza Macieja Grzywa w powstawanie publikacji określam jako 50 %,

obejmował on: przygotowanie koncepcji badania, opracowanie metodyki badania, wykonanie eksperymentów, opracowanie i analiza wyników, przygotowanie figur, napisanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej Tomasza Macieja Grzywa.

Magdalena Justyniarska

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 16.12.2021

Mgr Paulina Pomper

OŚWIADCZENIE

Jako współautor pracy pt. „Potent but transient immunosuppression of T-cells is a general feature of CD71+ erythroid cells" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Przeprowadzenie analiz aktywności arginazy 1 oraz arginazy 2.

Mój udział procentowy w przygotowaniu publikacji określam jako 0.5%.
Wkład Tomasza Macieja Grzywy w powstawanie publikacji określam jako 50 %,

obejmował on: przygotowanie koncepcji badania, opracowanie metodyki badania, wykonanie eksperymentów, opracowanie i analiza wyników, przygotowanie figur, napisanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej Tomasza Macieja Grzywy.

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 16.12.2021

Dr Marcin M. Grzybowski

OŚWIADCZENIE

Jako współautor pracy pt. „Potent but transient immunosuppression of T-cells is a general feature of CD71+ erythroid cells" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Przeprowadzenie analiz aktywności arginazy 1 oraz arginazy 2.

Mój udział procentowy w przygotowaniu publikacji określam jako 0.5%.
Wkład Tomasza Macieja Grzywy w powstawanie publikacji określam jako 50 %,

obejmował on: przygotowanie koncepcji badania, opracowanie metodyki badania, wykonanie eksperymentów, opracowanie i analiza wyników, przygotowanie figur, napisanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej Tomasza Macieja Grzywy.

.....

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 16.12.2021

Dr n. chem. Roman Błaszczyk

OŚWIADCZENIE

Jako współautor pracy pt. „Potent but transient immunosuppression of T-cells is a general feature of CD71+ erythroid cells" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Opracowanie i zapewnienie inhibitora arginazy OAT-1746.

Mój udział procentowy w przygotowaniu publikacji określam jako 0.5%.
Wkład Tomasza Macieja Grzywy w powstawanie publikacji określam jako 50 %,

obejmował on: przygotowanie koncepcji badania, opracowanie metodyki badania, wykonanie eksperymentów, opracowanie i analiza wyników, przygotowanie figur, napisanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej Tomasza Macieja Grzywy.

Roman Błaszczyk

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 16.12.2021

Dr Michał Węgrzynowicz

OŚWIADCZENIE

Jako współautor pracy pt. „Potent but transient immunosuppression of T-cells is a general feature of CD71+ erythroid cells" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Hodowla oraz zapewnienie myszy *Arg2*$^{-/-}$.

Mój udział procentowy w przygotowaniu publikacji określam jako 0.5%.
Wkład Tomasza Macieja Grzywy w powstawanie publikacji określam jako 50 %,

obejmował on: przygotowanie koncepcji badania, opracowanie metodyki badania, wykonanie eksperymentów, opracowanie i analiza wyników, przygotowanie figur, napisanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej Tomasza Macieja Grzywy.

.....

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 16.12.2021

Dr n. med. Agnieszka Tomaszewska

OŚWIADCZENIE

Jako współautor pracy pt. „Potent but transient immunosuppression of T-cells is a general feature of CD71+ erythroid cells" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Zebranie oraz zapewnienie komórek macierzystych z krwi obwodowej.

Mój udział procentowy w przygotowaniu publikacji określam jako 0.5%.
Wkład Tomasza Macieja Grzywy w powstawanie publikacji określam jako 50 %,

obejmował on: przygotowanie koncepcji badania, opracowanie metodyki badania, wykonanie eksperymentów, opracowanie i analiza wyników, przygotowanie figur, napisanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej Tomasza Macieja Grzywy.

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 16.12.2021

Prof. dr hab. Grzegorz Basak

OŚWIADCZENIE

Jako współautor pracy pt. „Potent but transient immunosuppression of T-cells is a general feature of CD71+ erythroid cells" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Zebranie oraz zapewnienie komórek macierzystych z krwi obwodowej.

Mój udział procentowy w przygotowaniu publikacji określam jako 0.5%.
Wkład Tomasza Macieja Grzywy w powstawanie publikacji określam jako 50 %,

obejmował on: przygotowanie koncepcji badania, opracowanie metodyki badania, wykonanie eksperymentów, opracowanie i analiza wyników, przygotowanie figur, napisanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej Tomasza Macieja Grzywy.

.....

KIEROWNIK
Katedry i Kliniki Hematologii,
Transplantologii i Chorób Wewnętrznych
prof. dr hab. med. Grzegorz W. Basak

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 16.12.2021

Prof. dr hab. Jakub Gołąb

OŚWIADCZENIE

Jako współautor pracy pt. „Potent but transient immunosuppression of T-cells is a general feature of CD71+ erythroid cells" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Koncepcja projektu, zapewnienie finansowania projektu, zaprojektowanie i kierowanie projektem, udział w przygotowaniu manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 20%.
Wkład Tomasza Macieja Grzywa w powstawanie publikacji określam jako 50 %,

obejmował on: przygotowanie koncepcji badania, opracowanie metodyki badania, wykonanie eksperymentów, opracowanie i analiza wyników, przygotowanie figur, napisanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej Tomasza Macieja Grzywa.

.....

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 16.12.2021

Prof. dr hab. Dominika Nowis

OŚWIADCZENIE

Jako współautor pracy pt. „Potent but transient immunosuppression of T-cells is a general feature of CD71+ erythroid cells" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Zapewnienie finansowania projektu, udział w doświadczeniach *in vivo*, zaprojektowanie i kierowanie projektem, udział w przygotowaniu manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 20%.
Wkład Tomasza Macieja Grzywa w powstawanie publikacji określam jako 50 %,

obejmował on: przygotowanie koncepcji badania, opracowanie metodyki badania, wykonanie eksperymentów, opracowanie i analiza wyników, przygotowanie figur, napisanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej Tomasza Macieja Grzywa.

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*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Analiza bibliometryczna dorobku publikacyjnego

WARSZAWSKI UNIWERSYTET MEDYCZNY
MEDICAL UNIVERSITY OF WARSAW
Biblioteka Główna

BIBG/Punktacja/ 906 /2021/KK

Warszawa, 29 grudnia 2021 r.

ANALIZA BIBLIOMETRYCZNA PUBLIKACJI
PANA TOMASZA MACIEJA GRZYWY
WCHODZĄCYCH W SKŁAD CYKLU PUBLIKACJI STANOWIĄCYCH ROZPRAWĘ DOKTORSKĄ

Lp.	Opis bibliograficzny	Impact Factor	MEiN
	Artykuły		
1.	**Grzywa T**, Sosnowska A, Rydzyńska Z, Lazniewski M, Plewczynski D, Klicka K, Małecka-Giełdowska M, Rodziewicz-Lurzynska A, Ciepiela O, Justyniarska M, Pomper P, Grzybowski M, Blaszczyk R, Wegrzynowicz M, Tomaszewska A, Basak G, Gołąb J, Nowis D. Potent but transient immunosuppression of T-cells is a general feature of CD71 + erythroid cells. Communications Biology. 2021;4(1):1384. [Rodzaj publikacji: praca oryginalna]	6,268	20
2.	**Grzywa T** [aut. koresp.], Nowis D, Gołąb J. The role of CD71+ erythroid cells in the regulation of the immune response. Pharmacology & Therapeutics. 2021;228:107927. [Rodzaj publikacji: praca poglądowa]	12,310	200
3.	**Grzywa T**, Justyniarska M, Nowis D, Gołąb J. Tumor Immune Evasion Induced by Dysregulation of Erythroid Progenitor Cells Development. Cancers. 2021;13(4):870. [Rodzaj publikacji: praca pogladowa]	6,639	140
Liczba punktów:		**25,217**	**360**
	Książki		
1.	-		
	Rozdziały w książkach		
1.	-		

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BIBG/Punktacja/995/2021/KK

Warszawa, 29 grudnia 2021 r.

ANALIZA BIBLIOMETRYCZNA CAŁOKSZTAŁTU DOROBKU PUBLIKACYJNEGO
PANA TOMASZA MACIEJA GRZYWY
W POSTĘPOWANIU O NADANIE STOPNIA NAUKOWEGO DOKTORA

Lp.	Opis bibliograficzny	Impact Factor	MEiN
I. Artykuły opublikowane w czasopismach naukowych lub w recenzowanych materiałach z konferencji międzynarodowych ujętych w aktualnym wykazie MEiN[1]			
1.	Ścieżyńska A, Sobiepanek A, Kowalska P, Soszyńska M, Łuszczyński K, **Grzywa T**, Krześniak N, Góźdź A, Włodarski P, Galus R, Kobiela T, Malejczyk J. A Novel and Effective Method for Human Primary Skin Melanocytes and Metastatic Melanoma Cell Isolation. Cancers. 2021;13(24):6244. [Rodzaj publikacji: praca oryginalna]	6,639	140
2.	**Grzywa T**, Sosnowska A, Rydzyńska Z, Lazniewski M, Plewczynski D, Klicka K, Małecka-Giełdowska M, Rodziewicz-Lurzynska A, Ciepiela O, Justyniarska M, Pomper P, Grzybowski M, Blaszczyk R, Wegrzynowicz M, Tomaszewska A, Basak G, Gołąb J, Nowis D. Potent but transient immunosuppression of T-cells is a general feature of CD71 + erythroid cells. Communications Biology. 2021;4(1):1384. [Rodzaj publikacji: praca oryginalna]	6,268	20
3.	Sosnowska A, Chlebowska-Tuz J, Matryba P, Pilch Z, Greig A, Wolny A, **Grzywa T**, Rydzyńska Z, Sokołowska O, Rygiel T, Grzybowski M, Stanczak P, Błaszczyk R, Nowis D, Gołąb J. Inhibition of arginase modulates T-cell response in the tumor microenvironment of lung carcinoma. OncoImmunology. 2021;10(1):1956143. [Rodzaj publikacji: praca oryginalna]	8,110	140
4.	Klicka K, **Grzywa T***, Klinke A, Mielniczuk A, Włodarski P. The Role of miRNAs in the Regulation of Endometrial Cancer Invasiveness and Metastasis - A Systematic Review. Cancers. 2021;13(14):3393. * równorzędny pierwszy autor [Rodzaj publikacji: praca oryginalna]	6,639	140
5.	**Grzywa T** [aut. koresp.], Nowis D, Gołąb J. The role of CD71+ erythroid cells in the regulation of the immune response. Pharmacology & Therapeutics. 2021;228:107927. [Rodzaj publikacji: praca poglądowa]	12,310	200

[1] Wykaz sporządzony zgodnie z przepisami wydanymi na podstawie art. 267 ust. 2 pkt 2 lit. b Ustawy z dnia 20 lipca 2018 r. - Prawo o szkolnictwie wyższym i nauce (Dz. U. z 2018 r., poz. 1668 z późn. zm.). Wykaz stanowi załącznik do komunikatu MEiN z 2 grudnia 2021 r. w sprawie wykazu czasopism naukowych i recenzowanych materiałów z konferencji międzynarodowych.

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6.	**Grzywa T**, Koppolu A, Paskal W, Klicka K, Rydzanicz M, Wejman J, Płoski R, Włodarski P. Higher Mutation Burden in High Proliferation Compartments of Heterogeneous Melanoma Tumors. International Journal of Molecular Sciences. 2021;22(8):3886. [Rodzaj publikacji: praca oryginalna]	5,924	140
7.	**Grzywa T**, Justyniarska M, Nowis D, Gołąb J. Tumor Immune Evasion Induced by Dysregulation of Erythroid Progenitor Cells Development. Cancers. 2021;13(4):870. [Rodzaj publikacji: praca pogladowa]	6,639	140
8.	Materna-Kiryluk A, Pollak A, Gawalski K, Szczawinska-Poplonyk A, Rydzynska Z, Sosnowska A, Cukrowska B, Gaperowicz P, Konopka E, Pietrucha B, **Grzywa T**, Banaszak-Ziemska M, Niedziela M, Skalska-Sadowska J, Stawiński P, Śladowski D, Nowis D, Płoski R. Mosaic IL6ST variant inducing constitutive GP130 cytokine receptor signaling as a cause of neonatal onset immunodeficiency with autoinflammation and dysmorphy. Human Molecular Genetics. 2021;30(3-4):226-233. [Rodzaj publikacji: praca oryginalna]	6,150	140
9.	Pełka K, Klicka K, **Grzywa T**, Gondek A, Marczewska J, Garbicz F, Szczepaniak K, Paskal W, Włodarski P. miR-96-5p, miR-134-5p, miR-181b-5p and miR-200b-3p heterogenous expression in sites of prostate cancer versus benign prostate hyperplasia-archival samples study. Histochemistry and Cell Biology. 2021;155(3):423-433. [Rodzaj publikacji: praca oryginalna]	4,304	100
10.	**Grzywa T**, Klicka K, Włodarski P. Regulators at Every Step-How microRNAs Drive Tumor Cell Invasiveness and Metastasis. Cancers. 2020;12(12):3709. [Rodzaj publikacji: praca poglądowa]	6,639	140
11.	**Grzywa T**, Klicka K, Paskal W, Dudkiewicz J, Wejman J, Pyzlak M, Włodarski P. miR-410-3p is induced by vemurafenib via ER stress and contributes to resistance to BRAF inhibitor in melanoma. PLoS ONE. 2020;15(6):e0234707. [Rodzaj publikacji: praca oryginalna]	3,240	100
12.	**Grzywa T**, Sosnowska A, Matryba P, Rydzynska Z, Jasiński M, Nowis D, Gołąb J. Myeloid Cell-Derived Arginase in Cancer Immune Response. Frontiers in Immunology. 2020;11:938. [Rodzaj publikacji: praca pogladowa]	7,561	140
13.	Rak B, Maksymowicz M, **Grzywa T**, Sajjad E, Pękul M, Włodarski P, Zieliński G. Pituitary tumours - a large retrospective single-centre study of over 2300 cases. Experience of a tertiary reference centre. Endokrynologia Polska. 2020;71(2):116-125. [Rodzaj publikacji:praca oryginalna]	1,582	70
14.	**Grzywa T**, Klicka K, Rak B, Mehlich D, Garbicz F, Zieliński G, Maksymowicz M, Sajjad E, Włodarski P. Lineage-dependent role of miR-410-3p as oncomiR in gonadotroph and corticotroph pituitary adenomas or tumor suppressor miR in somatotroph adenomas via MAPK, PTEN/AKT, and STAT3 signaling pathways. Endocrine. 2019;65(3):646-655. [Rodzaj publikacji: praca oryginalna]	3,235	100
Liczba punktów:		**85,240**	**1710**
II. Artykuły opublikowane przed 1.01.2019 r. w czasopismach ujętych w wykazie czasopism MNiSW z dnia 25.01.2017 r., o ile czasopismo uzyskało co najmniej 10 pkt.			
15.	**Grzywa T**, Paskal W, Włodarski P. Intratumor and Intertumor Heterogeneity in Melanoma. Translational Oncology. 2017;10(6):956-975. [Rodzaj publikacji: praca pogladowa]	3,071	30
Liczba punktów:		**3,071**	**30**

III. Pozostałe artykuły		
brak	-	-
Liczba punktów:	-	-
Punktacja łączna (cz. I- III):	**88,311**	**1740**
IV. Monografie naukowe/rozdziały w monografiach wydane przez wydawnictwa ujęte w wykazie MNiSW[2] lub jednostki organizacyjne podmiotów, których wydawnictwa są ujęte w tym wykazie		
brak		
V. Pozostałe monografie lub rozdziały w monografiach		
brak		
VI. Patenty		
brak		

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[2] Wykaz sporządzony zgodnie z przepisami wydanymi na podstawie art. 267 ust. 2 pkt 2 lit. a Ustawy z dnia 20 lipca 2018 r. - Prawo o szkolnictwie wyższym i nauce (Dz. U. z 2018 r., poz. 1668 z późn. zm.). Wykaz ogłoszony komunikatem MNiSW z dnia 29 września 2020 r. w sprawie wykazu wydawnictw publikujących recenzowane monografie naukowe.

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