Development and function of human innate immune cells in a humanized mouse model

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Mice repopulated with human hematopoietic cells are a powerful tool for the study of human hematopoiesis and immune function in vivo. However, existing humanized mouse models cannot support development of human innate immune cells, including myeloid cells and natural killer (NK) cells. Here we describe two mouse strains called MITRG and MISTRG, in which human versions of four genes encoding cytokines important for innate immune cell development are knocked into their respective mouse loci. The human cytokines support the development and function of monocytes, macrophages and NK cells derived from human fetal liver or adult CD34+ progenitor cells injected into the mice. Human macrophages infiltrated a human tumor xenograft in MITRG and MISTRG mice in a manner resembling that observed in tumors obtained from human patients. This humanized mouse model may be used to model the human immune system in scenarios of health and pathology, and may enable evaluation of therapeutic candidates in an in vivo setting relevant to human physiology.

Small-animal models such as mice are frequently used for in vivo studies of mammalian, especially human, immune responses. However, fundamental differences in immune function exist between species1,2 and frequently, knowledge gained from mouse studies cannot be translated to humans.

One promising approach for studying human immune function in vivo is to use immunodeficient mice transplanted with human hematopoietic stem and progenitor cells2,3. However, the development and function of several human immune cell types, such as monocytes, macrophages and NK cells, is largely defective in currently available models of humanized mice2. More specifically, human monocytes and macrophages are present at low frequency4,5, and although a report showed that these cells are functional4, another report identified functional impairments and an immature phenotype of human monocytes6. The maturation, function and homeostasis of human NK cells are also defective in existing humanized mice7,8. These limitations highlight a need to develop humanized mice that model a more complete and functional human innate immune system.

The defects in human innate immune cell development in existing humanized mice are most likely due to limited reactivity of mouse cytokines with corresponding human cytokine receptors9. Several strategies to attempt to circumvent this issue by delivering human cytokines to the mouse host have been described10,11; some rely on administered exogenous cytokines7 or cytokine-encoding plasmids5,12, whereas others use introduced transgenes encoding human cytokines13–15. However, high systemic concentrations of cytokines can result in artifactual effects such as the mobilization and exhaustion of hematopoietic stem cells13 or supraphysiological cell frequencies.

The approach of knocking in human cytokine genes to replace their mouse counterparts has the advantage of ensuring appropriate tissue-, cell- and context-specific expression of the human cytokine10. Furthermore, in the scenario of homozygous human cytokine knock-in mice, if the human cytokine is not fully reactive with the corresponding mouse cytokine receptor, mouse cell populations dependent on signaling from that cytokine may exhibit numerical or functional defects; these defects confer an additional competitive advantage on transplanted human cells10. This knock-in gene replacement strategy has been used to “humanize” several cytokine-encoding genes. For example, humanization of the gene encoding thrombopoietin (Thpo, also known as Tpo) had resulted in enhanced maintenance of functional human hematopoietic stem cells capable of multilineage differentiation, of sustaining long-term high engraftment in the bone marrow (BM) and of serial transplantation16; humanization of the genes encoding interleukin 3 (IL3) and granulocyte-macrophage colony-stimulating factor 2 (GM-CSF; Csf2) had lead to the development of functional human alveolar macrophages17; and humanization of the Csf1 gene, which encodes M-CSF, had resulted in increased numbers of human monocytes or macrophages in multiple tissues18. Although each of these gene replacements improved the development and function of individual cell types (Supplementary Table 1),

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they did not result in a complete and robust human myelomonocytic system in a mouse.

Modeling of the complete human monocyte and macrophage compartment is important because monocytes and macrophages are important in tissue homeostasis, inflammation and tumorigenesis, and in the response to infectious agents. Two general classes of macrophages have been defined on the basis of their gene expression profile, secretome composition and effector activity: the classically activated M1 subtype that displays proinflammatory and microbicidal activities, and the alternatively activated M2 subtype characterized by immunoregulatory, antiparasite and tissue-repair roles. That said, this dichotomy is probably an oversimplification, and a spectrum of functionally distinct macrophage subsets likely exists. Regardless, the M1–M2 paradigm of macrophage differentiation is relevant to a number of human pathologies, including cancer.

For example, M1-like tumor-infiltrating macrophages show tumoricidal activity, whereas M2-like macrophages in the tumor microenvironment promote tumor growth by providing proliferative, antiapoptotic and proangiogenic signals; these signals also enable cancer-cell egress from primary tumors and formation of metastases. Clinical observations indicate that myeloid cells infiltrate several types of tumors, and in most cases, high densities of infiltrating macrophages correlate with poor patient prognosis.

To develop a humanized mouse model to study this and other human macrophage-related phenomena, we hypothesized that a synergy between multiple humanized cytokines would enable the full recapitulation of human myeloid development and function in the mouse. Therefore, we generated immunodeficient RAG2<sup>−/−</sup>II2rg<sup>−/−</sup> mice in which the genes encoding human M-CSF<sup>10</sup>, human interleukin 3 (IL-3) and GM-CSF<sup>17</sup>, and human thrombopoietin<sup>16</sup> are knocked in to their respective mouse loci; these mice are named MITRG for the encoded proteins M-CSF, IL-3/GM-CSF and TPO in RAG2<sup>−/−</sup>II2rg<sup>−/−</sup> background. MITRG mice also bear a bacterial artificial chromosome (BAC) transgene encoding human SIRPa<sup>28</sup>. SIRPa binds to CD47 and the resulting signal suppresses phagocytosis of CD47-expressing cells. Because human CD47 constitutively expressed on human cells binds efficiently to the mouse BAC transgene–encoded human SIRPa, this transgene enables mouse phagocytes to ‘tolerate’ and not engulf engrafted human cells<sup>28,29</sup>. MITRG and MISTRG mice are highly permissive for human hematopoiesis. In particular they harbor functional human myelomonocytic cells exhibiting subset diversity and numbers unprecedented in all previous described humanized mouse models and closely resembling those in humans. Using these mice, we found that human monocytes act as a source of IL-15 trans-presentation, and support the development and function of human NK cells. We show that human myeloid cells infiltrated a human tumor grafted onto these mice; these tumor-infiltrating cells exhibited an immunosuppressive M2-like phenotype, and their presence correlated with tumor growth. By providing a more complete and functional model of the human innate immune system, MITRG and MISTRG mice may enable application of humanized mice to new areas of translational research.

**RESULTS**

**Hematopoietic engraftment in MITRG and MISTRG mice**

We sub lethally irradiated newborn MISTRG mice and their MISTRG littermates (lacking the human SIRPA transgene), and transplanted these mice with human fetal liver–derived CD34<sup>+</sup> cells, following a standard protocol<sup>27</sup> (Online Methods). RAG2<sup>−/−</sup>II2rg<sup>−/−</sup> mice that have the same genetic background (129 × Balb/c N2) but lack all the humanized alleles, and commercially available nonobese diabetic severe combined immunodeficient II2rg<sup>−/−</sup> (NSG) mice served as controls.

Blood engraftment (percentage of human CD45<sup>+</sup> cells (hCD45<sup>+</sup> cells) among all CD45<sup>+</sup> cells (including human and mouse CD45<sup>+</sup>)) in NSG recipients was higher than in RAG2<sup>−/−</sup>II2rg<sup>−/−</sup> recipients, as previously reported<sup>28,30</sup>. The percentage of blood hCD45<sup>+</sup> cells was similar in MISTRG, MITRG and NSG mice, which suggested that humanization of the four cytokine genes overcomes the need to induce phagocytic tolerance through SIRPa–CD47 cross-reactivity<sup>28,29,31</sup>, possibly owing to defects in the mouse innate response caused by the absence of mouse cytokines. We selected mice with at least 10% hCD45<sup>+</sup> cells in the blood for subsequent experiments (Supplementary Fig. 1b). In bone marrow (BM), percentages of hCD45<sup>+</sup> cells averaged ~90% in MITRG and MISTRG recipients (Fig. 1a,c and Supplementary Fig. 1c–e), and the high efficiency of engraftment in the BM was also independent of SIRPa–CD47 interaction. MISTRG mice can also be engrafted with hCD34<sup>+</sup> cells isolated from cord blood or from peripheral blood of adult donors after mobilization by G-CSF (Supplementary Fig. 2).

To test the capacity of humanized cytokines to support human hematopoiesis in more competitive conditions, we transplanted human fetal CD34<sup>+</sup> cells into nonirradiated MISTRG mice. This protocol resulted in hCD45<sup>+</sup> cells in the blood and BM of all recipients, and half of the mice showed chimerism as high as the highest levels measured in recipients engrafted after X-ray preconditioning (Fig. 1d,e). These results show that humanization of multiple cytokines creates a microenvironment in which human hematopoiesis can almost completely displace mouse hematopoiesis in BM and obviate the need for pathology-inducing irradiation.

**Functional human myeloid cells in MITRG and MISTRG mice**

Next we assessed the capacity of MITRG and MISTRG mice to support human myelopoiesis. Human myeloid cells (hCD33<sup>+</sup>) were present in significantly higher proportions in the blood and BM of MITRG and MISTRG mice compared to RAG2<sup>−/−</sup>II2rg<sup>−/−</sup> and NSG recipients (Fig. 2a and Supplementary Fig. 3a–c). The increased proportion of myeloid cells in MITRG and MISTRG mice resulted in a blood composition resembling that of human blood, which is rich in myeloid cells and radically different from that of lymphoid-rich mouse blood<sup>12</sup> (Fig. 2b and Supplementary Fig. 3d). We observed a similar composition in the blood of MISTRG mice engrafted without irradiation (Supplementary Fig. 3e). Human myeloid cells were also present in high numbers (exceeding those in NSG recipients by approximately tenfold) in nonlymphoid tissues such as lung, liver and colon of MITRG and MISTRG mice, as shown by immunohistochemistry (hCD68<sup>+</sup> cells; Fig. 2c) and flow cytometry (hCD33<sup>+</sup>; Supplementary Fig. 3f,g). Although both human monocytes (CD33<sup>+</sup>SSC<sup>−/−</sup>CD66<sup>+</sup>) and neutrophils (CD33<sup>+</sup>SSC<sup>−/−</sup>CD66<sup>+</sup>) were present in the BM of MITRG and MISTRG mice (Supplementary Fig. 4a,b), the human myeloid cell populations in peripheral blood of MITRG and MISTRG mice were composed mostly of monocytes (Supplementary Fig. 4c); this observation suggests that terminal differentiation, egress from the BM or peripheral survival of human neutrophils is still suboptimal in this mouse environment. MISTRG mice also supported the development of human eosinophils (Siglec-8<sup>+</sup>) and basophils (FceRI<sup>+</sup>) in the BM and blood (Supplementary Fig. 4d–g), and to a lesser extent in the lung (data not shown). Human dendritic cells (including conventional and plasmacytoid dendritic cells) were also present in MISTRG mice and underwent maturation after in vivo stimulation with lipopolysaccharide (LPS), which suggested that these cells were functional (Supplementary Fig. 5).

In humans, three subsets of monocytes have been phenotypically and functionally described<sup>19,32</sup> based on differential expression of...
CD14 and CD16 (CD14⁺CD16−, CD14⁺CD16+ and CD14dimCD16+). All three subpopulations were present in the blood as well as lymphoid and nonlymphoid tissues (for example, lung and liver) of MITRG and MISTRG mice (Fig. 2d,e and Supplementary Fig. 6a,b). In contrast, NSG mice harbored a lower frequency of total myeloid cells and very few CD14dimCD16+ cells. Staining with additional markers including CD33, CD11b, CD115, CD62L and CX3CR1 indicated that in most cases the monocyte subpopulations found in MISTRG mice closely resembled their counterparts in human peripheral blood and BM (Fig. 2f and Supplementary Fig. 7).

Next we assessed the function of human monocytes in MITRG and MISTRG mice. Human CD14⁺CD16− and CD14⁺CD16+ monocytes isolated from BM of MISTRG mice produced tumor necrosis factor alpha (TNFα) and IL-6 in response to stimulation with LPS (TLR4 agonist) and R848 (TLR7/8 agonist) (Fig. 3a,b). In addition, CD14⁺CD16− and CD14⁺CD16+ cells from MITRG mice phagocyted GFP-expressing Escherichia coli in vitro; CD14dimCD16+ monocytes had limited phagocytic ability (Fig. 3c), which reflected the physiological properties of the corresponding subpopulations in human blood12. After injection with LPS or infection with Listeria monocytogenes (Listeria) or influenza A, we detected higher amounts of human TNFα, IL-6 and interferon β (IFN-β) in the tissues of MITRG or MISTRG mice compared to NSG mice (Fig. 3d–f). These results demonstrate that the human monocyte subsets that develop in MITRG and MISTRG mice are functional in vitro and in vivo.

Drawbacks of the high overall human engraftment levels as well as human myeloid cell function are defective development of mouse red blood cells (RBCs), particularly after irradiation, and phagocytosis of these mouse RBCs by human macrophages. As the development of human RBCs is also inefficient, human hematopoietic cell engraftment in MITRG and MISTRG mice promotes progressive destruction of mouse RBCs and anemia ultimately ensues; the incidence of anemia in these mice correlates with engraftment levels higher than ~60% hCD45⁺ cells in the blood. This leaves a period of ~2–3 weeks to perform experiments on healthy mice before the development of anemia (Online Methods). For experiments requiring longer periods of analysis, the onset of anemia can be delayed, and the lifespan of engrafted MITRG and MISTRG mice can be extended. One way this can be achieved is by injecting human cells into recipient mice without irradiating the recipient mice. Indeed, nonirradiated MISTRG mice with human CD45⁺ cell engraftment levels of 60–80% in the blood were healthy and did not show any signs of anemia 12 weeks after transplantation (for example, the mice in Fig. 1e). Alternatively, a lower number of human fetal liver CD34⁺ donor cells can be injected; on a population basis, this leads to lower engraftment levels. Lastly, adult human CD34⁺ cells, which engraft less efficiently than human
fetal liver CD34+ cells, can be used. Notably, even with lower over-
all engraftment levels, MITRG and MISTRG mice retain all of their
advantages over NSG mice, such as superior myelopoiesis.

**Functional human NK cells in MISTRG mice**

By producing cytokines, myeloid cells can support the development
and differentiation of other immune cells. As such, we investigated
whether the improved human myeloid compartment in MISTRG
mice could be a source of human cytokines, such as IL-15 trans-
presented by IL-15Rx, which are essential for development of human
NK cells. Human mRNA transcripts encoding IL-15 and IL-15Rx
were more than tenfold more abundant in MITRG and MISTRG
compared to NSG liver and lung (Fig. 4a and Supplementary Fig. 8a).
Next we measured the abundance of human mRNA encoding IL-15
and IL-15Rx in purified human cell populations. Expression of
human mRNA encoding IL-15Rx was higher in human myeloid
cells (hCD33+) than nonmyeloid cells (hCD33−) (Fig. 4b). Human
CD14+CD16+ monocytes expressed particularly high amounts of
mRNA encoding IL-15 and IL-15Rx (Fig. 4b). Next we confirmed
eexpression of human IL-15Rx protein on the surface of human
myeloid cells from MISTRG mice by flow cytometry (Supplementary
Fig. 8b). Although human CD14+CD16+ monocytes did not express
higher amount of surface IL-15Rx than CD14+CD16− monocytes,
high background staining made it difficult to accurately compare
surface IL-15Rx expression between the two subsets.

Based on these findings, we analyzed the development of human
immune cells dependent on IL-15 trans-presentation, such as NK cells,
in MITRG and MISTRG mice. Efficient development of human NK
cells in existing humanized mouse models requires injection of exoge-

uous human IL-15 and IL-15Rx;12 because mouse IL-15 and IL-15Rx
are not sufficient to support human NK cells in vivo. As previously
reported,7,8,12 we observed very few human NK cells (hNKp46+CD3−)
in engrafted NSG mice (Fig. 4c,d and Supplementary Fig. 9a,b).
In contrast, we readily detected human NK cells (at tenfold

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**Figure 2** MITRG and MISTRG mice support efficient myeloid cell
development in lymphoid and nonlymphoid tissues. (a) Percentages of human
myeloid cells (hCD33+) among human hematopoietic cells (hCD45+) in the blood
of the indicated recipient mice, engrafted as newborns by intrahepatic injection of fetal liver
CD34+ cells after X-ray preconditioning. Each symbol represents
an individual mouse, and red bars indicate mean values
(n = 20–113 mice; statistical analysis is shown in Supplementary
Fig. 3a). (b) Composition of human white blood cells in the same mice as in a (n = 20–113 mice/group; n = 8 human
donors; error bars, s.e.m.). (c) Immunohistological staining of human myeloid cells (hCD68+) in nonlymphoid tissues
of the indicated recipient mice. Scale bars, 20 µm. Images are
representative of at least three mice analyzed per group. (d,e)
Flow cytometry analysis (d) and frequencies (e) of human monocyte
subsets, identified by expression of CD14 and CD16 among hCD45+CD33+ cells in the blood of recipient mice (n = 8–12 mice/group; error bars, s.e.m.).
Data are representative of the data shown in e (8–12 mice/group, combined from four independent experiments). Dot plots in d are gated on CD33+SSC−
cells to show the subset distribution among monocyctic cells. (f) Human monocytes in the blood of MISTRG recipients and human monocytes from a human
donor were stained with the indicated antibodies. Staining with isotype control antibodies is shown in red and specific antibodies in blue.
higher numbers compared to NSG mice) in multiple tissues of engrafted MISTRG mice (Fig. 4c,d and Supplementary Fig. 9a,b). With the exception of BM, MISTRG mice contained fewer human NK cells than MISTRG mice, most likely owing to the previously reported requirement for human SirPα for the survival of human NK cells in the periphery\(^1\). The hNKp46\(^+\)hCD3\(^+\) cells in MISTRG mice represented bona fide NK cells because like human NK cells they expressed the NK cell surface markers CD94, CD161 and killer inhibitory receptors (KIRs) (Supplementary Fig. 10a,b). Expression of the maturation marker CD16 was higher on the surface of NK cells from MISTRG mice compared to NSG mice, which suggested that human IL-15 and IL-15Rα also support maturation of NK cells in MISTRG mice (Supplementary Fig. 10c,d).

It is not known which cell(s) are responsible for trans-presentation of IL-15 in vivo in humans, but human myeloid cells can support human NK cell proliferation in vitro\(^2\). To test whether trans-presentation of human IL-15 by human monocytes or macrophages underlies the improved human NK cell development in MISTRG mice, we treated MISTRG mice with liposome-encapsulated clodronate to deplete phagocytic cells. Depletion of phagocytic cells significantly reduced the number of human NK cells, but not human T cells, in MISTRG mice (Fig. 4e,f), which suggested that human monocytes...
or macrophages are indeed a critical source of trans-presented IL-15 in MISTRG mice. However, we cannot formally exclude other possible explanations for the enhanced human NK cell development in MISTRG mice, such as direct effects of the knocked-in human cytokines on NK cells or secondary interactions between NK cells and other human or mouse cell types.

Next we examined the function of human NK cells in MISTRG mice. NK cells defend against pathogens by killing cells that lack surface MHC class I expression35 and by producing IFN-γ. Compared to human NK cells from NSG mice, NK cells from MISTRG mice expressed higher amounts of the lytic granule protein perforin (Fig. 5a,b) and exhibited significantly enhanced cytotoxic activity against human cells lacking MHC class I in vivo (Fig. 5c). In addition, expression of mRNA encoding human IFN-γ was more than tenfold higher in the liver of MISTRG than NSG mice 2 d after Listeria infection (Fig. 5d), and more NK cells from Listeria-infected MISTRG mice than NSG mice produced higher amounts of human IFN-γ (without ex vivo restimulation) (Fig. 5e,f). NK cells in MISTRG mice also showed more degranulation (measured by plasma membrane exposure of CD107a) after Listeria infection (Fig. 5f). These findings indicate that the efficient human myeloid cell development in MISTRG mice enables efficient development, differentiation and function of human NK cells.

Figure 5  Human NK cells in MISTRG mice are fully functional. (a,b) Intracellular perforin expression by human liver NK (hNKp46^+hCD3^+) and T cells (hCD3^+) from engrafted NSG and MISTRG mice (n = 3, unpaired Student’s t-test). MFI, mean fluorescence intensity. (c) Violet-labeled LCL721.221 (HLA class I-negative) and LCL721.45 (HLA class I-positive) cells were injected intravenously in a 1:1 ratio, and the proportions of HLA class I positive or HLA class I negative cells among Violet-labeled cells recovered 12 h later in the spleen, were used to calculate specific NK cell-mediated lysis (n = 8, unpaired Student’s t-test). (d) Quantitative RT-PCR analysis of human IFNG mRNA expression in the liver of NSG and MISTRG mice 2 d after Listeria infection (n = 8–9, unpaired student’s t-test). Expression was normalized to mouse Hprt. (e,f) Frequency (e) and representative flow cytometry analysis (f) of IFN-γ- expressing and degranulating (CD107a^+) human liver NK cells from either uninfected or Listeria-infected NSG and MISTRG mice (n = 4–11; one-way ANOVA). In b–e, each symbol represents an individual mouse. Black bars represent the mean. Results are representative of or combined from two experiments.

T and B lymphocyte function in MISTRG and MISTRG mice
Whereas the human cytokines expressed in MISTRG were principally aimed at improving the human innate immune cell development, we also characterized adaptive immune cells (T and B lymphocytes) in MITRG and MISTRG mice. Human T and B cells were present in MITRG and MISTRG mice at lower frequencies than in NSG mice, owing to the relative increase in myeloid cells in MITRG and MISTRG mice (Fig. 2b, and Supplementary Figs. 11a and 12a). B cells mostly displayed an immature phenotype, as shown by the expression of the CD10, CD24 and CD38 markers, in both NSG and MISTRG recipients (Supplementary Fig. 11b,c). Likely as a consequence of this B cell immaturity,37 humoral immune responses were low in both strains of mice (Supplementary Fig. 11d). The majority of CD4 and CD8 T cells in the blood of NSG and MISTRG displayed a naive surface phenotype (CCR7^+CD45RA^+; Supplementary Fig. 12b,c). Human T cells in MISTRG mice were functional because like human NK cells they produced IFN-γ in response to Listeria infection (Supplementary Fig. 12d,e).

Tumor infiltration in MITRG and MISTRG mice
In addition to their roles in infection and inflammation, monocytes and macrophages can acquire immunosuppressive functions important for the resolution of inflammation and for tissue repair. These anti-inflammatory properties, if exhibited by tumor-infiltrating macrophages, can provide a survival advantage to evolving tumors.22,23 We thus examined the influence of human myeloid cells on tumor development in MITRG and MISTRG mice. We used the human melanoma cell line Me275 as a tumor model.38 In agreement with clinical observations showing that myeloid cells infiltrate tumors in several solid tumors,23–26 we detected more human myeloid cell infiltration in tumors in MITRG and MISTRG mice than in NSG mice, as shown by the expression of human PTPRC mRNA (encodes CD45) and ITGAM mRNA (encodes CD11b) in subcutaneously grafted Me275 tumors (Fig. 6a). Cells expressing the macrophage markers CD163 and CD14 were abundant in tumors in MISTRG mice and in tumors from human patients but were almost undetectable in the tumors in NSG mice (Fig. 6b,c, and Supplementary Fig. 13). CD163^+ cells also expressed low levels of HLA-DR and high levels of CD206 (Fig. 6b,d), an immunophenotype generally associated with M2-like macrophages.

We hypothesized that M2-like macrophages infiltrating tumors in MISTRG mice may promote tumor growth. Supporting this hypothesis, the tumors in engrafted MITRG and MISTRG mice, which are heavily infiltrated by human CD163^+ HLA-DR^low CD206^+ macrophages, were significantly larger than tumors in NSG mice, which are not infiltrated by human macrophages; tumors in engrafted NSG mice are similar in size to tumors in non-engrafted NSG or MITRG and MISTRG mice (Fig. 6e,f). One way macrophages support tumor growth is by producing cytokines or enzymes that promote vascularization and immune suppression. VEGF is one such molecule39,40, and to test
whether it was involved in tumor growth in MISTRG mice, we treated the mice with the human-VEGF inhibitor Avastin. This treatment completely inhibited the tumor growth in engrafted MITRG and MISTRG mice (Fig. 6f), suggesting that engrafted cells, potentially including myeloid cells, in MITRG and MISTRG mice support tumor growth through a mechanism that involves VEGF activity. These results show that MITRG and MISTRG mice recapitulate the role of human macrophages in tumor development and fulfill a critical need for models that enable study of the interaction between human tumors and human macrophages in vivo.

DISCUSSION

Despite their great promise for translational research, existing humanized mouse models do not permit efficient development of human innate immune cells. The MITRG and MISTRG model described here overcomes this major limitation. As such, MITRG and MISTRG mice may uniquely enable study of fundamental questions regarding the biology of the human immune system, in vivo modeling of human diseases and preclinical in vivo testing of new drug candidates.

The high efficiency of human hematopoietic cell engraftment (for example, human cells almost completely replace mouse cells in BM) and robust development of diverse subsets of human innate immune cells in MITRG and MISTRG mice is due to the synergistic effect of cytokines that support successive steps and multiple lineages of human hematopoiesis as well as to the secondary provision of additional cytokines by human cells themselves. The fact that MISTRG can be efficiently engrafted without irradiation illustrates the advantage of the combined replacement of multiple mouse cytokine genes with their human counterparts. The absence of mouse cytokines results in defects in mouse phagocytic cells (in particular mouse hematopoietic stem cells) and induces a state of genetic preconditioning.

MITRG and MISTRG mice will facilitate the engraftment of hematopoietic cells derived from the peripheral blood and/or BM of humans with disease. This will allow the reconstitution of a patient-derived immune system and may permit the study of low-proliferative, primary human hematopoietic malignancies, such as myelodysplastic syndromes or myeloproliferative neoplasia, that thus far have proven difficult to efficiently engraft immunodeficient mice.41,42.

Figure 6 Infiltration and growth of a tumor in MISTRG mice. (a) Human melanoma cell line Me275 was subcutaneously implanted in the flank of engrafted or non-engrafted NSG, MITRG and MISTRG mice. Where indicated, mice were treated with the VEGF inhibitor Avastin every 2 d, starting on the day of tumor injection. The tumors were measured and dissected for analysis 11 d later. Infiltration of human hematopoietic cells in the tumor was determined by the expression of PTPRC mRNA (encoding CD45) and ITGAM mRNA (encoding CD11b) (n = 6–7; unpaired Student’s t-test). (b, d) Representative immunohistochemistry images of human myeloid cell markers in tumors from NSG mice, MITRG mice and human patients. Data are representative of 5–7 samples/group, from three independent experiments (b) and 3 samples/group, from two independent experiments (d). Scale bars, 100 µm. (c) Quantification of the density of CD163+ cells in tumors (n = 3 samples/group, 3 slides counted/sample). (e, f) Representative pictures (e) and volume (f) of the tumors in the indicated groups of mice (n = 7–24 mice/group). Student’s t-test (a) or one-way ANOVA (c, f) followed by Tukey post-hoc test (* P < 0.05).
Furthermore, a human immune system can be combined with co-transplantation of diseased tissues from the same donor; this may be relevant to autoimmune diseases and cancer. This will create a personalized model in which the interaction of the patient's immune system with cancer cells or autoimmune target tissues can be studied in vivo. Moreover, the effect of drugs on the immune system and co-transplanted tissues can be evaluated preclinically, and treatments could be tailored to the individual patient in a personalized manner. An implication of our tumor xenograft experiments is that the outcome of antitumor drug treatment is markedly affected by the presence or absence of tumor-infiltrating human immune cells. Therefore, the role of nontumor cells, including but not limited to human myeloid cells, should be considered and carefully evaluated in the screening of candidate antitumor drugs.

MITRG and MISTRG mice will also enable study of the human innate immune system in acute and chronic inflammation as well as in infectious disease. The function of monocytes or macrophages is highly dependent on in vivo contexts that cannot be easily modeled in vitro. For example, CD16+ monocytes, which develop in the MITRG model but not in other humanized mouse models, are expanded in humans in inflammatory settings including those caused by HIV infection and atherosclerosis. However, the functional importance of CD16+ monocytes in these settings is unknown; MITRG and MISTRG mice may enable the investigation of causal relationships between CD16+ monocytes and human inflammatory disease. Macrophages are also prominent in humans infected with bacteria including Mycobacterium tuberculosis. Furthermore, models of the adaptive immune system function in MITRG and MISTRG mice (see below) should enable study of the interaction between human macrophages, CD4+ T cells, and M. tuberculosis in vivo. Standard nonhumanized mice do not represent this complex human disease faithfully. Other fundamental questions that may be answered using the MITRG and MISTRG mouse model include the ontogenic origin of tissue macrophages versus monocyte-derived macrophages, the trafficking of these cells in response to inflammation or infection, and the mechanisms of differentiation and effector function of M1 versus M2 macrophages.

Finally, MISTRG mice will allow study of the role of human NK cells in vivo, for example, in the context of cancer and viral infections. Although MITRG mice represent a marked improvement over existing humanized mouse models, they also have two drawbacks. First, as mentioned earlier, development and survival of both mouse and human RBCs is suboptimal in MITRG and MISTRG mice. This problem will need to be addressed in the future by genetic strategies to either protect mouse RBCs from phagocytosis by human cells or to support the development and survival of human RBCs.

The other limitation of MITRG and MISTRG mice, similarly to other models, is their relatively weak adaptive immune responses, with low cytotoxic and humoral immune responses, particularly a low frequency of somatic hypermutation and class-switching in B cells. Adaptive immunity might be improved by several approaches, including expression of human MHC elements in mice or use of the BM-liver-thymus approach in which human fetal tissues are transplanted along with human CD34+ cells. The BM-liver-thymus approach results in more robust adaptive immune responses because T cells develop and are selected in a human thymic tissue. A non–mutually exclusive approach would be humanizing additional cytokine genes in recipient mice. By combining multiple strategies, humanized mouse models may fully recapitulate functional human innate and adaptive immune responses. Such mouse models may enable an even wider variety of basic and preclinical research applications.

**METHODS**

Methods and any associated references are available in the online version of the paper. 

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**AUTHOR CONTRIBUTIONS**

A.R., T.W. and J.M. designed and performed experiments and analyzed results. T.S., S.V.G., L.L.T., Y.S. and F.M. performed experiments. S.V. provided reagents. A.K.P. designed experiments and analyzed results. M.G.M. and R.A.F. conceived the project and supervised its participants and interpreted its results. A.R., T.W., A.K.P., M.G.M. and R.A.F. wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.
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**ONLINE METHODS**

**Mice.** The knock-in replacement (from ATG to stop codon) of individual loci (encoding thrombopoietin (TPO), IL-3/GM-CSF or M-CSF) in the Rag2<sup>-/-</sup> IIrg<sup>-/-</sup> 129×Balb/c (N2) genetic background was performed using Velocigene Technology in collaboration with Regeneron Pharmaceuticals, as reported previously<sup>16–18</sup>. Human SIRPα expression was achieved by BAC transgenesis in the same genetic background<sup>28</sup>. We intercrossed these strains by conventional breeding to obtain MITRG (CSF<sub>1/+</sub> IL3<sub>2/+</sub> THPo<sub>2/+</sub> Rag2<sup>-/-</sup> IIrg<sup>-/-</sup>) and MISTRG (CSF<sub>1/+</sub> IL3<sub>2/+</sub>SIRPA<sub>W</sub> THPo<sub>2/+</sub> Rag2<sup>-/-</sup> IIrg<sup>-/-</sup>) mice. We maintained them under specific pathogen-free conditions with continuous treatment with enrofloxacin in the drinking water (Baytril, 0.27 mg/ml). MITRG and MISTRG mice were viable for 1 year or more and had normal fertility at least until 8 months of age. NSG mice were obtained from The Jackson Laboratory.

MITRG and MISTRG mice have been deposited at The Jackson Laboratory.

**Human progenitor cell isolation and injection.** Recipient mice were engrafted with human hematopoietic stem and progenitor cells as previously described<sup>16,19,27,28</sup>. Briefly, human fetal liver samples were cut in small fragments, treated for 45 min at 37 °C with collagenase D (Roche, 100 ng/ml), and a cell suspension was prepared. Human CD34<sup>+</sup> cells were purified from fetal liver samples or from cord blood by density gradient centrifugation (Lymphocyte Separation Medium, MP Biomedicals) followed by positive immunomagnetic selection with anti-human CD34 microbeads (Miltenyi Biotec). Cells were frozen in FBS containing 10% DMSO and kept in liquid nitrogen. G-CSF–mobilized adult CD34<sup>+</sup> cells were obtained from Yale University, Yale University Hospital or University Hospital Zurich, with donors’ written informed consent. All human studies were approved by the Yale University Human Investigation Committee or the Cantonal ethics committee of Zurich, Switzerland.

Newborn mice (within first 2 d of life) were sublethally irradiated (X-ray irradiation with X-RAD 320 irradiator, PXi; Rag2<sup>-/-</sup> IIrg<sup>-/-</sup> 2× 180 cGy 4 h apart; NSG, 1 × 100 cGy; MITRG and MISTRG, 1 × 150 cGy) and 100,000 CD34<sup>+</sup> cells in 20 µl of PBS were injected into the liver with a 22-gauge needle (Hamilton Company). Unless otherwise specified, all mice were transplanted with CD34<sup>+</sup> cells isolated from fetal liver (FL). In specific experiments (Fig. 1d,e), 200,000–300,000 FL–CD34<sup>+</sup> cells were injected into nonirradiated MITRG newborn recipients. The mice were bled 7–9 weeks later and the percentage of human CD45<sup>+</sup> cells was measured by flow cytometry. Mice in which human CD45<sup>+</sup> cells represented at least 5% (Rag2<sup>-/-</sup> IIrg<sup>-/-</sup> or 10% (NSG, MITRG and MISTRG mice) of the total (mouse and human combined) CD45<sup>+</sup> populations were selected for further experimentation. The mice were killed or used for experiments 9–12 weeks after transplantation.

The postengraftment lifespan is ~10–12 weeks (for MISTRG mice) and ~12–16 weeks (for MITRG mice) when the mice are transplanted with 100,000 FL–CD34<sup>+</sup> cells after sublethal irradiation (150 cGy). The first clinical signs of anemia directly correlated with high engraftment levels, and they generally appeared when engraftment levels reached ~60% human CD45<sup>+</sup> cells in peripheral blood. Adult CD34<sup>+</sup> cells were less potent than fetal CD34<sup>+</sup> cells in that human engraftment levels did not exceed 20–30% in the peripheral blood of mice injected with adult CD34<sup>+</sup> cells. As a consequence, mice remained alive and healthy for up to 22 weeks after engraftment with adult CD34<sup>+</sup> cells. Irradiation also contributes to anemia, as mice engrafted without irradiation remained healthy for up to 12 weeks even with 60–80% human CD45<sup>+</sup> cells in the blood.

Mice (both males and females) of comparable engraftment levels (percentage of human CD45<sup>+</sup> cells in the blood and relative lineage composition) that were engrafted with cells from the same donor(s) were separated randomly into the experimental groups. The results presented are combined from at least two independent experiments, with a total of 5–12 mice/experimental group; each independent experiment was performed with mice transplanted with CD34<sup>+</sup> cells from different donors.

As MITRG and MISTRG mice are similar in many aspects, for some experiments we used both MITRG and MISTRG mice engrafted with matched fetal liver samples and with comparable engraftment levels.

All animal experimentations were performed in compliance with Yale Institutional Animal Care and Use Committee protocols.

**Immunophenotypic analysis of human cell populations.** To prepare white blood cells from engrafted mice, heparinized blood was treated twice with ammonium-chloride-potassium (ACK) lysis buffer to eliminate RBCs. For the spleen and BM, single-cell suspensions (flushed from the femur and tibia in the BM) were treated with ACK lysis buffer. Liver and lung leukocytes were isolated by mechanically dissociating and digesting tissues with 100 U/ml collagenase IV and 0.02 mg/ml DNase I (Sigma) for 1 h at 37 °C, followed by density gradient centrifugation.

For FACS analysis, antibodies against the following antigens were used: mouse antigens: CD45 (clone 30-F11); human antigens: CD1c (BDCA1, clone L161), CD3 (UCHT1), CD4 (OKT4), CD8 (HIT8a) CD10 (H11a), CD16 (ICRF44), CD11c (B-Ly6, BD Biosciences), CD14 (M5E2), CD16 (3G8), CD19 (HB19), CD20 (2H7), CD24 (ML5), CD33 (WM53), CD38 (HIT2), CD45 (H100), CD45RA (H110), CD62L (DREG-56), CD66 (ASL-32), CD9 (D94), CD107a (HA43), CD115 (9–42D–14E), CD123 (6H6), CD141 (BDA3, M80), CD161 (HP-3G10), CD303 (BDC2A, 201A), NKP46 (9E2), IL-15Rα (I74A4), CR2 (150503, BD Biosystems), CX3CR1 (2A9–1), FcRn (AER-37), HLA-ABC (W6/32), HLA-DR (L243), IFN-γ (B72) KIR3DL1/S1 (HP-MA4), KIR2DL2/L3 (DX27), KIR3DL1 (DX9), perforin (d9g) and Siglec-8 (7C9); and human lineage cocktail: CD3, CD15, CD19, CD56 and NKP46.

All antibodies were obtained from Biologend, unless otherwise specified. Data were acquired with FACSDiva on an LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software.

For histological analysis, spleen, lung, liver and colon tissues were fixed overnight in IHC zinc fixative (BD Biosciences) and embedded in paraffin. Sections were stained with anti-human CD68 (clone PG-M1) followed by a HRP-conjugated secondary antibody and revealed with the peroxidase substrate 3, 3′-diaminobenzidine.

**Phagocytosis assay in vitro.** E. coli expressing GFP were grown in LB medium overnight at 37 °C to an OD at 600 nm of 1.5–1.8, at which point the bacteria were diluted and grown for 1–2 h to an OD<sub>600</sub> of ~1.0. The E. coli were washed three times with PBS and incubated with WBCs from MITRG mice for 4 h at 37 °C in a volume of 200 µl with about 2 × 10<sup>5</sup> E. coli per 1 × 10<sup>7</sup> WBCs. After the incubation, the cells were washed with PBS and analyzed by flow cytometry.

**TLR stimulation in vitro and infection in vivo.** Human monocyte subsets were isolated from the BM of engrafted mice. Briefly, BM cells were recovered and pooled from the hind legs and the spine of six mice. Human CD3<sup>+</sup> cells were enriched by magnetic isolation (EasySep CD3 selection kit, StemCell Technologies). CD1<sup>+</sup> CD16<sup>+</sup> and CD1<sup>+</sup> CD14<sup>+</sup> subsets were purified on a FACSAria cell sorter (BD Biosciences). 100,000 cells in 200 µl medium were cultivated overnight in the presence of the TLR4 ligand LPS (E. coli 0111:B4, Sigma-Aldrich, 100 ng/ml) or the TLR7/8 ligand R848 (Invigen, 10 µg/ml). For in vivo stimulation, 35 µg of LPS (E. coli 0111:B4, Sigma-Aldrich) in 100 µl PBS were injected intraperitoneally and the serum was collected 90 min later. For dendritic cell analysis, 10 µg of LPS was injected intraperitoneally and the mice were killed 6 h later.

Mice were infected with 3 × 10<sup>3</sup> colony-forming units (CFU) of L. monocytogenes (strain 10403S) by intravenous injection. 48 h after infection, sera and tissues were collected for ELISA and quantitative (q)PCR, respectively. Cytokine concentrations (human TNFα, IL-6 and IL-1β) in mouse serum and in culture supernatants were measured using ELISA MAX Standard kits (Biolegend), following the manufacturer’s instructions. Liver lymphocytes from uninfected or infected mice were incubated at 37°C in 5% CO<sub>2</sub> for 4 h in medium containing monensin (GolgiStop, BD Biosciences) and anti-human CD107α. Cells were then stained for surface antigens (mCD45, hCD45, hNKP46 and hCD3), permeabilized using Cytofix/Cytoperm kit (BD Biosciences), and stained for intracellular human IFN-γ.

Mice were infected intranasally with 2 × 10<sup>6</sup> plaque-forming units (PFU) of influenza A/PR8 (H1N1) virus, and lungs were collected on day 3 postinfection for qPCR analysis.

**Depletion of phagocytic cells in vivo.** Phagocytic cells were depleted by intravenous retroorbital injection of 100 µl of clodronate-loaded liposomes<sup>50</sup>.
Clodronate-liposomes were injected three times daily and human NK cells in mouse liver were analyzed 24 h after the last injection.

**Quantitative reverse transcription–PCR.** Total RNA was extracted from tissues or purified cells with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions and used for cDNA synthesis with the SuperScript First-Strand Synthesis System (Invitrogen). Quantitative reverse transcription (RT)-PCR was performed on a 7500 Fast Real-Time PCR system with primer-probe sets purchased from ABI (hIFNBI, Hs01077958_s1; hIL15, Hs01003716_m1; hIL15RA, Hs00542604_m1; hPTPRC, Hs04189704_m1; hITGAM, Hs00355885_m1). Expression values were calculated using the comparative threshold cycle method and normalized to mouse Hprt (Mm00446968_m1) or human HPRT (Hs99999909_m1), as indicated.

**In vivo NK cell cytotoxicity assays.** Human NK cell cytotoxicity *in vivo* was determined following a previously reported protocol\(^8\), LCL721.221 (HLA class I–negative) and LCL721.45 (HLA class I–positive) cells were mixed in a 1:1 ratio, labeled with CellTrace Violet (Invitrogen) and injected intravenously (1 × 10\(^7\) cells/mouse) into engrafted NSG or MISTRG mice. Mice were killed 12 h later and a single cell suspension of the spleen was prepared and analyzed by flow cytometry. The proportions of HLA class I–positive and HLA class I–negative cells among Violet-labeled cells were measured and specific lysis was calculated as (MHC class I\(^+\) – MHC class I\(^−\)) × 100 / MHC class I\(^+\).

**Immunization.** Ovalbumin (Sigma-Aldrich, 100 µg/mouse) was emulsified in Complete Freund’s adjuvant (Difco, primary immunization) or Incomplete Freund’s adjuvant (secondary immunization 2 weeks later). The emulsion was injected intraperitoneally. The mice were bled before the first immunization (pre-immune serum) and 1 week after the second immunization. The presence of ovalbumin-specific human IgM in the serum was measured by ELISA, using a biotinylated anti-IgM antibody (BD Biosciences) followed by horseradish peroxidase-conjugated streptavidin (Vector Laboratories).

**Tumorigenesis.** The human melanoma cell line Me275 (ref. 38; provided by P. Romero, Lausanne, Switzerland), which was mycoplasma-free (assessed by MycoAlert assay, Lonza), was grown to ~90% confluency and the cells (~7 million cells per mouse) were injected subcutaneously under anesthesia in the flank of the mouse. For some experiments, the mice were treated every other day, starting on the day of tumor implantation, with the human VEGF-specific antibody Avastin (Roche; 100 µg intravenously). The size of the tumors was measured, in a blinded manner when possible, 11 d later and the volume calculated using the following formula: volume = 0.5 × length\(^2\) × width.

**Statistical analysis.** Statistical analysis was performed with the GraphPad Prism 6 software, using one-way ANOVA followed by Tukey post-hoc test, two-tailed unpaired Student’s *t*-test. Similar s.d. were determined within each of those tests. The size of the groups was determined based on the results of preliminary experiments, in order to achieve statistical significance, but without specific statistical analysis to determine prespecified group size.

**Clodronate-liposomes mediated depletion of macrophages:** mechanism of action, preparation of liposomes and applications. *J. Immunol. Methods* **174**, 83–93 (1994).

Van Rooijen, N. & Sanders, A. Liposome mediated depletion of macrophages:
Corrigendum: Development and function of human innate immune cells in a humanized mouse model

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In the version of this article initially published, the cells labeled Me290 were Me275 cells. Both Me275 and Me290 are human metastatic HLA-A201+ melanoma cell lines, and both of them were obtained from the Ludwig Cancer Institute. Therefore, the mislabeling does not affect the conclusions of the paper. The error has been corrected in the HTML and PDF versions of the article.