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Long Term Human Reconstitution and Immune Aging in NOD-Rag (–)- γ chain (–) Mice

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Abstract

Aging of the human immune system is characterized by a gradual loss of immune function and a skewing of hematopoiesis toward the myeloid lineage, a reduction in the lymphocytic lineage, and progressive increases in senescent memory T cells at the expense of naïve T cells. Both the innate and the adaptive branches of the immune system are affected, including neutrophils, macrophages, dendritic cells and lymphocytes. Mice, the most common research model, although inexpensive, do not necessarily reflect the human immune system in terms of its interaction with infectious agents of human origin or environmental factors. This study analyzed whether a human immune system contained within the NOD-Rag (–)- γ chain (–) mouse model could realistically be used to evaluate the development and therapy of aging-related diseases. To that end lightly irradiated NOD-Rag (–)- γ chain (–) mice were injected intra-hepatically on day 1 of life with purified cord blood-derived CD34+ stem and progenitor cells. Multiple mice were constructed from each cord blood donor. Mice were analyzed quarterly for age-related changes in the hematopoietic and immune systems, and followed for periods up to 18–24 months post-transplant. Flow cytometric analyses were performed for hematopoietic and immune reconstitution. It was observed that NOD-Rag (–)- γ chain (–) mice could be “humanized” long-term using cord blood stem cells, and that some evidence of immune aging occurred during the life of the mice.

Keywords

cord blood; CD34; stem cells; NRG mice

INTRODUCTION

Aging of the human immune system is characterized by a gradual loss of immune function and a skewing of the hematopoietic cells toward the myeloid (CD14) lineage, with a reduction in the lymphocytic lineage (T, B and NK cells), and progressive increases in senescent memory T cells at the expense of naïve T cells (CD3+45RO+28–; Dorshkind et al, 2009; Gomez et al, 2005; Maue et al, 2009). Both the innate (Gomez et al, 2005) and the adaptive (Dorshkind et al, 2009) branches of the immune system are affected, including neutrophils (PMN), macrophages, dendritic cells (DC) and lymphocytes. The hematopoietic

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stem cell (HSC) population is also detrimentally affected by aging as reflected by its inability to maintain both hematopoiesis and lymphopoiesis (Rossi et al, 2005). Mice, the most common research model, although inexpensive, do not necessarily reflect the human immune system in terms of its interaction with infectious agents of human origin or environmental factors. Therefore, a major challenge for immunology and gerontology is to develop a model that functionally mimics human immune system development, function and senescence, which is relatively inexpensive, and can be manipulated to explore the intricacies of aging. Thus there is a need for a humanized animal model in which triggers of immune aging and treatment variables can be manipulated in order to delineate optimal therapeutic regimes. The model analyzed in this study built upon work reported in the literature developing humanized mice (Traggiai et al, 2004; Brehm et al, 2010; Shultz et al, 2010), and expanded these studies to include aged humanized animals.

In aging there is a progressive decline in tissue and organ function along with a loss of stem cell numbers and/or function (Rossi et al, 2005; Hattiangady and Shetty, 2008; Zhou et al, 2008; Min et al, 2011; Sowa et al, 2012). As individuals age there is an increased incidence of myeloid cancers but it is unknown if any of these age-related changes are due to intrinsic or extrinsic factors. Although polycomb group proteins are more likely to become methylated with age it is unclear if this observation is a cause or effect of aging (Teschendorff et al, 2010). However, recent work (Mayak et al, 2010; Conboy et al, 2005) has shown that HSC aging can be reversed by exposure to a younger environment implying that non-cell intrinsic local and systemic factors are involved, which may be evolutionarily conserved (e.g. IGF-1 and Notch signals). Similar “rejuvenation” results have been seen with muscle as well (Conboy et al, 2003). It is currently unknown if a similar result would be obtained with the innate and adaptive immune systems, both of which decline with age. If so, aging might best be treated by targeting the extrinsic environmental factors rather than cell intrinsic factors (Kovacs et al, 2009).

Over the past four years a number of novel humanized mouse strains have been developed in an attempt to provide model systems to address some of these issues, albeit there is some debate as to whether one model is better than another. Many of these models have utilized purified umbilical cord blood CD34⁺ stem cells injected into newborn (triply) immunodeficient mice which then reconstitute the animal with some or all parts of the human innate and adaptive immune systems. There have been questions as to whether a truly functional (as opposed to phenotypic) human immune system develops, whether it represents a diverse and properly restricted adaptive immune system (focused on recognition of human HLA molecules), and if all parts of the innate immune system are intact. Data has been published to support and refute each of these claims. (Traggiai et al, 2004; Brehm et al, 2010; Shultz et al, 2010; Liu et al, 2010; Watanabe et al, 2009; Melkus et al, 2006; Marodon et al, 2009; Freitas et al, 1989; Strowig et al, 2010; Chang et al, 2012). Essentially no work has been published with regard to the use of these systems to study aging and its effects on the human hematopoietic and immune systems (Maue et al, 2009; Weng et al, 2009).

The intent of the current study was to determine the effects of host aging on the humanized blood and immune system contained within NOD-Rag1^{null}- γ chain^{null} (NRG) mice constructed from a single stem cell donor, as measured by flow cytometric analyses. We serially sampled the same mice over a period of time of 1 year or longer. It was observed that NRG mice could be “humanized” long-term using cord blood stem cells, and that such animals do exhibit some signs of immune system aging.

MATERIALS AND METHODS

Mice

Humanized mice were created using a variation of the NOD-Rag1^{null}- γ chain^{null} (NRG) model as described in the literature (Chicha et al, 2005). Briefly, female mice homozygous for both the Rag1^{null} and IL2r γ ^{null} (common gamma chain null) mutations were bred with male mice homozygous for the Rag1 knockout mutation and hemizygous for the X-linked IL2r γ ^{null} mutation, as obtained from Jackson Labs (Bar Harbor, ME). Mice were bred at the University of Arizona animal facilities, an AAALAC-approved institution.

Cord blood collections and CD34 purifications

Cord blood (CB) samples were collected, processed and cryopreserved as previously described (Harris et al, 1994) under an IRB-approved protocol. CD34⁺ CB stem cells were enriched using magnetic beads columns (StemCell Technologies, Vancouver, Canada), as per the manufacturer's instructions, to 95% or greater purity.

Transplantation

Newborn mice were irradiated in a 3–4h interval (2 $\times$ 2 Gy) using a Cesium or Cobalt source. At 4–12h post-radiation, mice were transplanted intra-hepatically with CD34⁺ (>95% purity) human cord blood stem cells (30 μ l in PBS, 2–4 $\times$ 10⁵ cells/mouse) collected from healthy, disease-free mothers and infants. The mice were anesthetized, the liver was visualized through the skin, and cord blood stem cells are directly injected into the organ. This procedure was performed by the EMSS (Experimental Mouse Shared Service) of the Arizona Cancer Center at the University of Arizona. Mononuclear cells from the cord blood collection used for transplantation were frozen and stored for later use in future experiments. Engraftment of human cells was ascertained by flow cytometric (FACS) analyses of peripheral blood obtained from each transplanted mouse.

FACS Analysis

The humanized mice were characterized 90–120 days post-transplant by the presence of human immune cells as determined by flow cytometry using heparinized blood obtained from the sub-mandibular pouch (i.e., presence of CD45⁺, CD14⁺, CD3⁺, CD19⁺, CD16⁺, CD11c⁺ populations, etc.) of each animal. Samples were analyzed either with a FACScan flow cytometer (BD, Franklin Lakes, NJ), with single laser emitting at 488 nm, a LSRII using lasers emitting at 633nm and 532 nm. Data was analyzed and displayed with FACS Diva software or Cytologic. A minimum of 20,000 gated events were analyzed for each sample. Antibodies were obtained from Biolegend (San Diego, CA), BD (Franklin Lakes, NJ) and Caltag Laboratories (Burlingame, CA). Gating was performed on viable cells as based on forward and side scatter parameters. Human cells were identified based on anti-human CD45 mAb binding. In addition comparable anti-mouse leukocyte mAbs were added to each tube to identify any possible contribution of mouse leukocytes to overall mAb binding. Red blood cells were lysed using standard tris-ammonium chloride buffer prior to staining and analysis.

RESULTS

Long term engraftment and survival parameters

NRG mice were transplanted with CB CD34⁺ cells on d1 of life as described in Materials and Methods. At 90–120d post-transplant mice were analyzed for the presence of human cells in the peripheral blood. As shown in Figure 1 the level of human cell engraftment varied from less than 10% of normal murine blood levels to greater than 90%, with the

average engraftment being approximately 30% of expected normal blood cell numbers. It was observed that initial human blood cell engraftment in the NRG mice was not correlated with CD34⁺ cell dose.

Liu et al reported that robust human engraftment of the NSG mouse strain was correlated with progenitor cell dose as evaluated at later time points up to 19 weeks (Liu et al, 2010). As noted above we did not find that initial human blood cell engraftment was correlated with CD34⁺ cell dose (above a threshold dose) and further, we observed that long term survival of the transplanted NRG mice also was not correlated with cell dose (see Figure 2), although there was a trend for mice transplanted with higher CD34⁺ cell doses to survive 300 days or longer.

Engrafted mice (N=66) were followed over a period of up to 600 days with each mouse being serially examined on a monthly or quarterly basis. As shown in Figure 2, 35 of 66 mice survived more than 150d (>21 weeks) and 25 of the mice survived for more than 1 year. Those mice that died prematurely (before 100 days) were found upon autopsy to have succumbed to a red cell anemia, as has been reported for other types of humanized mice (data not shown, personal communication, L Shultz, Jackson Laboratories). Why this phenomenon did not affect all humanized mice is not known.

Hematopoietic and Immune Reconstitution

A select group of mice that had survived for 1 year or more post-transplant were chosen for further analysis. These mice were engrafted from a single cord blood stem cell donor, allowing for analysis of variability in the creation of these “syngeneic” animals. As shown in Figure 3, these mice rapidly engrafted with human leukocytes. Human CD19⁺ B cells were present early after transplant in the peripheral blood, which declined with time after transplant, as has been reported for clinical human stem cell transplants (Atkinson, 1990), reaching a stable plateau at approximately 20 weeks post-transplant. Myeloid cells (CD14⁺ cells) were also present at low numbers early after transplant and generally remained at low levels (2–10%) thereafter. As B cell numbers declined, human T cells (CD3⁺ cells) increased and remained elevated for periods of time of more than 1 year post-engraftment.

Further analysis of the human T cells present in the peripheral blood of the humanized NRG mice (see Figure 4) revealed that CD8⁺ T cells were the first subset to appear after engraftment followed by a rapid expansion of CD4⁺ T cells over time. Whether this imbalance in the CD4/CD8 ratio can be explained by differences in homeostatic proliferation of the T cell subsets is not known at the current time. That is, a higher fraction of CD4⁺ T cells and a smaller fraction of CD8⁺ T cells were present, but without absolute numbers it remains unclear whether the observation could have been due to replacement or simply the development and expansion of the CD4 T cell compartment. There did not appear to be any significant phenotypic differences between mice reconstituted with stem cells from the same donor.

Immune System Aging

All engrafted animals were kept in HEPA-filtered caging systems under SPF conditions and were fed sterilized food and water. Therefore, we expected to find that most if not all human T cells present in the animals were of the naïve subset (i.e., expressing CD45RA), which is what was observed for the first 4–6 months following engraftment. Subsequently, as shown in Figure 5 (representative of mice constructed from a single donor), T cells expressing the memory phenotype (i.e., CD45RO⁺) dominated in the peripheral blood of the humanized mice at later time points. It remains to be determined whether these are truly memory T cells due to inadvertent stimulation with various microbial agents (and whether of

the central or effector memory cell phenotype), or if the phenotypic change is a result of homeostatic proliferation. If this phenotype was due to the latter possibility, why it occurred so late in time after engraftment is unknown as cellularity was generally constant over this time period. The change in phenotype was seen in both CD4⁺ and CD8⁺ T cells (Figures 6 and 7, respectively). Interestingly, as shown in Figure 8, there was an increase in the percentage of CD3⁺CD28⁻ T cells over time, which might be indicative of aging driven, rather than homeostatic-driven, changes in the T cell populations.

DISCUSSION

Recently, based on the original work by Traggiai et al (2004) considerable effort has gone into construction of a variety of humanized mouse strains to provide animal models to interrogate the human immune system. The NRG mouse was originally described by Pearson et al (2008) as a radioresistant version of the previously derived NSG mouse model (Shultz et al, 2005). These investigators reported that human immune cell engraftment was similar to that reported with the NSG mouse model (Brehm et al, 2010) at early time periods (12–16 weeks). Their work delineated the human engraftment of adult mice after 12 weeks after injection, demonstrating T cell, B cell and myeloid reconstitution in various lymphoid organs. However, these investigators did not follow these mice long term and no information is available regarding stable hematopoiesis.

Many of the humanized models recapitulate some, if not all, of the development of the human blood and immune systems. However, the majority of these studies have been terminated well before any epigenetic and other effects of host aging might be noticed. Aging of the human blood and immune systems is characterized by a gradual loss of immune function, a skewing of the hematopoietic cells toward the myeloid lineage with a reduction in the lymphocytic lineage, and progressive increases in senescent memory T cells at the expense of naïve T cells. The reasons for these effects are not clear, but there is evidence that persistent viral infections, particularly those of the herpetic type, such as cytomegalovirus (CMV) are in part responsible, since clonal expansion of memory immune cells with receptors specific for CMV antigens is characteristic of this phenomenon (Kovacs et al, 2009). Both the innate and the adaptive branches of the immune system are affected, including neutrophils (PMN), macrophages, dendritic cells and lymphocytes (Traggiai et al, 2004). The hematopoietic stem cell (HSC) population is also detrimentally affected by aging as reflected by an inability to maintain both hematopoiesis and lymphopoiesis (DiCarlo et al, 2009). Significantly, when old HSC or old PMN are placed into a young mouse, the cells behave as young cells. Conversely, young HSC or young PMN when placed in an old mouse behave as aged cells (Conboy et al, 2005). These results indicate the importance of extrinsic factors in the aging of the functional immune system. If true, it should be more feasible to alter the microenvironment of the aged individual than to rewire the hematopoietic and immune progenitor cell genome. Thus, intervention should be possible.

Further, the studies referenced above have generally not compared the humanized blood and immune systems to the original donor(s) used to construct the strains to determine if indeed a faithful recapitulation has occurred. Finally, if multiple animals were constructed from a single donor, no reports have been published comparing these “syngeneic” animals to each other to determine if indeed each animal were essentially identical to the other (or not). In this study each of these possibilities was analyzed. The goal of this study was to address several of these overarching questions. First, is hematopoietic reconstitution from the same HSC donor similar in multiple recipients? Is reconstitution from different HSC donors also similar? Does reconstitution faithfully recapitulate the hematopoietic system observed in the HSC donor? Answering these questions might allow for the identification of molecular mechanisms important in these processes, and determine how accurately these humanized

models reflect the human situation. Second, are the effects of aging on the blood and immune systems more cell-intrinsic or -extrinsic in terms of importance? That is, in the aged mouse does the human immune system show signs of aging as might be expected from that reported for elderly human patients, or do these systems appear to remain neonatal in nature, independent of the aging of the recipient animal?

Transplantation of CD34⁺ cord blood cells into day 1 NRG mice resulted in stable long term engraftment of the mice with human blood and immune cells. T cells, B cells, myeloid cells and neutrophils were observed over long periods of time, easily detectable for periods of up to 600 days post-transplant. There did not seem to be significant differences between mice engrafted with CD34⁺ cells from the same donor. Mice engrafted with CD34⁺ cells from different donors showed minimal discrepancies when compared to each other (data not shown). Long term engraftment resulted in an abundance of CD4⁺ T cells at the expense of the CD8⁺ T cell subset. B cells appeared earlier than T cells and then declined, while myeloid cells were stably present at low levels at all time points. Previous investigators using the NSG model, but not the NRG model, found after 20–25 weeks (140–175 days), large numbers of naïve B and myeloid cells early after reconstitution with mostly “central memory” CD4⁺ T cells (CD45RO⁺, CD62L⁺, CCR7⁺) later and few CD8 T cells late after reconstitution (Andre et al, 2010). It appeared that immune system aging might be occurring in in our NRG model in that not only was there an increase in CD45RO⁺ T cells with time, but there was also an increase in CD3⁺CD28[−] T cells with time. This latter T cell population is thought to be a dysfunctional T cell that accumulates in the elderly, having reduced TCR diversity and function (Weng et al, 2009). These T cells are thought to arise from repeated stimulation and the CD8⁺CD28[−]CD27[−] T cells are thought to be terminally differentiated.

In this study we attempted to answer a series of questions pertaining to the humanized mouse model. First, is hematopoietic reconstitution from the same stem cell donor similar in multiple recipients? The answer appears to be yes. Is reconstitution from different HSC donors also similar? Yes, reconstitution appears to be similar although not identical. Does reconstitution faithfully recapitulate the hematopoietic system observed in the stem cell donor? Yes, in the sense that all aspects of the immune system seem to be represented similarly. Thus, this model seems to be a fairly accurate reflection of the human situation.

Second, are the effects of aging on the blood and immune systems more cell-intrinsic or -extrinsic in terms of importance? That is, in the aged mouse does the human immune system show signs of aging as might be expected from that reported for elderly human patients, or do these systems appear to remain neonatal in nature? It appears that the human immune system does display accelerated aging due to cell extrinsic factors in the aged mouse host. Although we cannot make direct comparisons to the aged stem cell donor (as the donor is at most 2–3 years old currently) there is evidence of T cell aging (in terms of phenotype) over time.

It should be noted that the reconstituted animals were not directly challenged immunologically to ascertain the status of the resident immune systems. A conscious decision was made to manipulate the animals as little as possible over the extended time of the experimentation to avoid loss of animals as well as accidental stimulation of the immune systems. However, it was possible to move the transplanted animals out of the ultra-clean animal rooms into normal mouse housing without infectious incident, which seems to imply the presence of a functional immune system. This aspect of immune aging is currently under investigation in separate experimentation.

As an aside, this model system, with its abundance of long term CD4⁺ T cells and a stable myeloid population might be ideal for the study of HIV and its treatment, particularly in the

elderly population, as others have done in similar model systems (Berges et al, 2006; Watanabe et al, 2007; Sun et al, 2007). Previous humanized models have been limited by the time that infected animals can be studied or have required the intravenous injection mature T cells to observe peripheral blood immune system effects. The current NRG model system solves those problems.

Finally, these results should have a broad impact as it should be applicable to the current 20% of the population that is considered aged, a population which continues to grow annually (Kovacs et al, 2009). Immune senescence has been well documented to be a contributor to frailty and mortality risk in the elderly, who respond less well than the young to immunizations, and are at increased risk of sepsis and death with a variety of bacterial and viral infections. This model system should allow for experimental manipulations to improve our understanding of the aging human immune system, as well as for investigation of novel interventions to improve the function of the aging immune system. Such an understanding should have significant benefits in terms of dealing with infectious disease and improving methods for vaccination in the elderly.

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Abbreviations

CB	cord blood
NRG	NOD/RAG $^{-/-}$ /IL2R γ^{null}
PMN	polymorphonuclear cells
HSC	hematopoietic stem cells

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Figure 1. Engraftment versus CD34+ cell dose

Newborn NRG mice were irradiated and injected intra-hepatically with the indicated dose of purified CD34⁺ cord blood stem cells (x-axis) as described in Materials and Methods. Engraftment was determined at days 90–120 by flow cytometry for the presence of human T, B and myeloid cells. Total human leukocyte cell counts were determined and compared to normal murine blood leukocyte cell counts (y-axis). Each symbol represents an individual mouse. The trend line indicated no significant correlation of cell dose with engraftment.

Figure 2. Long term survival of transplanted NRG mice versus CD34+ cell dose

Newborn NRG mice were irradiated and injected intra-hepatically with the indicated dose of purified CD34⁺ cord blood stem cells (y-axis) as described in Materials and Methods. Engraftment was determined at days 90–120 by flow cytometry. Mice were then followed for up to 600 days for survival and presence of human cells in the peripheral blood by FACS (x-axis). The trend line indicated no significant correlation between cell dose and long term survival. Each symbol represents an independent animal.

Figure 3. FACS analysis of immune reconstitution versus time after transplant

Newborn NRG mice were irradiated and injected intra-hepatically with 100,000 purified CD34⁺ cord blood stem cells as described in Materials and Methods. At the indicated time points (x-axis) peripheral blood was obtained and analyzed by FACS for the presence of human T (CD3), B (CD19) and myeloid (CD14) cells. Each line represents an individual animal created from a single cord blood donor. Data are shown as the percentage of total peripheral blood lymphocytes expressing CD3, CD19 or CD14 at each time point (y-axis).

Figure 4. FACS analysis of T cell reconstitution versus time after transplant

Newborn NRG mice were irradiated and injected intra-hepatically with 100,000 purified CD34⁺ cord blood stem cells as described in Materials and Methods. At the indicated time points (x-axis) peripheral blood was obtained and analyzed by FACS for the presence of human CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells. Each line represents an independent animal created from a single cord blood donor. Data are shown as the percentage of total CD3⁺ cells that also expressed either CD4 or CD8 at each time point (y-axis).

Figure 5. FACS analysis of T cell subsets versus time after transplant

Newborn NRG mice were irradiated and injected intra-hepatically with 100,000 purified CD34⁺ cord blood stem cells as described in Materials and Methods. At the indicated time points (x-axis) peripheral blood was obtained and analyzed by FACS for the presence of human CD3⁺CD45RA⁺ and CD3⁺CD45RO⁺ T cells. Each line represents an independent animal created from a single cord blood donor. Data are presented as the percentage of total human T cells that expressed either CD45RA or CD45RO at each time point (y-axis).

Fig. 6. FACS analysis of CD4 Subsets time after transplant

Newborn NRG mice were irradiated and injected intra-hepatically with 100,000 purified CD34⁺ cord blood stem cells as described in Materials and Methods. At the indicated time points (x-axis) peripheral blood was obtained and analyzed by FACS for the presence of human CD3⁺CD4⁺CD45RA⁺ and CD3⁺CD4⁺CD45RO⁺ T cells. Each line represents an independent animal created from a single cord blood donor. Data are presented as the percentage of CD3⁺CD4⁺ T cells that also expressed either CD45RA or CD45RO at each time point (y-axis).

Fig. 7. FACS analysis of CD8 T cell subsets versus time after transplant

Newborn NRG mice were irradiated and injected intra-hepatically with the indicated dose of purified CD34⁺ cord blood stem cells as described in Materials and Methods. At the indicated time points (x-axis) peripheral blood was obtained and analyzed by FACS for the presence of human CD3⁺CD8⁺CD45RA⁺ and CD3⁺CD8⁺CD45RO⁺ T cells. Each line represents an independent animal created from a single cord blood donor. Data are presented as the percentage of peripheral blood CD3⁺CD8⁺ T cells that also expressed either CD45RA or CD45RO at each time point for each animal (y-axis).

Figure 8. FACS analysis of Putative T cell aging in Humanized Mice with Time after Transplant
 Newborn NRG mice were irradiated and injected intra-hepatically with the indicated dose of purified CD34⁺ cord blood stem cells as described in Materials and Methods. At the indicated time points (x-axis) peripheral blood was obtained and analyzed by FACS for the presence of human CD3⁺CD28⁺ and CD3⁺CD28⁻ T cells. Each line represents an independent animal created from a single cord blood donor. Data are presented as the percentage of total CD3⁺ T cells in the peripheral blood that did not express CD28 at each time point (y-axis).