Published in final edited form as:

Transl Immunol. 2013 March ; 28(0): 112–119. doi:10.1016/j.trim.2013.01.001.

Maintenance of Host Leukocytes in Peripheral Immune Compartments Following Lethal Irradiation and Bone Marrow Reconstitution: Implications for Graft Versus Host Disease

Elizabeth M. Staley^{a,b,c}, Scott M. Tanner^c, Joseph G. Daft^c, Andrea L. Stanus^c, Steven M. Martin[#], and Robin G. Lorenz^{c,d,*}

^aDepartment of Medicine, University of Alabama at Birmingham

^bDepartment of Epidemiology, University of Alabama at Birmingham

^cDepartment of Pathology, University of Alabama at Birmingham

^dDepartment of Microbiology, University of Alabama at Birmingham

Abstract

Bone marrow reconstitution is utilized as a tool for disease treatment and as a research technique to elucidate the function of bone marrow derived cells. Clinically successful engraftment is indicated by the development of a functioning immune repertoire. In research, reconstitution is considered successful if >85% of splenic leukocytes are of donor origins. Previous work suggests that splenic reconstitution may not be indicative of reconstitution in the mucosa. We sought to evaluate mucosal reconstitution in animals following a standard bone marrow eradication and reconstitution technique. Bone marrow was harvested from adult B6.SJL donor mice (CD45.1) and injected via either the retro-orbital or intraperitoneal route into lethally irradiated B6 (CD45.2) adult or neonatal recipients respectively. Expression of CD45 by flow cytometry was used to calculate reconstitution with respect to immune compartment and cell type. In reconstituted adult animals 93.2±1.5% of splenic leukocytes expressed the donor CD45.1 antigen thus meeting the standard definition of reconstitution, however only 58.6±13.6% of intestinal lamina propria lymphocytes and 52.4±16.0% of intestinal intraepithelial lymphocytes were of donor origin, confirming splenic reconstitution fails to represent peripheral immune reconstitution. T-cells in the gastrointestinal tract are the most poorly reconstituted, while B-cells appear to be almost universally replaced by donor cells. The inadequate mucosal reconstitution was not corrected by evaluating later timepoints or by performing the bone marrow transfer during the neonatal period. This demonstration that substantial host T-cells remain in the intestinal mucosa after a “successful” bone marrow transplantation should cause a re-evaluation of data from research bone marrow chimera experiments, as well as the mechanisms for complications after clinical bone marrow transplantation.

© 2012 Elsevier B.V. All rights reserved.

* Address correspondence to: Dr. Robin G. Lorenz, Department of Pathology, University of Alabama at Birmingham, 1825 University Blvd., SHEL 602, Birmingham, AL 35294-2182. Phone: 205-934-0676. Fax: 205-996-9113. rlorenz@uab.edu.

[#]Current Address: Sequoia Sciences, Saint Louis, MO

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The authors have no conflict of interest to declare.

Keywords

Bone Marrow Reconstitution; Mucosal Immunology; Chimera; Neonates; Graft versus Host Disease; Intestine

1. Introduction

Historically bone marrow reconstitution has been utilized as either a clinical tool for the treatment of malignancies and immune deficiency syndromes, or as a research technique to elucidate the function of immune cells derived from a hematopoietic lineage (Sanders, 1997). Treatment typically requires that patients first undergo myeloablative therapy to deplete hematopoietic precursor cells, and then infusion/reconstitution with allogenic donor bone marrow (Petersen, 2007). In human populations, success of engraftment is indicated by survival secondary to the development of a functioning, potentially cancer free, immune repertoire. However, in patients receiving both myeloablative and non-myeloablative therapy prior to reconstitution with matched bone marrow, chimerism has been observed in peripheral blood immune cell populations (van Leeuwen et al., 1994a; van Leeuwen et al., 1994b; Imamura et al., 2003).

This mixed chimerism has been associated with graft rejection and graft versus host disease (GVHD) in patient populations (Hill et al., 1986; Shlomchik et al., 1999; Imamura et al., 2003). In GVHD, donor cells recognize host minor histocompatibility antigens that are displayed by residual host cells (Korngold and Sprent, 1978; den Haan et al., 1995). These donor cells then initiate an inflammatory response, which ultimately results in effector cell infiltration into the gastrointestinal tract, skin, eyes and liver. Chronic GVHD occurs in as many as 20% of transplants involving histocompatible siblings, and 40% of matched unrelated donor transplants; thus posing a significant threat to transplant success (Sanders, 2002).

In research studies animals are typically reconstituted with donor cells expressing alternate leukocyte specific cell markers, which allow for quantification of host versus donor cell expression. Engraftment may then be assessed at sacrifice as a function of splenic leukocytes expressing donor specific cell markers. Reconstitution is commonly considered successful if researchers attain greater than 85% expression of donor specific leukocytes in the spleen. However, it has long been suspected that splenic reconstitution is an inaccurate assessment of reconstitution in peripheral immune compartments, and more specifically in mucosal tissue (Blazar et al., 2000). Immune defense in peripheral tissue, particularly in the gastrointestinal tract, has been increasingly being recognized as a regulator of systemic immunity (Noverr and Huffnagle, 2004). The gastrointestinal mucosal immune system is composed of both organized lymphoid tissues, such as mesenteric lymph nodes (MLN) and Peyer's patches (PP), and diffuse, non-organized lymphoid tissues, which include the intestinal epithelial lymphocyte (IEL) compartment and the lamina propria (LP). Mucosal immune responses are initiated in the MLN and PP, while the IEL and LP are effector sites of the mucosal immune system (McCracken and Lorenz, 2001).

While a wide variety of cells of both host and donor origin have been implicated in graft rejection and GVHD; very little is known about host versus donor cell type predominance in leukocyte subsets in peripheral tissue following standard bone marrow reconstitution. Maintenance of host leukocytes in peripheral immune compartments could have a profound impact on research involving chimeric animals, as well on studies pertaining to the treatment of GVHD. It was our purpose to determine what percentage of host versus donor leukocytes are maintained in mucosal immune compartments (particularly in the gastrointestinal tract)

following a standard bone marrow reconstitution protocol. Furthermore, we sought to identify the predominant host immune cell subtypes maintained in the periphery following reconstitution.

2. Materials and Methods

2.1. Animals

C57BL/6J (B6 (Cd45.2, H-2B)) and B6.SJL-Ptprc^aPepc^b/BoyJ (B6.SJL (CD45.1, H-2B)) were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were bred and maintained under specific pathogen free (SPF) conditions in Thoren Isolator racks (Hazleton, PA) under positive pressure and were fed autoclaved NIH-31 rodent diet (Harlan Teklad, Madison, WI), and sterile drinking water ad libitum. SPF conditions at UAB include absence of the following organisms, as determined by serological screening: mouse parvoviruses, including MPV-1, MPV-2, and minute virus of mice; mouse hepatitis virus, Theiler's murine encephalomyelitis virus; mouse rotavirus (epizootic diarrhea of infant mice), Sendai virus; pneumonia virus of mice; reovirus; *Mycoplasma pulmonis*; lymphocytic choriomeningitis virus; mouse adenovirus; ectromelia (mousepox) virus; K polyoma virus; and mouse polyoma virus. Testing and other methods were as described at <http://main.uab.edu/Sites/ComparativePathology/surveillance/>. All experiments were approved by the Institutional Care and Use Committee of the University of Alabama at Birmingham.

2.2. Bone Marrow Isolation and Reconstitution

To generate a bone marrow suspension for transfer, B6.SJL (CD45.1) or B6 (CD45.2) donor animals were anesthetized with isoflurane and euthanized by cervical dislocation. Tibia and femurs were harvested and stripped of fat and muscle. Marrow was flushed from bone using a 26-gauge needle into HBSS+ (HBSS, 25mM Hepes, 1% fetal calf serum, and 1% Penicillin-Streptomycin)(Fisher-Scientific, Pittsburgh, PA). The cell suspension was mechanically dispersed by passage through the same needle, and filtered using a 70 μ M Nitex membrane to remove debris (Tetko, Elmsford, NY). Cells were washed twice in R10 (RPMI, 10% fetal calf serum, 1% Glutamax®, and 0.5% Gentamycin) (Fisher-Scientific). Cells were incubated for 45 minutes at 37°C with anti-Thy1.2 antibody followed by a 45-minute incubation in rabbit complement to deplete activated T-cells (Cedarlane, Burlington, Ontario Canada). Reconstitution was performed as previously described, in brief cells were washed twice in R10, and counted (Staley et al., 2011). Cells were resuspended in PBS at 100ul/recipient (approximately 3×10^6 cells/animal and injected retro-orbitally or intraperitoneally respectively into lethally irradiated adult (8 weeks) or neonatal (day 7) recipients. Animals injected retro-orbitally were anesthetized with isoflurane prior to injection. Animals were treated with Bactrim in drinking water for 10 days following irradiation to prevent infection during immune recovery.

B6 (CD45.2) or B6.SJL (CD45.1) recipient adults and B6 (CD45.2) recipient neonates were exposed to 9 Grays (Two doses of 4.5G administered 4 hours apart) of X-ray radiation using the X-RAD 320 Irradiator prior to reconstitution with donor bone marrow (Precision X-Ray Inc. N. Branford, CT). Lethal dose was determined in previous experiments from our lab, and was determined to be a minimum of 7 Gray for adult mice, and 8.5 Gray for neonatal animals. Mice were reconstituted within 4 hours of exposure to radiation. Animals were maintained for 8- or 11-weeks following reconstitution to establish engraftment. Animals were then sacrificed via cervical dislocation and tissue was harvested and prepared for cellular isolation.

2.3. Tissue Harvest and Cell Isolation

Tissue from spleen (SP), MLN, and PP was mechanically disrupted between sterile ground glass slides. This single cell suspension was then washed in PBS and filtered to remove debris. Erythrocytes were lysed using ammonium chloride. Cells were again washed and stored on ice in HBSS+ overnight. Both small and large intestinal tissue was stripped of fat, cut lengthwise, and flushed with PBS to remove feces. Tissue was then cut into 1 cm fragments and placed in HBSS containing 0.1M EDTA (Sigma-Aldrich, St. Louis, MO). Tissue was shaken for 40 minutes at 37°C. Supernatant containing IELs was then removed and the EDTA was inactivated with HBSS+. IELs were washed in PBS and resuspended in HBSS+ and stored on ice overnight. The remaining intestinal tissue was washed in PBS, and then shaken for 40 minutes in HBSS containing 0.5% collagenase IV (Sigma-Aldrich). This preparation was filtered and resuspended in 40% Percoll, and layered across a 70% Percoll gradient (Fisher-Scientific). Cells were spun at 1800 RPM for 20 minutes with no break. LP cells were then extracted from the 40%/70% interface and resuspended in HBSS+ and stored on ice overnight.

2.4. Flow Cytometry

Cells were washed in FACs Wash solution (PBS, 0.5% bovine serum albumin), resuspended in staining buffer (HBSS, 1% BSA, and 1mg/ml human IgG), and labeled with fluorochrome tagged antibodies for the lymphocyte markers CD4 (primarily helper T-cells), CD8 (primarily cytotoxic T-cells), FoxP3 (Tregs), CD11c (predominately dendritic cells (DC)), CD11b (predominately macrophages), CD19 (B-cells), NKp46 (natural killer (NK) cells), TCR $\alpha\beta$ (T-cell receptor $\alpha\beta$), and TCR $\gamma\delta$ (T-cell Receptor $\gamma\delta$), to better assess post-irradiation maintenance of host derived cells in mucosal compartments (BD Biosciences®, San Diego, CA). Cells were also assessed for expression of CD45.1 (B6.SJL derived leukocyte marker), and CD45.2 (B6 derived leukocyte marker). All flow cytometry was conducted using a Becton Dickinson FACSCalibur (BD Biosciences) and events were collected using CellQuest software. All analysis was conducted utilizing FlowJo software (Tree Star, Inc., Ashland, OR).

2.5 Statistical Analysis

Statistical analysis was performed using the Prism® program available from Graph Pad® (La Jolla, CA). Statistical analysis on flow cytometry data was performed using the Kruskal-Wallis test, and Dunn's multiple comparison analysis.

3. Results and Discussion

3.1. Leukocyte Origin in Peripheral Immune Compartments

3.1.1. Adult Bone Marrow Reconstitution in Peripheral Immune Compartments

—To determine the percentage of donor leukocytes present in peripheral tissue following radiation and a syngenic bone marrow reconstitution, 8-week old adult B6 animals (CD45.2, H-2B) were exposed to a lethal dose of total body X-ray radiation, and were subsequently reconstituted with bone marrow from B6.SJL animals (CD45.1, H-2B). Tissue was harvested 8-weeks after the bone marrow reconstitution and flow cytometric analysis was preformed utilizing fluorescently tagged antibodies targeted to panCD45 and CD45.2 expression on leukocytes from peripheral lymphoid organs. In addition, antibodies targeted to CD45.1 expression were also independently utilized to confirm that all panCD45+ cells were either of donor (CD45.1) or host (CD45.2) origin (data not shown). The data demonstrates that by conventional definition, all of the adult animals were successfully reconstituted. 93.2±1.5% of splenic leukocytes (PanCD45+) expressed the donor CD45.1 molecule (Fig. 1A,B). Successful reconstitution was also demonstrated in both MLNs

(92.6±4.1%), and PPs (93.7±2.6%). However the expression of the donor CD45.1 molecule was significantly reduced in the diffuse, non-organized gastrointestinal immune tissues, with only 52.4±16.0% of IELs and 58.6±13.6% of LP leukocytes expressing the donor CD45.1 molecule. This result indicates that only ~50% of the leukocytes in gastrointestinal tissue are of donor origin following bone marrow reconstitution. The percentage of leukocytes expressing donor cell markers and total cell numbers are shown in Tables 1 and 2.

To confirm that this incomplete reconstitution in the diffuse, non-organized lymphoid compartments of the gastrointestinal tract was not due to simply a delayed peripheral reconstitution, a second experiment with longer recovery after bone marrow reconstitution was performed. Eight week old adult B6.SJL mice (CD45.1, H-2B) were lethally irradiated and subsequently reconstituted with bone marrow from B6 mice (CD45.2, H-2B). These mice were sacrificed 11-weeks after transfer. They demonstrated a similar level of donor reconstitution as the animals from the 8-week reconstitution experiment (spleen-94.7%; PP-96.0%; IEL-64.8%; and LP-57.8%). These results indicate that incomplete reconstitution in the diffuse gastrointestinal lymphoid compartments is seen at two different reconstitution timepoints and is therefore not simply due to delayed reconstitution kinetics. In addition, this incomplete reconstitution is seen regardless of the strain origin of the donor lymphocytes, as it is observed both when donor B6 (into B6.SJL) or donor B6.SJL (into B6) bone marrow progenitors are used for the reconstitution.

3.1.2. Neonatal Bone Marrow Reconstitution in Immune Compartments—It has been shown in patient populations that a younger age at bone marrow transplantation correlates significantly with increased host hematopoiesis (mixed chimerism post transplantation) (van Leeuwen et al., 1994b). To ascertain whether a younger age at reconstitution would affect the percentage of donor derived leukocytes present in mucosal immune tissue, post-natal day 7 B6 (CD45.2) mice were lethally irradiated and reconstituted with B6.SJL (CD45.1) bone marrow as described above. Interestingly, animals reconstituted in the neonatal period consistently demonstrated lower expression of donor CD45.1+ leukocytes (i.e. increased retention of host immune cells) in all tissues except the LP when compared to animals reconstituted as adults. The percent of neonatal leukocytes expressing donor CD45.1 was 86.9±5.0% in the SP, 79.8±7.6% in the MLN, 91±6.3% in the PPs, 43±20.8 in IELs and 71.2±11.9% in the LP (Fig. 1B). Interestingly the increased expression of donor leukocytes demonstrated in LP tissue was not significantly representative of any one immune cell subtype, however the percentage of TCRαβ⁺, CD19⁺, CD8⁺, FoxP3⁺, and CD11b⁺ cells expressing donor antigen was increased in this tissue (Table 3). This data indicates that in our model increased age at reconstitution correlates with increased overall expression of donor leukocytes in most peripheral immune compartments following reconstitution, and therefore decreased mixed chimerism. However, it should be noted that in the neonatal model, much like in the adult, the percentage of leukocytes of donor origin was dramatically reduced in the diffuse, non-organized lymphoid tissue. The percentage of neonatal leukocytes expressing donor antigen is summarized in Table 1, total number of cells is summarized in Table 3.

3.2 Origins of Adaptive Immune Cells in Peripheral Immune Compartments Following Reconstitution

3.2.1. B-cell and T-cell Origin in Peripheral Tissue following Reconstitution—

As the adaptive immune response has been classically implicated in the development of GVHD, and as our previous experiments clearly demonstrated a difference in leukocyte reconstitution in mucosal immune compartments, we chose to look at host versus donor origins in B- and T-cells following bone marrow reconstitution of adult animals.

B-cells were identified based on expression of the B-cell marker CD19 and expression of the donor CD45.1 marker was used to assess host vs. donor origin. In adult animals 8-weeks after bone marrow transfer, B-cells demonstrated near total reconstitution in all immune compartments analyzed, with approximately 90% or more of B-cells expressing donor leukocyte markers (Fig. 2A, Table 1). Similar results were seen using anti-IgM to identify B-cells in mice 11-weeks after reconstitution (spleen-99.4%; PP-99.6%; LP=98.1%). Animals reconstituted during the neonatal time period in general demonstrated slightly reduced reconstitution with donor CD19⁺ cells when compared to adults in all tissue except the LP (Table 1). However similar overall trends were observed, with 96.8±2.7% of SP, 89.3±9.4% of MLN, 87.1±21.5% of PP, 79.1±20.5 of IELs, and 92.7±4.3% of LP CD19⁺ cells expressing donor CD45.1 antigen (Fig. 2B).

T-cell populations were identified by looking for expression of αβ and γδT-cell markers, as both types of T-cells are represented in gastrointestinal tissue where donor cell reconstitution is reduced. In adult animals 8-weeks after bone marrow transfer, T-cell populations, specifically αβT-cells, demonstrated the greatest variability in reconstitution of diffuse, non-organized lymphoid tissue. Only 32.8±2.5% of LP and 49.7±9.4% of IEL αβT-cells were of donor origin, while greater than 50% of αβT-cells in organized lymphoid tissue expressed donor antigen (64±2.6% PP, 82.9±1.8% MLN, and 77.8±1.7% SP) (Fig. 2C, Table 1). This data clearly indicates that αβT-cell populations in diffuse lymphoid tissue demonstrate significant resistance to reconstitution following irradiation and bone marrow reconstitution in adult animals.

In neonatal animals similar results were seen in that expression of CD45.1 on αβT-cells was increased in organized lymphoid tissue (SP 62.6±8.3%, MLN 69.5±7.1%, and PP 57.8±17.2%) when compared to diffuse lymphoid tissue (IEL 44.6±16.9% and LP 40.4±12.4%). Neonatal animals demonstrated reduced reconstitution of αβT-cells in every tissue type analyzed except LP (Fig 2D).

In adult animals a greater percentage of γδT-cells demonstrated reconstitution with all tissue types showing greater than 70% of γδT-cells expressing donor CD45.1 (79.1±7.9% LP, 70.5±7.1% IEL, 93.7±4.8% PP, 89.5±4.1% MLN, and 92.5±2.9% SP) (Fig. 2C). Of note a significantly higher percentage of γδT-cells in organized lymphoid tissue (SP and PP) demonstrated reconstitution when compared to γδT-cells in the IEL.

Neonatal animals similarly demonstrated greater reconstitution of γδT-cells than αβT-cells, albeit at lower percentages than seen in adults. Furthermore, in neonatal animals reconstitution of γδT-cells was greater in organized lymphoid tissue (SP 77.3±8.6%, MLN 84.4±3.5%, and PP 80.6±16.6%) than in diffuse lymphoid tissue (IEL 69.7±19.7% and 70.8±4.2%)(Fig 2D).

3.2.2. Reconstitution of T-Cell Subsets in Peripheral Immune Tissue—Given the dramatically decreased percentage of αβT-cells of donor origin in peripheral immune compartments following bone marrow reconstitution, we chose to further analyze T-cells subsets by first looking at expression of the T-cell markers CD4 and CD8 on host and donor leukocytes. In adult animals we found that in more centrally located organized lymphoid tissue such as the SP and the MLN approximately 75% of both CD4⁺ and CD8⁺ cells express the donor CD45.1 marker. However, in peripheral lymphoid tissue co-expression of the donor CD45.1 molecule was dramatically reduced. Only 57.4±9.9% of CD4⁺ and 57.3±15.3% of CD8⁺ leukocytes in PPs were of donor origin. Donor CD45.1 expression was even further reduced in diffuse intestinal immune tissues as 53.4±17.5% of CD4⁺ and 48.2±16.4 of CD8⁺ cells co-expressed the donor CD45.1 marker in IELs; and only 42.7±9.2% of CD4⁺ and 48.8±18.7% of CD8⁺ cells expressed the donor marker in the LP 8-

weeks after bone marrow transfer. In animals 11-weeks after reconstitution, only 40.9% of CD8⁺ IELs expressed the donor CD45 marker, while 37.7% of CD8⁺ LP lymphocytes and 47.0% of CD4⁺ LP lymphocytes expressed the donor CD45 marker. This data demonstrates that in gastrointestinal immune compartments greater than half of the T-cells present following irradiation and reconstitution are actually of host origin (Fig. 3A).

Animals reconstituted during the neonatal time point displayed similar results. Greater than 60% of CD4 and CD8 cells in organized lymphoid tissue (SP, MLN, and PP) expressed donor antigen. However, only 47.5±15.0% and 43.6±12.4% of CD4 cells and 31.2±21.0% and 51.1±14.7% of CD8 cells expressed donor cell CD45.1, in the IEL and LP respectively. This data again confirms that even in neonatal animals, which consistently reconstitute at a lesser rate than adults, reconstitution in organized lymphoid tissue is greater than diffuse lymphoid tissue.

We further chose to investigate the T-cell environment, by determining whether the CD4⁺FoxP3⁺ Tregs present in peripheral immune tissue in our chimeric animals were predominantly of host or donor origin. In our model of reconstitution we found that following radiation and reconstitution CD4⁺FoxP3⁺ expressing Tregs are predominantly of donor origin in central immune compartments. Approximately 54.9±5.2% of SP and 64.4%±4.5% of MLN CD4⁺FoxP3⁺ Tregs co-express the donor CD45.1 marker. Expression of donor CD45.1 was again dramatically reduced in peripheral mucosal immune compartments. Only 21.3±8.9% of PP, 34.6±10.2% of IEL, and 23.6%±6.3% of LP Tregs were of donor origins (Fig 3C). These data clearly demonstrate that Tregs exhibit profoundly mixed chimerism in immune compartments following adult bone marrow reconstitution. In neonatally reconstituted animals, the percentage of CD4⁺FoxP3⁺ Tregs of donor origin are significantly less in the MLN (48.0±14.0%), when compared to the adult reconstitution, but are a significantly higher percentage in the PP (36.7±11.2%) (Fig. 3D).

3.3 Origins of Innate Immune Cells following Bone Marrow Reconstitution

Given the profound difference in cellular origins of adaptive immune cells in peripheral immune tissue following reconstitution, we chose to look specifically at populations of innate immune cells to distinguish cellular origin following reconstitution. We chose to look at co-expression of the markers CD11b (which is predominately found on monocytes/macrophages, but can also be expressed in granulocytes, NK cells, CD5⁺ B-cells and a subset of DCs), CD11c (primarily a marker of dendritic cells, but can be weakly expressed on some NK cells, B-cells, and T-cells) and NKp46 (CD335, NK cells).

CD11b⁺ cells demonstrated very little post-reconstitution chimerism in all tissues examined, with greater than 80% of cells co-expressing the donor leukocyte markers (90.9±2.8% SP, 84.9±8.7% MLN, 87.1±9.4% PP, 88.6±13.2% IEL, and 81.9±8.7% LP) in adult reconstitutions (Fig 4A) and similar levels of reconstitution in neonatal reconstitutions (Fig. 4B). NKp46⁺ cells were also predominantly of host origin in all peripheral immune compartments. 92.8±1.3% of SP, 78±8.2% of MLN, 91.4±4% of PP, 69.4±28% of IEL, and 79.8±7.5% of LP NK cells in adult reconstituted animals were found to co-express the donor leukocyte marker (Fig. 4E) and similar numbers were seen in the majority of tissues from neonatal reconstituted mice (Fig. 4F). The two exceptions were that significantly less NKp46⁺ cells were found in the neonatal spleen (65.8±11.7) and PP (77.0±13.9) (Table 1). However, CD11c⁺ cells did demonstrate some variability of reconstitution in peripheral immune compartments. Approximately 80% of CD11c⁺ SP, MLN, and PP co-expressed CD45.1 (SP 86.9±4.9%, MLN 79.6±9.7%, PP 83.1±5.1%), while only 57.3±16.6% of IEL and 69.5±18.8% of LP CD11c⁺ cells expressed donor leukocyte markers in adult reconstitutions (Fig. 4C). A similar trend was seen in the neonatally reconstituted mice (Fig. 4D). This data suggests that incomplete chimerism following reconstitution is not limited to

T-cells, but also includes CD11c⁺ cells. Therefore following reconstitution, a large percentage of donor immune cells involved in both the adaptive and innate immune response are being maintained in peripheral immune sites.

4. Conclusions

Initially the purpose of our study was to examine the expression of host vs. donor molecular markers on leukocytes in peripheral and mucosal immune compartments following bone marrow reconstitution. We found that following a standard bone marrow reconstitution protocol leukocytes in diffuse, non-organized gastrointestinal immune tissue (LP and IEL) express a mixed chimerism, as roughly only half the leukocytes in these tissues express molecular markers confirming host origination. We then further demonstrated that this mixed chimerism is present in peripheral immune compartments regardless of age at reconstitution or duration of reconstitution. Animals reconstituted at neonatal time points (Day 7) actually demonstrate increased chimerism, or rather increased retention of host cell markers on leukocytes in peripheral immune tissue in almost every tissue type, and in every leukocyte subtype analyzed.

In order to more clearly define this chimerism we further classified the leukocyte subtypes in peripheral immune tissue, which maintained expression of host molecular cell markers. We found that in immune cells of the adaptive immune response, B-cells demonstrate near total reconstitution (>90%) in all immune compartments; however mixed chimerism is prominent in T-cells, particularly those expressing the TCRαβ receptor. We found that almost half of all CD4⁺ and CD8⁺ T-cells in mucosal immune compartments (PP, LP and IEL) maintain host cell origins. Interestingly, CD4⁺FoxP3⁺ Tregs show the most resistance to reconstitution. CD4⁺FoxP3⁺ T-cells in mucosal tissue were predominantly of host origin. We next examined the origin of leukocyte subtypes in cells of the innate immune response. We found that like B-cells, CD11b⁺ and NKp46⁺ cells demonstrate near total reconstitution (>70–80%) in all immune compartments analyzed. However, CD11c⁺ cells demonstrated some mixed chimerism in peripheral immune compartments with only 57% of CD11c⁺ IELs expressing donor leukocyte markers.

The development of bone marrow chimeras has long been utilized as a research tool enabling scientists to analyze the impact of various immune cell types, or the expression of proteins in those cell types on the development of disease (Panwala et al., 1998; Staley et al., 2011). However these data may carry an inherent flaw in that conventional methods to derive bone marrow chimeras appear to inadequately reconstitute cells in peripheral immune compartments. Our data suggests that contaminating host cells are being maintained in peripheral immune tissue and thus are potential confounders to the interpretation of results. Furthermore, GVHD has been proposed to be the development of disease in response to immune cells derived from engrafted bone marrow instigating an inflammatory response directed at host tissue. Our data would argue that the issue is more complicated than this, as substantial percentage of cells present in gastrointestinal immune tissue (one of the many sites of GVHD development) may be of host origin. These findings indicate that in the future, reconstitution of mucosal compartments needs to be separately investigated from splenic or peripheral blood reconstitution for adequate interpretation of research and clinical data.

Acknowledgments

We would like to thank Jamie McNaught, Mason Harris, and Daniel Harmon for their invaluable advice and assistance.

Funding Sources

This work was supported in part by the NIH grants R01 DK059911 and P01 DK071176; the UAB Digestive Diseases Research Development Center Grant DK064400; the Juvenile Diabetes Research Foundation grant 36-3008-930; and the Crohn's and Colitis Foundation grant 26971. EMS received salary support from the Cancer Research Experience for Students Internship (R25 CA076023). SMT received partial salary support from a Crohn's and Colitis Student Research Fellowship Award, from the UAB-Hughes Med to Grad Fellowship, and from the UAB Carmichael Fellowship. Aspects of this project were conducted in biomedical research space that was constructed with funds supported in part by the NIH grant C06RR020136.

References

- Blazar BR, Lees CJ, Martin PJ, Noelle RJ, Kwon B, Murphy W, Taylor PA. Host T cells resist graft-versus-host disease mediated by donor leukocyte infusions. *J Immunol*. 2000; 165:4901–4909. [PubMed: 11046015]
- den Haan JM, Sherman NE, Blokland E, Huczko E, Koning F, Drijfhout JW, Skipper J, Shabanowitz J, Hunt DF, Engelhard VH, et al. Identification of a graft versus host disease-associated human minor histocompatibility antigen. *Science*. 1995; 268:1476–1480. [PubMed: 7539551]
- Hill RS, Petersen FB, Storb R, Appelbaum FR, Doney K, Dahlberg S, Ramberg R, Thomas ED. Mixed hematologic chimerism after allogeneic marrow transplantation for severe aplastic anemia is associated with a higher risk of graft rejection and a lessened incidence of acute graft-versus-host disease. *Blood*. 1986; 67:811–816. [PubMed: 3511988]
- Imamura M, Tsutsumi Y, Miura Y, Toubai T, Tanaka J. Immune reconstitution and tolerance after allogeneic hematopoietic stem cell transplantation. *Hematology*. 2003; 8:19–26. [PubMed: 12623423]
- Korngold R, Sprent J. Lethal graft-versus-host disease after bone marrow transplantation across minor histocompatibility barriers in mice. Prevention by removing mature T cells from marrow. *J Exp Med*. 1978; 148:1687–1698. [PubMed: 363972]
- McCracken V, Lorenz R. The gastrointestinal ecosystem: a precarious alliance among epithelium, immunity and microbiota. *Cell Microbiol*. 2001; 3:1–11. [PubMed: 11207615]
- Noverr MC, Huffnagle GB. Does the microbiota regulate immune responses outside the gut? *Trends Microbiol*. 2004; 12:562–568. [PubMed: 15539116]
- Panwala CM, Jones JC, Viney JL. A novel model of inflammatory bowel disease: mice deficient for the multiple drug resistance gene, *mdr1a*, spontaneously develop colitis. *J Immunol*. 1998; 161:5733–5744. [PubMed: 9820555]
- Petersen SL. Alloreactivity as therapeutic principle in the treatment of hematologic malignancies. Studies of clinical and immunologic aspects of allogeneic hematopoietic cell transplantation with nonmyeloablative conditioning. *Danish medical bulletin*. 2007; 54:112–139. [PubMed: 17521527]
- Sanders JE. Bone marrow transplantation for pediatric malignancies. *Pediatr Clin North Am*. 1997; 44:1005–1020. [PubMed: 9286297]
- Sanders JE. Chronic graft-versus-host disease and late effects after hematopoietic stem cell transplantation. *International journal of hematology*. 2002; 76(Suppl 2):15–28. [PubMed: 12430895]
- Shlomchik WD, Couzens MS, Tang CB, McNiff J, Robert ME, Liu J, Shlomchik MJ, Emerson SG. Prevention of graft versus host disease by inactivation of host antigen-presenting cells. *Science*. 1999; 285:412–415. [PubMed: 10411505]
- Staley EM, Dimmitt RA, Schoeb TR, Tanner SM, Lorenz RG. Critical Role For P-Glycoprotein Expression in Hematopoietic Cells In The Fvb.Mdr1a^{-/-} Model of Colitis. *Journal of pediatric gastroenterology and nutrition*. 2011
- van Leeuwen JE, van Tol MJ, Joosten AM, Schellekens PT, van den Bergh RL, Waaijer JL, Oudeman-Gruber NJ, van der Weijden-Ragas CP, Roos MT, Gerritsen EJ, et al. Relationship between patterns of engraftment in peripheral blood and immune reconstitution after allogeneic bone marrow transplantation for (severe) combined immunodeficiency. *Blood*. 1994a; 84:3936–3947. [PubMed: 7949150]
- van Leeuwen JE, van Tol MJ, Joosten AM, Wijnen JT, Verweij PJ, Khan PM, Vossen JM. Persistence of host-type hematopoiesis after allogeneic bone marrow transplantation for leukemia is significantly related to the recipient's age and/or the conditioning regimen, but it is not associated with an increased risk of relapse. *Blood*. 1994b; 83:3059–3067. [PubMed: 8180403]

Highlights

- Splenic leukocyte reconstitution after bone marrow transfer is not indicative of peripheral immune reconstitution.
- Reconstitution of neonatal animals correlates with increased chimerism.
- 50% of gastrointestinal T-cells remain of host origin following reconstitution.

Figure 1. Bone Marrow Transfer in both Adult and Neonatal Mice Results in Incomplete Reconstitution of Diffuse, Gastrointestinal Lymphoid Tissue Compartments

Representative flow cytometric analysis of leukocytes from animals reconstituted in adulthood. Shown is the analysis of cells in immune compartments (SP, MLN, PP, IEL, and LP) co-expressing PanCD45 and donor CD45.2 (A). Reciprocal data was seen using PanCD45 and host CD45.1 (data not shown), indicating that all CD45⁺ cells expressed either CD45.1 or CD45.2. Graphical representation of the percentage of leukocytes (PanCD45⁺) in SP, MLN, PP, IEL, and LP co-expressing donor antigen (CD45.1) in neonatal and adult animals 8 weeks post-reconstitution (B). n=15 adult animals, and n=8

neonatal animals. *Indicates statistical significance when compared to adult or neonatal spleen $p < 0.05$.

Figure 2. T-cells, but not B-cells, are Incompletely Reconstituted in Peripheral Immune Compartments

Percentage of CD19⁺ (A,B) and TCRαβ⁺ or TCRγδ⁺ cells (C,D) co-expressing the donor CD45.1 molecule. Experiments were conducted in adult animals (A, C) or neonatal animals (B, D) 8 weeks post-reconstitution. N=15 adult animals for CD19⁺ labeling, and 7–10 for αβ/γδ T-cell labeling, N=8 neonatal animals. *Indicates statistical significance of $p < 0.05$.

Figure 3. Regulatory T-cells are Incompletely Reconstituted in both Adult and Neonatally Reconstituted Peripheral Immune Compartments
 Percentage of CD4⁺ and CD8⁺ (A,B) and CD4⁺ FoxP3⁺ T-regulatory (C,D) cells of donor origin. Experiments were conducted in adult animals (A, C) or neonatal animals (B, D) 8 weeks post-reconstitution. N=7–10 adult animals, N=8 neonatal animals. *Indicates statistical significance of p<0.05.

Figure 4. CD11b⁺ and NKP46⁺, but not CD11c⁺ Innate Immune Cells are Adequately Reconstituted in Peripheral Immune Compartments

Percentage of CD11b⁺ (A,B) CD11c⁺ (C,D) and NKP46⁺ (E,F) cells co-expressing donor leukocyte markers following reconstitution. Experiments were conducted in adult animals (A, C, E) or neonatal animals (B, D, F) 8 weeks post-reconstitution. N=7–10 adult animals. N=8 neonatal animals. *Indicates statistical significance of $p < 0.05$.

Table 1

Percentage of Cells Expressing Donor CD45.1.

		SP	MLN	PP	IEL	LP
Pan CD45	Adult	93.2±1.5% *	92.6±4.1% *	93.7±2.6%	52.4±16.0%	58.6±13.6% *
	Neonate	86.9±5.0%	79.8±7.6%	91.0±6.3%	43.0±20.8%	71.2±11.9%
CD19+	Adult	98.8±0.8%	97.1±3.1% *	97.5±1.6%	93.3±2.5%	88.0±7.4%
	Neonate	96.8±2.7%	89.3±9.4%	87.1±21.5%	79.1±20.5%	92.7±4.3%
CD4+	Adult	76.7±2.7%	75.6±8.8% *	57.4±9.9%	53.5±17.5%	42.7±9.2%
	Neonate	64.5±9.0%	67.2±9.2%	60.4±13.4%	47.5±15.0%	43.6±12.4%
CD8+	Adult	73.4±6.0%	74.3±6.0% *	57.3±15.3%	48.2±16.4% *	48.8±18.7%
	Neonate	61.1±11.8%	67.1±8.2%	60.1±9.9%	31.2±21.0%	51.1±14.7%
CD4+TCRαβ+	Adult	77.8±1.7%	82.9±1.8% *	64.0±2.6%	49.7±9.4%	32.8±2.5%
	Neonate	62.6±8.3%	69.5±7.1%	57.8±17.2%	44.6±16.9%	40.4±12.4%
CD4+TCRγδ+	Adult	92.5±2.9% *	89.5±34.1% *	93.7±4.8%	70.5±7.1%	79.1±9.1% *
	Neonate	77.3±8.6%	84.4±3.5%	80.6±16.6%	69.7±19.7%	70.8±4.2%
CD4+FoxP3+	Adult	54.9±5.2%	64.4±4.5% *	21.3±8.9% *	34.6±10.2%	23.6±6.8%
	Neonate	46.8±11.7%	48.0±14.0%	36.7±11.2%	40.5±17.5%	26.9±7.5%
CD11c+	Adult	86.0±4.9% *	76.6±9.7%	83.1±5.9%	57.3±16.6%	69.5±18.8% *
	Neonate	79.7±4.96%	74.9±8.5%	77.2±16.9%	52.0±27.0%	45.6±24.3%
CD11b+	Adult	91.0±2.8%	85.0±8.7% *	87.1±9.4%	88.6±13.2% *	81.9±8.7%
	Neonate	88.6±2.2%	74.3±5.7%	78.5±14.3%	55.0±15.0%	85.4±4.0%
NKP46+	Adult	92.8±1.3% *	78.0±8.2%	91.4±4.0% *	69.4±28.0%	79.8±7.5%
	Neonate	65.8±11.7%	70.5±6.8%	77.0±13.9%	90.6±6.3%	72.6±19.3%

* Indicates statistical significance when compared to values from animals reconstituted in neonates.

Table 2

Total Number of Adult Leukocyte Co-expressing Donor CD45.1.

	SPLN	MLN	PP	IEL	LP
PanCD45	7.31E+07 ± 2.79E+07	2.30E+06 ± 1.25E+06	5.75E+05 ± 2.27E+05	7.95E+06 ± 7.00E+06	1.67E+06 ± 4.37E+06
CD19+	4.93E+07 ± 2.59E+07	1.36E+06 ± 7.70E+05	5.78E+05 ± 1.28E+05	7.24E+05 ± 2.94E+05	2.25E+05 ± 1.60E+05
CD4+	1.23E+07 ± 5.16E+06	5.83E+05 ± 5.89E+05	4.61E+04 ± 2.30E+04	1.61E+06 ± 1.99E+06	3.96E+05 ± 8.49E+05
CD8+	4.80E+06 ± 2.26E+06	2.40E+05 ± 2.69E+05	1.90E+04 ± 1.21E+04	5.83E+06 ± 5.21E+06	5.71E+05 ± 1.52E+06
CD4+ TCRαβ+	1.91E+07 ± 9.61E+06	1.60E+06 ± 4.43E+05	7.40E+04 ± 2.21E+04	1.46E+06 ± 1.09E+06	6.14E+06 ± 1.40E+06
CD4+ TCRγδ+	9.60E+05 ± 5.60E+05	4.49E+04 ± 1.37E+04	1.61E+04 ± 6.40E+03	8.57E+05 ± 6.31E+05	3.39E+05 ± 7.85E+05
CD4+ FoxP3+	9.28E+06 ± 2.17E+07	2.95E+05 ± 5.14E+05	1.57E+05 ± 3.59E+05	3.67E+05 ± 6.42E+05	9.15E+04 ± 1.15E+05
CD11c+	6.00E+06 ± 1.18E+07	1.16E+05 ± 8.97E+07	3.65E+04 ± 1.63E+04	2.50E+06 ± 2.93E+06	2.01E+05 ± 2.18E+05
CD11b+	2.07E+06 ± 1.95E+06	8.22E+04 ± 3.08E+04	1.40E+04 ± 7.45E+03	1.02E+06 ± 5.71E+05	2.20E+06 ± 6.61+06
NKP46+	3.21E+06 ± 1.41E+06	1.64E+05 ± 7.31E+04	5.81E+04 ± 1.74E+04	7.42E+05 ± 2.11E+05	1.24E+06 ± 2.58E+06

Table 3

Total Number of Neonatal Leukocytes Co-expressing Donor CD45.1.

	SPLN	MLN	PP	IEL	LP
PanCD45	2.07E+07 ± 9.5E+06	2.53E+06 ± 3.80E+06	5.22E+05 ± 4.19E+05	1.56E+06 ± 1.11E+06	1.23E+06 ± 1.12E+06
CD19	1.39E+07 ± 4.94E+06	1.15E+06 ± 1.36E+06	5.57E+05 ± 4.75E+05	8.14E+05 ± 7.98E+05	5.96E+05 ± 5.02E+05
CD4+	2.99E+06 ± 1.44E+06	6.62E+05 ± 7.48E+05	7.44E+04 ± 7.39E+04	1.46E+05 ± 1.16E+05	2.37E+05 ± 2.21E+05
CD8+	1.62E+06 ± 7.86E+05	4.98E+05 ± 5.97E+05	2.14E+04 ± 1.42E+04	6.18E+05 ± 3.11E+05	8.21E+04 ± 8.24E+04
CD4+ TCRαβ+	5.56E+06 ± 4.05E+06	8.11E+05 ± 2.05E+05	1.62E+05 ± 7.35E+04	6.63E+05 ± 2.98E+05	1.15E+06 ± 1.25E+06
CD4+ TCRγδ+	6.37E+05 ± 3.04E+05	9.89E+04 ± 7.54E+04	3.53E+04 ± 2.81E+04	1.18E+06 ± 1.12E+06	3.04E+05 ± 3.22E+05
CD4+ FoxP3+	4.14E+05 ± 1.08E+05	4.38E+04 ± 1.18E+04	3.29E+04 ± 3.59E+04	1.17E+05 ± 5.13E+04	1.41E+05 ± 1.38E+05
CD11c+	7.85E+05 ± 5.34E+05	3.99E+04 ± 3.56E+04	9.08E+04 ± 1.21E+05	1.82E+06 ± 1.55E+06	2.17E+05 ± 1.77E+05
CD11b+	1.65E+06 ± 4.29E+05	2.55E+05 ± 3.32E+05	5.47E+04 ± 4.94E+04	7.35E+05 ± 1.59E+05	2.83E+05 ± 2.16E+05
NKP46+	8.82E+05 ± 1.11E+06	2.11E+05 ± 3.18E+05	3.41E+04 ± 2.01E+04	7.23E+05 ± 5.41E+05	7.64E+04 ± 5.10E+04