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Journal

Annals of Surgery, 263(1)

ISSN

0003-4932

Authors

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Publication Date

2016

DOI

10.1097/sla.0000000000001123

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Ann Surg. Author manuscript; available in PMC 2019 June 05.

Published in final edited form as:

Ann Surg. 2016 January ; 263(1): 199–204. doi:10.1097/SLA.0000000000001123.

The Response to Burn Injury in Mice with Human Hemato-Lymphoid Systems

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Abstract

Objective: To develop an animal model of injury that more closely represents the human inflammatory cell response to injury.

Summary Background Data: Because the mouse inflammatory response to burn injury cannot account for the contribution of human-specific genes, animal models are needed to more closely recapitulate the human inflammatory response and improve the translational impact of injury research. To this end, we hypothesized that the human inflammatory cell response to injury could be selectively assessed after severe burn injury using humanized mice.

Methods: NOD-Scid-IL2Rγ^{null} mice were transplanted with human hematopoietic $CD34^+$ progenitor cells, their engraftment confirmed and then subjected to 30% TBSA steam burn injury. Blood, bone marrow, and lung tissue were collected 4 hours following injury and human inflammatory cell mobilization analyzed using flow cytometry and immunohistochemistry.

Results: Burn injury caused mobilization of human inflammatory cells into the systemic circulation. Next, the burn injury was accompanied by evidence of histologic lung injury and concomitant mobilization of human $CD45^+$ immune cells into the lung that were associated with increased trafficking of human $CD11b^+$ myeloid cells.

Conclusion: These experiments are the first to demonstrate the suitability of humanized mice for injury research. They offer the possibility to address very specific research questions that are not amenable to traditional mouse models of injury, for example, the emerging role of certain human-specific genes that are either unrepresented or totally absent, from the mouse genome.

Mini-Abstract

Improved animal models are needed to better understand the human inflammatory cell response to injury. Here, we measure the mobilization of human inflammatory cells after burn injury in mice with human hemato-lymphoid systems. These experiments are the first to demonstrate the suitability of humanized mice for injury research.

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INTRODUCTION

While there are many genes that humans share with other species, there are some that are unique to humans. The presence of these human-specific genes might explain the unique biology of humans and could transform our understanding of the human response to injury and disease. While still a matter of significant contention,[1–4] the differences seen when comparing the injury response between humans and murine models underscore the fact that no preclinical model can completely recapitulate the complexity of the human injury response.[5] Furthermore, they raise significant questions regarding the translational potential of current preclinical modeling in regards to characterizing the human immune response, identifying potential therapeutics,[6] and advancing the field of burn injury research, in particular.

To assess the human contribution to a human-specific inflammatory response to cutaneous burn injury, we exploited significant advances in mouse modeling of HIV-AIDS.[7–9] Mice with a human hemato-lymphoid immune system,[10–12] referred here as 'humanized', are generated by engraftment of human $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) in NOD-Scid-IL2Rγ^{null} (NSG) mice.[13] We reasoned that these humanized mice might help study the contribution of human immune cells and human-specific genes to the inflammatory response to burn injury. With this in mind, we performed a series of experiments to first demonstrate that humanized NSG mice recapitulate a human response to burn injury[2] and then demonstrate a mobilization of human immune cells into the circulation and trafficking to the lung, a particularly well-defined site of distal organ injury in the systemic inflammatory response (SIRS) to cutaneous burn injury.[1]

METHODS

Mice with Human Hemato-lymphoid Systems

NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}*/SzJ* (NSG) mice were reconstituted with human $CD34^+$ HSPCs by Jackson Labs (#715557, Bar Harbor, ME) as previously described. Briefly, 3 week old NSG female mice where subjected to whole body sub-lethal irradiation and injection with 10^5 human fetal liver $CD34^+$ HSPCs[14, 15]. At 12 weeks post-intravenous injection the engraftment of human $CD45^+$ cells in peripheral blood was confirmed by flow cytometry with only mice having at least 25% human $CD45^+$ cells being used for studies 12–15 weeks post-injection. These mice are referred to as humanized NSG mice (hNSG) in the manuscript.

Animal Model of Burn Injury

Humanized NSG mice were subjected to burn injury at 15–18 weeks of age. Mice were placed under general anesthesia using inhaled isofluorane. Animals underwent dorsal fur clipping with an electric clipper prior to 30% total body surface area (TBSA) dorsal steam burn for 7 seconds using a template designed to estimate 30% TBSA based on the Walker-Mason burn model as previously described.[16–18] The mortality of this model is < 5%. Following burn, animals received a subcutaneous injection of normal saline containing buprenorphine (0.05 mg/kg) in a non-burned area for fluid resuscitation and pain control.

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Animals were recovered from anesthesia and returned to their cages where they were provided food and water ad libitum. Tissue was harvested at 4 hours post-injury. Humanized mice subjected to burn injury were compared to un-injured humanized mice. We also compared humanized NSG mice to NSG mice that were not transplanted with human $CD34^+$ HSPCs as an important control group for these studies. All results were repeated in independent experiments.

Flow Cytometry

Animals were euthanized at 4 hours post-injury for tissue harvest. Blood was obtained via cardiac puncture and immediately placed on ice. Serum was obtained through centrifugation and stored at −80°C for cytokine analysis. Bone Marrow was harvested by dissecting the soft tissue and muscle from bilateral femurs. The distal end of the femur was transected and flushed with 2ml of PBS. Leukocytes were prepared for FACS from heparinized blood by lysis with ammonium chloride lysing buffer (Becton Dickson, San Jose, CA) for 15 minutes. Lung tissue cells were isolated from normal saline perfused whole lung collected at 4 hours following burn. The lung tissue was minced and placed in enzyme solution Collagenase A/ Dispase II and incubated at 37°C for 20 minutes. The lung tissue was then passed through a 70 μm filter, digestion was quenched with 5% FBS solution. Cells were analyzed by flow cytometry utilizing the following human-specific antibodies: CD45-FITC (clone H130, BD Pharmingen), CD11b-PE (clone CBRM1/5, eBioscience, San Diego, CA), CD14-APC (clone MSE2, BD Pharmingen), CD16-APC Cy7 (clone 3G8, BD Pharmingen), or CD3-PE Cy7 (clone SK7, BD Pharmingen). A Becton Dickson Accuri C6 Flow Cytometer was used for all analyses.

Detection of human-specific gene expression

To detect expression of human-specific genes in human $CD34^+$ HSPCs and in humanized NSG mice, we probed cDNA libraries prepared from mRNA that was isolated from purified human $CD34^+$ cells or from humanized mouse spleen and performed RT-PCR. For CHRFAM7A we used the following primers to avoid hybridization with mouse genes or the human 7 nicotinic acetylcholine receptor: Forward, 5’-CCTGATGTCACCTTCACAGT-3’, and Reverse, 5’-GAATCTGCAGGAAGCAGGAA-3’. TBC1D3 expression was evaluated using the following primers: Forward, 5’-GCATCGACCGGGACGTAAG-3’, and Reverse, 5’-CCTCCGGGTTGTACTCCTCAT-3’.[19] In control studies, the size of the anticipated amplified fragment was compared to the fragment amplified from a cDNA expression plasmid obtained from Origene and absent in unmodified NSG mice.

Lung Histology and Immunohistochemistry

Following sacrifice by cardiac puncture, the lung was perfused with PBS, then insufflated and the left mainstem bronchus clamped to maintain inflation of the left lung. The lung was then fixed in 4% paraformaldehyde followed by 70% alcohol for histologic evaluation. Lung sections were stained with hematoxylin and eosin (H&E) to visualize lung architecture. Immunohistochemistry for human CD45 was utilized to assess for the presence of human inflammatory cells in the lung following injury. Sections of lung tissue were fixed in paraffin and stained for human CD45 (clone H130, BD Pharmingen) using the Vector M.O.M.

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Immunodetection Kit (Vector Laboratories, Burlingame, CA). Sections were viewed an imaged using the Olympus FSX100 (Olympus, Center Valley, PA).

Study Approval

All animal experiments were approved by the University of California Animal Subjects Committee and were conducted in accordance with accepted guidelines for animal studies.

RESULTS

Human inflammatory cells are engrafted in NSG mice

To establish that 'humanized' mice can support a population of human immune cells capable of differentiating into lymphoid and myeloid cells, we performed a series of flow cytometry analyses. Humanized NSG mice (hNSG) were subjected to an analysis of the expression of human CD45, an antigen present on all human leukocytes, using a human-specific antibody ($hCD45^+$) or mouse-specific antibody ($mCD45^+$) antibodies to measure their relative expression in hNSG mice (Figure 1A). Flow cytometry analyses demonstrated an abundance of $hCD45^+$ leukocytes (27% of the cells were $hCD45^+mCD45^-$ from all viable erythrocyte-lysed bone marrow cells), and 13% of cells were $hCD45^-mCD45^+$ cells. Based on the detection of $hCD45^+$ cells in hNSG mice, we further analyzed bone marrow for markers of myeloid lineages (i.e. $CD16^+$, $CD14^+$, and $CD11b^+$) and lymphoid lineages (i.e. $CD3^+$) (Figure 1A). These results demonstrated engraftment of both human myeloid and lymphoid cells in this model. Next, we measured the presence of the human-specific genes CHRFAM7A[20, 21] and TBC1D3[22, 23], genes that are not present in the mouse genome, to further confirm the engraftment of human inflammatory cells in this mouse model. CHRFAM7A was identified in the human $CD34^+$ HPSCs as a positive controls(Figure 1B, left panel), while the expression of the human-specific genes CHRFAM7A and TBC1D3 was detected in splenic tissue harvested from hNSG mice. Neither gene was present in control NSG mice that were not transplanted with human $CD34^+$ HPSCs (Figure 1B, right panel).

Human immune cells are mobilized into the systemic circulation after burn injury

The reconstitution of NSG mice with human immune cells enables their selective tracking after burn injury using human specific antibodies. In one set of experiments, we analyzed changes in circulating immune cell populations of hNSG mice following severe, 30% total body surface area cutaneous burn injury. Flow cytometry analyses of control NSG mice that were not reconstituted with human $CD34^+$ HPSCs, (Figure 2A, left column) demonstrated the specificity of the anti-human antigen antibodies, and the detection and measurement of human transplanted cells. Human $CD45^+$ myeloid cells ($CD11b^+$, $CD14^+$, $CD16^+$) and human $CD3^+$ lymphoid cells are shown as bivariant dot plots (Figure 2A) detected from representative animals 4 hrs following sham vs. burn injury with representative histograms that compare the mobilization of each cell type (Figure 2B). These results establish that human myeloid and lymphoid cells are mobilized into the systemic circulation following burn injury.

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Human inflammatory cells are mobilized to sites of tissue injury

Cutaneous burn of the humanized mice causes significant lung injury similar to that observed in man and animal models.[4, 24] For example, there is an increase in the mobilization of $CD11b^+$ myeloid cells that, includes a 5-fold increase in the population of human $CD45+CD11b^+$ cells (Figure 3). We also performed immunohistochemical staining for human $CD45^+$ cells to demonstrate the recruitment of human inflammatory cells to the lung following severe burn. As shown in Figure 4, there is a significant increase in the recruitment of human $CD45^+$ cells to the lung of burn-injured animals compared to sham. Control NSG mice have no human HSPCs and, as expected, do not exhibit anti-human CD45 immunoreactivity. Furthermore, the changes in human leukocyte recruitment correlate with the increased histologic lung injury that follows burn injury.

DISCUSSION

Basic research studies using mouse models have been invaluable in understanding the immune response to injury. Specifically, they have allowed for controlled *in vivo* studies that helped define the molecules and signaling pathways that are critical in SIRS.[25, 26] Mouse models have also provided the opportunity to develop and test therapeutics aimed at limiting the inflammatory response to injury. Unfortunately, promising results in these mouse models have not yet translated to the clinical setting. Clearly, it would be ideal to study the injury response in controlled, *in vivo* studies using human cells and tissues. Research using human samples is often limited to studies performed with peripheral blood, restricted by the timing of informed consent, which prevents researchers from exerting rigorous experimental control on the timing and variables related to injury. For those reasons, we have turned to the emerging field of humanized mice to help address the limitations of mouse studies in understanding the human response to injury.

Humanized mice have been used for numerous clinical conditions including cancer,[27] sepsis[28], hepatitis,[29] allergy,[13] graft versus host disease,[7] and HIV/AIDS.[7–9, 12] Here, we add this application to burn injury and demonstrate the potential for utilizing mice transplanted with human hemato-lymphoid systems to facilitate the study of human-specific genes in the injury response *in vivo*.[27, 29] The fact that these human-specific genes are not represented in the mouse genome, and cannot be studied using traditional mouse models, further underscores the need for alternative models that more closely resemble the human injury response.[21] Although *in vitro* studies are extraordinarily useful and have been successfully used to study human-specific genes in human cells, they do not, and cannot, reiterate the molecular, cellular or systems complexity of *in vivo* injury.

The ability to study the contribution of human-specific genes is necessary to advance our understanding of the human response to injury and to determine the translational potential for therapies that have been well defined in classical animal models. For instance, the potential for vagus nerve stimulation to modulate the injury response has been well-characterized in standard murine models and has been proposed as a potential anti-inflammatory strategy that could be translated to the clinical setting[17, 18, 30, 31]. The anti-inflammatory effects of VNS are mediated by the α7 subunit of the nicotinic Acetylcholine (Ach) receptor (α7nAchR), where the protective effects of VNS are lost in α7nAchR

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knockout mice[32]. The recent discovery of the human-specific gene CHRFAM7A[33], which regulates α7nAchR signaling, suggests that vagal responsiveness may be fundamentally different in humans[1, 20]. The potential for CHRFAM7A to alter human inflammatory responses however cannot be studied in animals as it does not exist in their genome and no cognate gene is known. The significance of this problem is further underscored by Hedlund et al. who demonstrated that *cmah*, a taxonomically-restricted gene (TRG) that is absent from the human genome, confounds the murine injury response.[34] Characterizing the role of human-specific genes in mediating the immune response to injury, and defining how these genes potentially modulate pathophysiologic responses are critical to advance the field of injury research.

The technology to create mice with human hemato-lymphoid systems is not new[10, 11] and was originally developed to help study the emergence of human viruses that have no animal host (e.g. HIV). Just as there is no alternative way to study HIV *in vivo*, there is no alternative way to study the contribution of human-specific genes to the inflammatory response to injury *in vivo*. While humanized mice have been used to study the human immune cell response in a model of sepsis,[28] to our knowledge this is the first report describing humanized mice in cutaneous burn injury.

A combination of flow cytometry and immunohistochemical staining approaches were used to characterize the human inflammatory cell response to burn injury and we analyzed a specific human myeloid cell type in mouse lung tissue that would otherwise not readily be available from human burn patients. Accordingly, these tissues offer a complementary way to study the human immune response than more traditional mouse models of injury. For example, the human-specific immune response in tissues such as bone marrow, spleen, lung, gut, liver, and kidney cannot be routinely collected in human research so blood is routinely analyzed as a surrogate marker for systemic inflammation. While humanized mice do not recapitulate all aspects of human immunity and inflammation, they will undoubtedly bridge differences between mouse and humans. At a minimum, this approach can address the contribution of gross differences in myeloid vs. lymphoid cell lineage representation that characterize healthy normal mice vs. humans.[6]

On a final note, the translational success of injury research is dependent on preclinical models that can bridge the mechanistic basis of disease to the success of therapeutics in humans. Whereas traditional mouse models have been, and will continue to be important in dissecting signaling pathways and understanding the molecular events involved in the inflammatory response to injury, humanized mice offer a complementary approach that might more closely resemble the heterogeneity of man. If so, the use of humanized mice for injury research will enable the field of burn injury research to manipulate the human immune system, monitor human-specific responses, and target human-specific genes in ways that are otherwise not possible.

ACKNOWLEDGEMENTS

The authors would like to thank Drs. Vishal Bansal and Bruce Potenza for their thoughtful advice in this project, and Ann-Marie Hageny and James Putnam for their technical assistance. This work was supported by the American College of Surgeons C. James Carrico Faculty Research Fellowship (to T.W. Costantini), NIH Grant R01CA170140

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(to B.P. Eliceiri), and the Reinvestment Fund of the UC San Diego Division of Trauma, Surgical Critical Care, Burns and Acute Care Surgery.

Conflicts of Interest and Sources of Funding: This work was supported by the American College of Surgeons C. James Carrico Faculty Research Fellowship (to T.W. Costantini), NIH Grant R01CA170140 (to B.P. Eliceiri), and the Reinvestment Fund of the UC San Diego Division of Trauma, Surgical Critical Care, Burns and Acute Care Surgery. The authors report no conflicts of interest

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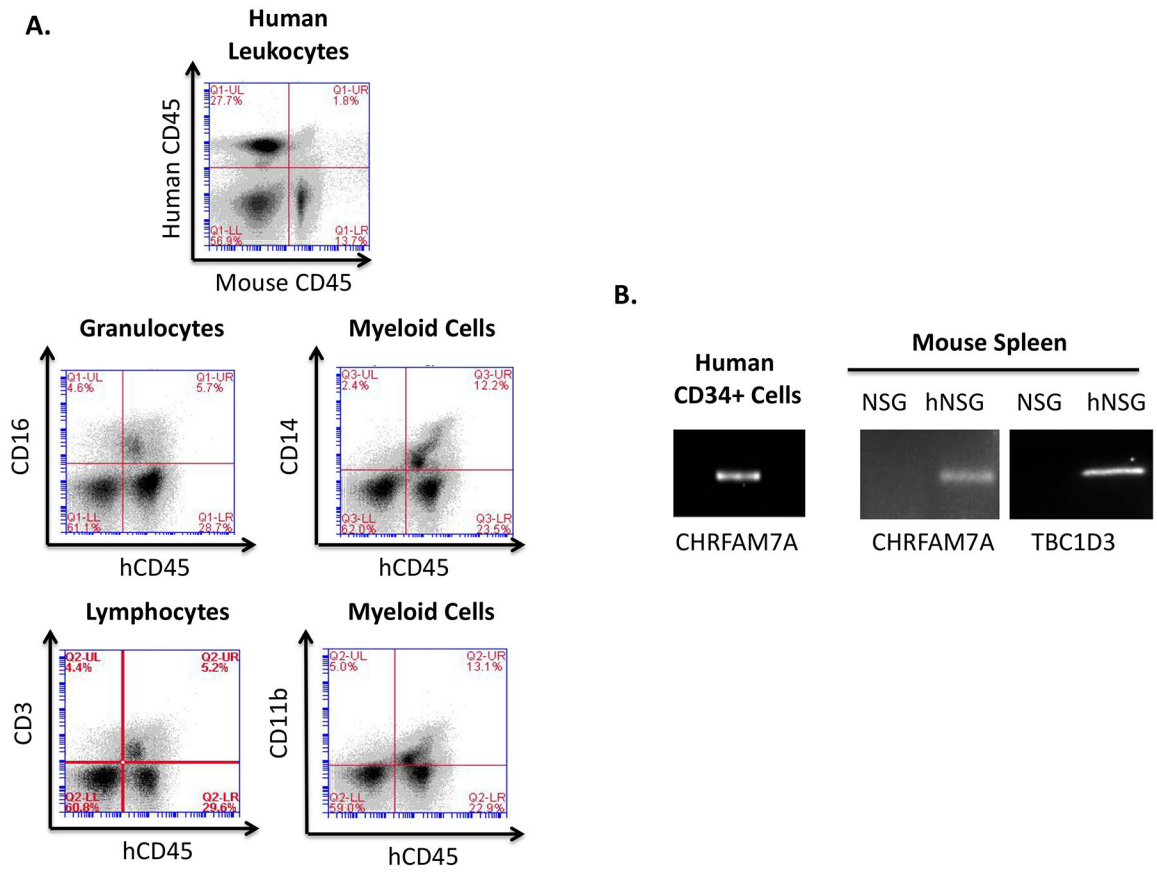

Figure 1:

Identification of human inflammatory cell types in bone marrow of NSG mice transplanted with human $CD34^+$ hematopoietic stem cells. (A) FACS was used to identify human vs. mouse $CD45^+$ leukocytes 4 hours post-burn. Human $CD45^+$ cells (hCD45) were further characterized to identify the population of human granulocytes ($hCD45^+CD16^+$), lymphocytes ($hCD45^+CD3^+$), and myeloid cells ($hCD45^+CD11b^+$, $hCD45^+CD14^+$). (B) PCR demonstrating the presence of the human-specific gene CHRFAM7A in the human CD34+ hematopoietic progenitor cells that were transplanted into NSG mice. PCR of spleen harvested from NSG mice transplanted with human CD34+ cells (hNSG) confirms engraftment of human inflammatory cells based on the presence of the human-specific genes CHRFAM7A and TBC1D3.

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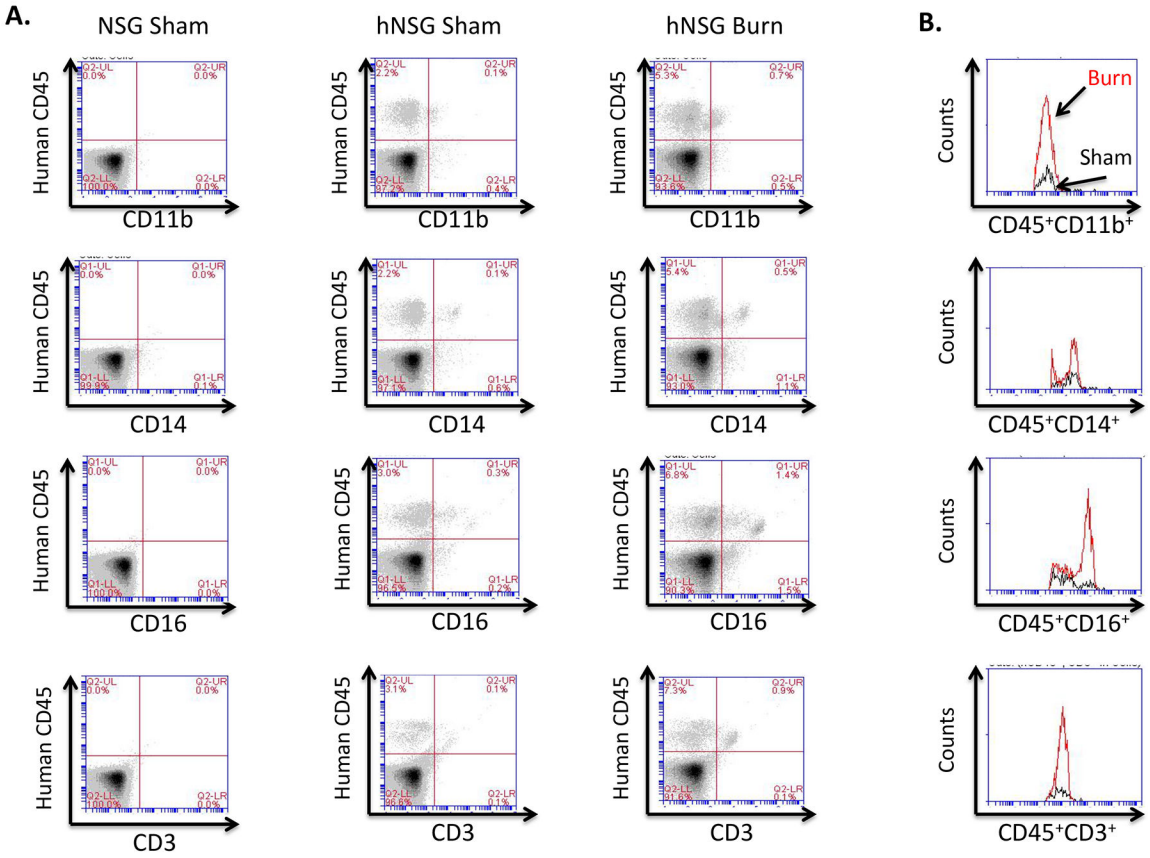

Figure 2:

Human immune cells are mobilized in the peripheral blood following injury. (A) Representative dot plots demonstrating characterization of human $CD45^+$ myeloid (CD11b, CD14, CD16) and lymphoid ($CD3^+$) cell populations. (B) Representative histograms demonstrate the mobilization of human myeloid and lymphoid cells after cutaneous burn injury. Black line = Sham, Red line = Burn.

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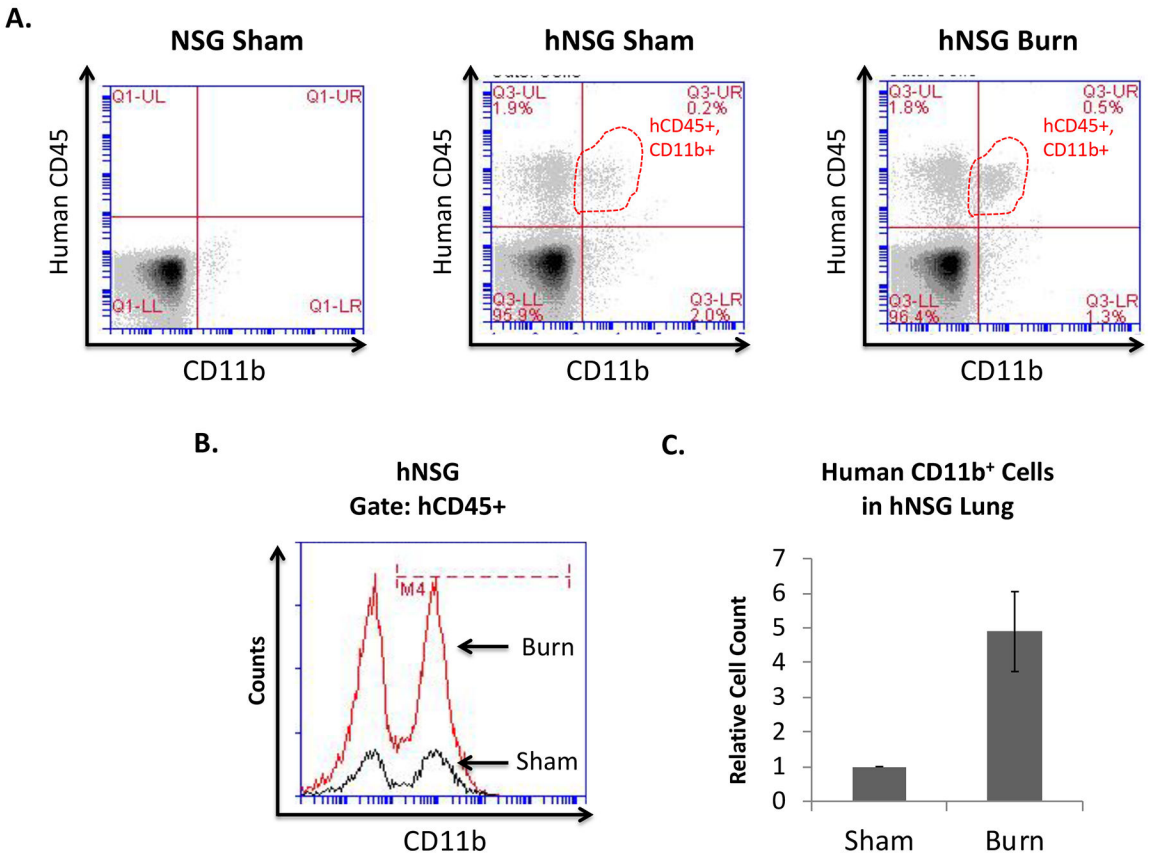

Figure 3:

Burn-induced acute lung injury is associated with recruitment of human inflammatory cells in mice with human hemato-lymphoid systems. (A) Representative flow cytometry dot plot demonstrating mobilization of human $CD45^{+}CD11b^{+}$ leukocytes to the lung. (B) Representative histogram demonstrating that human $CD11b^{+}$ inflammatory cells are recruited to the lung following injury. (C) Human $CD11b^{+}$ inflammatory cells are recruited to the lung after cutaneous burn injury. Quantification of flow cytometry results demonstrating increased mobilization of human $CD11b^{+}$ cells to the lung in burn-injured animals compared to sham. Black line = Sham, Red line = Burn

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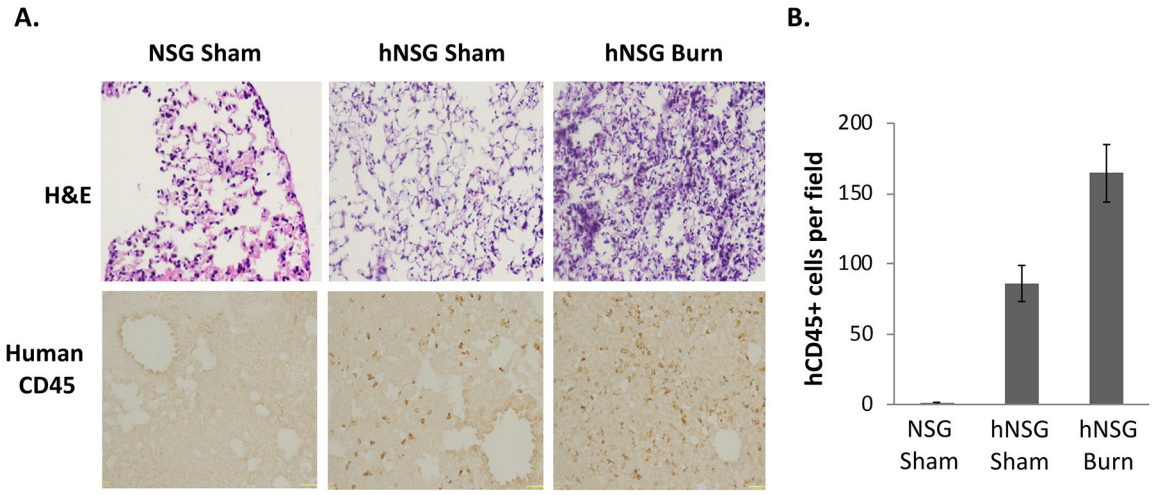

Figure 4:

Severe cutaneous burn injury causes histologic lung injury in humanized mice and is associated with recruitment of human $CD45^+$ leukocytes. (A) Lung tissue harvested following burn injury and stained with H&E or human CD45 to localize human inflammatory cells. (B) Quantification of human $CD45^+$ inflammatory cells from sections of lung stained for human CD45 using immunohistochemistry. There is increased mobilization of human $CD45^+$ stained cells following cutaneous burn injury compared to sham. There are no human $CD45^+$ cells seen in control NSG animals that were not transplanted with human hematopoietic stem and progenitor cells.