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Flt3 Ligand treatment attenuates T cell dysfunction and improves survival in a murine model of burn wound sepsis

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Abstract

Introduction—Sepsis is a leading cause of death among severely burned patients. Burn injury disrupts the protective skin barrier and causes immunological dysfunction. In our previous studies, we found that burn injury and wound infection causes a significant decline in lymphocyte populations, implying adaptive immune system dysfunction. In the present study, we examined the effect of treatment with Fms like tyrosine kinase-3 Ligand (Flt3L) on T cell phenotype and function in a model of burn wound sepsis. Flt3L is an essential cytokine required for hematopoietic progenitor cell development and expansion of both myeloid and lymphoid lineages. Flt3L has been shown to potentiate innate immune functions of dendritic cells and neutrophils during burn wound sepsis. However, the ability of Flt3L to improve T cell function during burn wound sepsis has not been previously evaluated.

Methods—Mice underwent 35% total body surface area scald burn and were treated with Flt3L (10µg) or vehicle daily via the intraperitoneal route starting one day after burn injury. On day 4 after burn injury, *Pseudomonas aeruginosa* was used to induce wound infection. Leukocytes in spleen and wound draining lymph nodes were characterized using flow cytometry. Bacterial clearance, organ injury and survival were also assessed.

Results—Flt3L treatment prevented the decline in splenic CD4⁺ and CD8⁺ T cells caused by burn injury and infection. Flt3L treatment also attenuated the decline in CD28 expression on CD4⁺ and CD8⁺ T cells and IFN γ production by CD8⁺ T cells in the spleen and wound draining lymph nodes. Furthermore, Flt3L decreased the levels of programmed death ligand 1 (PD-L1) expression on splenic dendritic cells and macrophages. Flt3 treatment improved systemic bacterial clearance, decreased liver and kidney injury, and significantly improved survival in mice with burn wound sepsis.

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Conclusion—Burn injury and associated sepsis causes significant loss of T cells and evidence of T cell dysfunction. Flt3L attenuates T cell dysfunction and improves host resistance to burn wound sepsis in mice.

Keywords

Sepsis; Fms like tyrosine kinase-3 (Flt3) ligand; burn; wound infection; sepsis; T cells; dendritic cells; macrophages

Introduction

Burn injury causes a loss of the protective skin barrier and renders patients susceptible to opportunistic infections. Impaired immune responses, both innate and adaptive, further increase the vulnerability to infections. Severe infection remains the leading cause of death among burn patients despite widespread use of antibiotics (1). The emergence of antibiotic resistant microorganisms presents a major obstacle to controlling infections in burn patients (1). Thus it is important to better understand the factors that predispose burn patients to infection and develop interventions to treat or prevent infections in this vulnerable population.

Recent studies from our laboratory demonstrate that both burn injury and associated wound sepsis causes significant adaptive immune system dysfunction (2). Our major findings included a significant loss of the blood lymphocytes and splenic T cells following burn injury and associated sepsis. Studies from other laboratories have also shown that cutaneous causes a suppression of splenic T cell cytokine production (3). Burn injury has also been shown to induce T cell apoptosis (4), a decreased ability of T cells to proliferate upon polyclonal and antigen specific stimulation, and to secrete Th1 cytokines, including interferon- γ (5). Additionally, antigen-presenting cell dysfunction after burns may also contribute to impaired adaptive immunity (6).

Human studies show that patients dying of sepsis have marked immunosuppression and low absolute lymphocyte counts are associated with higher mortality rates (7). Therefore, burn injury combined with sepsis is likely to severely compromise the ability to clear infections. Severe infections often lead to multi-organ injury, which contributes to the increased morbidity and mortality (8-10).

Fms-like tyrosine kinase-3 Ligand (Flt3L) represents a hematopoietic factor that can be used as an immunomodulatory agent. Flt3L is necessary of maintenance and expansion of early hematopoietic stem cells and it acts on a class III tyrosine kinase receptor, *Flt3R*, which is expressed predominantly on hematopoietic progenitor cells (including both myeloid and lymphoid) (11). Flt3L is typically a cell surface transmembrane protein that can also be proteolytically cleaved and released as a soluble protein. Both forms are biologically active (11). Flt3L has been shown to regulate the development of dendritic cells and a large expansion of dendritic cells has been observed in healthy human volunteers receiving Flt3L (12). Previous studies from our laboratory have demonstrated that one of the mechanisms by which Flt3L treatment confers protection against burn wound infection is the potentiation of innate immune responses (13-15). Specifically, we showed that Flt3L treatment causes a

potent expansion of splenic dendritic cells, which enhances neutrophil antimicrobial functions (13, 14). However, the effect of Flt3L on adaptive immune functions in burned and infected subjects has not been studied. A detailed analysis of CD4⁺ and CD8⁺ T cell function during burn injury and infection is also lacking. T cells are one of the critical effector cells of the adaptive immune system and T cell dysfunction contributes significantly to post-sepsis immunosuppression (7). Therefore, we sought to evaluate the effect of Flt3L on T cell functions during burn wound sepsis.

Materials and Methods

Mouse model of burn injury

All animal procedures were performed in accordance with the National Institutes of Health Guidelines and were approved by the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center. Ten to twelve week-old male BALB/c mice were purchased from ENVIGO (Indianapolis, IN). As described previously, a well-established mouse model of full-thickness cutaneous burn injury was used (2, 16). Buprenorphine (0.1 mg/kg, subcutaneously) was administered 30 minutes prior to burn injury for analgesia. Under general anesthesia (2.5 % isoflurane), the dorsal surface of mice was shaved and 1 mL normal saline was injected subcutaneously into the burn target area to prevent injury to the underlying tissues. An approximately 35 % total body surface area scald burn was induced by exposing the shaved dorsal area to 97-98°C water for 10 seconds, followed by intraperitoneal injection of 2 mL Lactated Ringers solution for fluid resuscitation. A second injection of buprenorphine (0.1 mg/kg, subcutaneously) was administered to all the mice at 8–12 hours after the burn procedure and continued twice daily after burn injury. The burn injured mice were housed individually in sterile cages and were provided sterile water and food. Sham mice underwent the same experimental procedure except that mice were exposed to room temperature water.

Burn Wound infection

The burn wound was inoculated topically with *Pseudomonas aeruginosa* obtained from American Type Culture and Collection (Manassas, VA; ATCC 19660). The culture was grown in tryptic soy broth and diluted in sterile saline solution prior to inoculation. On day 4 post burn, wound infection was induced by mice skin surface inoculation of 1×10^8 colony forming units (cfu) of *Pseudomonas aeruginosa*.

Flt3 Ligand (Flt3L) treatment protocol

Flt3L (10 µg in 200 µL sterile PBS with 0.1% bovine serum albumin) was administered via the intraperitoneal route daily starting one day post burn injury through day 4 and then every other day thereafter (Figure 1A). Recombinant Human Flt3L was purchased from Peprotech, Inc. The Flt3L dose was based on the previous studies from our laboratory which show that a 10 µg dose of Flt3L boosts innate immune cell functions and affords protection during burn wound infection (13, 14). An equivalent volume (0.2mL) of PBS (with 0.1% bovine serum albumin) was administered to the vehicle treated groups. Blood, spleen and wound draining lymph nodes were harvested on day 4 post burn (prior to infection and after three doses of Flt3L) and on day 2 following wound infection to assess lymphocyte numbers and

phenotype. Mice were monitored for 7 days post wound infection in survival studies. Figure 1A provides graphical representation of the Flt3L treatment time line.

Preparation of spleen and lymph node single cell suspensions

As described previously (2), single cell suspensions of splenocytes were prepared by gently pressing the spleen through 70 μ m cell strainer. The cells were centrifuged ($300 \times g$ for 10 minutes at 4°C) and red blood cells in the splenocyte pellet were lysed with Red Blood Cell Lysis Buffer (Sigma Life Sciences, St Louis, MO). Wound draining lymph nodes were dissociated by mincing. The cell counts, per spleen and pooled wound draining lymph nodes were measured using a TC20 cell counter (BioRad, Hercules, CA). Splenocytes and lymph node cells were then centrifuged ($300 \times g$ for 10 minutes at 4°C) and resuspended in PBS to achieve a concentration of 1×10^7 cells/mL, for further analysis using flow cytometry.

Flow cytometry

Leukocytes isolated from spleen and lymph nodes were resuspended in cold PBS (1×10^7 cells/mL) and incubated with 1 μ L/mL anti-mouse CD16/32 (eBiosciences, San Diego, CA) for 5 minutes to block non-specific Fc receptor-mediated antibody binding. One million cells were then transferred to polystyrene tubes and incubated with 0.5 μ g of fluorochrome-conjugated specific antibodies or isotype control antibodies (4°C, 30 minutes), followed by washing with 2 mL cold PBS and centrifugation ($300 \times g$ for 5 minutes). The cell pellet was then resuspended in 200 μ L cold PBS. Flow cytometry was performed using BD Accuri C6 instrument (BD Biosciences, San Diego, CA). Data were analyzed using Accuri C6 software. The following fluorochrome conjugated anti-mouse antibodies (eBiosciences, San Diego, CA) were used: anti-CD3-FITC, anti-CD4-PerCPCy5.5, anti-CD4-FITC, anti-CD8-PE, anti-CD8-FITC, anti-CD19-PE, anti-PD-1-FITC, anti-F4/80-FITC, anti-CD-28-APC, anti-Ly6C-PerCPCy5.5, anti-IFN γ -PE, anti-PD-L1-PE, anti-MHCII-Cy7, anti-CD11c-FITC, anti-CD80-PE, anti-CD86-APC, and respective isotype controls.

Measurement of bacterial counts

As described previously (2), serial dilutions of blood and lung tissue homogenates were grown on tryptic soy agar overnight to determine colony forming units (CFU) of *Pseudomonas aeruginosa* per ml of blood or per gram of tissue. Bacterial counts were measured on day 2 post wound infection.

Measurement of organ injury markers and complete blood count

Whole blood was harvested by carotid artery laceration under general isoflurane anesthesia. Blood was collected in K3EDTA tubes (Greiner Bio-One, Kremsmunster, Austria) and kept on ice until CBC and differential leukocyte counts analysis. CBC measurements were performed using a Forcyte veterinary hematology analyzer (Oxford Science, Oxford, CT). Remaining blood was centrifuged (4750 rpm for 15 minutes at 4°C) to collect plasma for measurement of liver injury markers – alanine aminotransferase (ALT) and aspartate amino transferase (AST); and kidney injury marker - blood urea nitrogen (BUN). These were measured using Vet Axcel Chemistry Analyzer in the Translational Pathology Shared Resource facility at Vanderbilt University.

Intracellular interferon-gamma (IFN- γ) measurement in CD4⁺ and CD8⁺ T cells

Single cell suspensions of splenocytes and lymph nodes were prepared as described above. One million cells were stimulated with PMA/Ionomycin (eBiosciences, San Diego, CA) for 5 hours at 37°C in RPMI media containing 10 % fetal calf serum, in a humidified incubator (5% CO₂, 95% air). Protein transport inhibitors (Brefeldin A+ Monesin) were added after one hour of incubation with PMA/Ionomycin (17). After 5 hours of stimulation with PMA/Ionomycin, the cells were harvested and labelled with fluorescence-conjugated antibodies to CD4 and CD8. Cells were then fixed and permeabilized with Cytotfix/Cytoperm Plus (BD Biosciences, 250 μ L / tube) for 20 minutes at 4°C, followed by washing with BD Perm/Wash solution. Anti-IFN γ -PE (clone XMG1.2, eBiosciences) was used to detect intracellular IFN γ production. Fluorochrome-conjugated isotype-specific IgGs served as controls. All samples were analyzed using an Accuri C6 flow cytometer (BD Biosciences, San Diego, CA). Data were analyzed using Accuri C6 software.

Survival Study

Following wound infection with *Pseudomonas aeruginosa*, mice were monitored at least three times a day for seven days (2). Additionally, the health of mice was also monitored by the animal care veterinary technicians on a daily basis. Mice were checked for signs of morbidity including decreased activity and response to stimuli combined with abnormal posturing and labored breathing. Core body temperature was also measured using a lubricated rectal temperature probe and digital thermometer (Physitemp Instruments INC., Clifton, NJ, USA), as an indicator of morbidity (8, 17). Mice displaying signs of morbidity and/or body temperature below 28°C, were humanely euthanized under isoflurane anesthesia as non-survivors. To assure proper euthanasia, the unconscious mice underwent cervical dislocation and mortality was confirmed.

Statistical analysis

Graph preparations and data analyses were performed using Prism 6.0 (GraphPad Software Inc., San Diego, CA). Data with three or more groups were analyzed using a one-way ANOVA followed by the Tukey multi-comparison post-hoc test. Comparison between two groups was analyzed using Students T test. All data values are presented as mean $\pm$ SEM, except for bacterial counts, for which median values are designated. The median values derived from bacterial clearance studies were evaluated using the non-parametric Mann Whitney test. A value of $p < 0.05$ was considered statistically significant for all experiments. Survival curves were analyzed using a Mantel-Cox log rank test.

Results

Flt3L improves systemic bacterial clearance, attenuates organ injury and improves survival following burn wound infection

Figure 1A describes the protocol used in all experiments. Mice received a cutaneous scald burn on day 0 and were treated with Flt3L or vehicle on days 1-4, 6, 8 and 10. *Pseudomonas aeruginosa* was applied to the burn wound on day 4 after burn injury (Figure 1A). This time point of inoculation represents a clinical scenario wherein patients typically develop

infection after burn injury (18). Following burn wound inoculation, the bacteria gradually colonize the injured area and spread systemically into surrounding tissues, blood and eventually to distant organs, namely the lungs (13). Immunological measurements were performed on days 4 and 6 post-burn in some mice and another cohort was followed for survival out to day 11. As shown in Figures 1B and 1C, significant numbers of *Pseudomonas* were present in the blood and lungs on day 2 after wound infection, demonstrating systemic spread. Flt3L significantly reduced the bacterial load in blood and lungs at day 2 post wound infection (Figure 1B and 1C). Flt3L also protected against kidney injury as indicated by reduction in the infection- induced rise in serum BUN concentrations (Figure 1D). Burn wound sepsis also led to a significant increase in serum ALT and AST concentrations, as compared to sham or burn only mice, implying liver injury, which were attenuated by FLT3L treatment (Figure 1E and 1F). Our model of burn wound sepsis caused 100% mortality in vehicle treated mice. Flt3L significantly improved survival (Figure 1G).

Burn injury plus wound infection causes a significant decline in blood leukocyte counts, which is attenuated by Flt3L treatment

Burn injury alone induced a significant decrease in blood absolute lymphocyte count, but not total white blood cell or blood neutrophil counts, on day 4 post-burn compared to sham controls (Figure 2A-2C). Flt3L treatment caused a further decline in lymphocyte count in burned mice but did not significantly affect total white blood cell or neutrophil counts (Figures 2A-2C). Burn wound sepsis caused further decreases in the total white blood cell, absolute lymphocyte and neutrophil counts in vehicle- treated mice, which were attenuated by Flt3L treatment (Figures 2A-2C).

Flt3L treatment attenuates decreased splenic CD4⁺ and CD8⁺ counts caused by burn and infection

As shown in Figures 3A-D, burn wound sepsis, but not burn injury alone, caused CD4⁺ and CD8⁺ T cell counts to significantly decrease in spleen and wound draining lymph nodes as compared to sham mice. Flt3L treatment significantly protected against the decline in CD4⁺ and CD8⁺ T cell counts in the spleen but not in wound draining lymph nodes (Figure 3A-3D).

Flt3L treatment preserves T cell IFN γ secretion in the spleen following burn wound sepsis

Measurement of IFN γ production offers a way to measure functional status of T cells (19). Although wound infection did not alter CD4⁺ T cell IFN γ production in spleen, Flt3L treatment significantly augmented CD4⁺ T cell IFN γ production in spleen. (Figure 4A). Burn wound sepsis caused a significant decline in the CD8⁺ T cell IFN γ production both in the spleen and the wound draining lymph nodes (Figure 4B and 4D) as compared to sham and burn alone groups. Flt3L treatment attenuated the decline in CD8⁺ T cell IFN γ production both in spleen and wound draining lymph nodes (Figure 4B and 4D).

Flt3L treatment attenuates the decline in CD28 expression on CD4⁺ and CD8⁺ T cells in the spleen and wound draining lymph nodes during burn wound sepsis

CD28 is a co-stimulatory molecule that interacts with the CD80/86 molecules on antigen presenting cells and is required for effective T cell activation (20). Burn injury alone caused a significant decline in CD28 expression on both CD4⁺ and CD8⁺ T cells in the spleen but not lymph nodes (Figures 5A-5D). Burn wound sepsis caused a significant decline in CD28 expression on the CD4⁺ and CD8⁺ T lymphocytes in the spleen and wound draining lymph nodes, as compared to sham and burn injured mice group (Figure 5A-5D). Flt3L treatment attenuated the decline in CD28 expression on both CD4⁺ and CD8⁺ T cells in the spleen and lymph nodes in mice with burn wound sepsis.

Burn wound infection or Flt3L treatment does not significantly alter the expression of Programmed Death 1 (PD-1) on T cells

Programmed death 1 receptor (PD-1) expressed primarily on T cells, is known to downregulate T cell functions during sepsis (21-24). As shown in Figure 6, burn injury alone or combined with wound infection (with or without Flt3L treatment) did not alter the expression levels of PD-1 on CD4⁺ or CD8⁺ T cells (Figure 6A - 6D). Flt3L treatment significantly decreased PD-1 expression on CD4⁺ T cells in mice receiving burn injury alone or burn plus infection (Figure 6A and 6C).

Flt3L treatment stimulates dendritic cell expansion and attenuates increased PD-L1 expression on splenic dendritic cells during burn wound sepsis

Burn injury alone or combined with wound infection did not affect CD11c⁺ dendritic cell numbers in the spleen (Figure 7A). Whereas, in the lymph nodes, burn injury alone caused to a significant decline in the CD11c⁺ dendritic cell counts as compared to sham controls (Figure 7D). Burn wound infection caused a further decline in the dendritic cell counts in the lymph nodes, with no change in the spleen (Figure 7A and 7D). In both spleen and lymph nodes, Flt3L treatment lead to a significant expansion of splenic dendritic cells in mice with burn alone or with wound infection and prevented the infection-induced decline in dendritic cell counts in the lymph nodes (Figure 7A and 7D). Burn wound sepsis caused a significant upregulation of cell surface PD-L1 expression on dendritic cells in the spleen, which was attenuated by Flt3L treatment (Figure 7B). Wound infection also caused a significant decline in MHCII expression on dendritic cells in the spleen, which was attenuated by Flt3L treatment (Figure 7C). PD-L1 and MHCII expression on dendritic cells in the lymph node cells remained unchanged in all the groups (Figure 7E and 7F).

CD80 and CD86, expressed by antigen presenting cells such as dendritic cells provide co-stimulatory signals necessary for effective T cell activation through interaction with CD28 (25). Burn injury and wound infection caused a significant increase in CD80 expression by dendritic cells, both in the spleen and lymph nodes (Figure 8A and 8C), which was attenuated by Flt3L treatment. On the other hand, burn injury and wound infection did not alter CD86 expression on dendritic cells in both spleen and the lymph node cells (Figure 8B and 8D).

Flt3 ligand treatment attenuates the increase in PD-L1 expression on macrophages and inflammatory monocytes in the spleen during burn wound sepsis

Burn wound sepsis caused a significant decline in the F4/80⁺ macrophage cell counts in the spleen as compared to sham and burn injured mice, with no change in the Ly6C⁺ inflammatory monocyte cell counts (Figure 9A and 9C). Flt3L treatment lead to a significant increase in the macrophage and inflammatory monocyte cell counts in the spleen post wound infection (Figure 9A and 9C). Burn wound sepsis caused a significant upregulation of surface PD-L1 expression on macrophages in the spleen and a trend towards increase in the inflammatory monocytes (not statistically significant), as compared to sham and burn injured mice groups (Figure 9B and 9D). Flt3L attenuated the increase in PD-L1 expression on both macrophages and inflammatory monocytes following burn wound sepsis (Figure 9B and 9D).

Discussion

Sepsis is the major cause of death among burn patients (26). A study by Mann *et al.* showed that burn patients have a very high prevalence of sepsis and associated poor outcomes (27). T cell depletion and dysfunction have been shown to be a major contributing factor to increased mortality in non-burned septic patients and immunosuppression caused by adaptive immune system dysfunction is increasingly being recognized as a significant phenomenon during sepsis (7). Therefore, immunomodulatory therapies that augment adaptive immune functions may have benefit in preventing and/or treating sepsis (28, 29). The impact of burn wound sepsis on T cell function has not been studied in detail. Our current study reveals that burn wound sepsis causes a significant decline in lymphocyte counts in peripheral blood, spleen and burn wound-draining lymph nodes, which were attenuated by treatment with Flt3L. Flt3L treatment also augmented T cell IFN γ production, facilitated expansion of dendritic cells, macrophages and monocytes as well as blunted sepsis-induced expression of PD-L1 by antigen presenting cells. These findings were associated with improved bacterial clearance, attenuated organ injury and augmented survival in mice treated with Flt3L.

We previously reported that burn injury and associated wound sepsis causes a significant decline in the circulating and splenic lymphocytes (2). In accordance with our previous findings, the present study demonstrates that burn injury alone and burn wound sepsis cause a significant decline in blood, splenic and lymph node T cells. The blood lymphocyte count was higher in the burn alone (non-infected) group as compared to the wound infected Flt3 ligand treated group. The blood lymphocyte counts might not be a good indicator of overall lymphocyte function *in vivo*. Organs such as spleen and lymph nodes serve as the major reservoir of lymphoid cells and the results seen in these organs are more reflective of the lymphocyte status *in vivo*. These organs also serve as the source of lymphocytes, which are trafficked to the sites of infection, upon antigen presenting cell stimulation. Our data shows that Flt3 Ligand treatment improves T cell function in these critical lymphoid organs and affords protection against burn wound sepsis. One of the mechanisms of immunosuppression during sepsis is known to be T cell apoptosis (7). Animal models have been shown to increase apoptotic cells in lymphoid tissues such as thymus, spleen and intestinal Peyer's

patches as early as 6 hours after burn injury (4). However, it is unclear if lymphocyte depletion and dysfunction increase host susceptibility to burn wound sepsis. We showed that burn injury and wound infection causes a significant decline in the CD4⁺ and CD8⁺ T cell function, as demonstrated by impaired IFN γ production, reduced cell surface CD28 expression and increased PD-1 expression (Figure 4 and 5). These findings were associated with significant systemic bacterial dissemination, liver and kidney injury and mortality. Treatment with Fms like tyrosine kinase-3 ligand (Flt3L) attenuated T cell dysfunction, improved systemic bacterial clearance, reduced organ injury and improved survival following burn wound sepsis.

Flt3L is known to mediate the expansion of hematopoietic progenitor cells (11). *In situ*, Flt3L protein is predominantly found in T cells and stromal fibroblasts of the bone marrow microenvironment and numerous studies have shown that Flt3L causes expansion of dendritic cells and acts as a dendritic cell growth factor (11). Previous studies from our laboratory have predominantly studied the alterations in the innate immune responses following burn wound infection and showed that Flt3L treatment improves survival during burn wound sepsis via expansion of dendritic cells (13) and modification of neutrophil responses (14). To our knowledge, the present study is the first to evaluate the ability of Flt3L to improve T cell function during burn wound sepsis.

Positive co-stimulatory signal through cell surface CD28 is required for optimal functioning of T cells and numerous studies have revealed that CD28 ligation is required for T cell proliferation, cell survival and production of IFN γ and interleukin-2, which are critical to combat infections (30). Inadequate CD28 co-stimulation causes T cell anergy and dysfunction. Previous studies show that CD28 expression is decreased in animal models of non-burn related sepsis and in patients with sepsis, causing T cell dysfunction (31). Our study is the first to report that burn injury and associated sepsis causes a significant decline in CD28 expression on CD4⁺ and CD8⁺ T cells in the spleen and wound draining lymph nodes. We hypothesize that reduced CD28 expression is one of the potential mechanisms leading to T cell dysfunction during burn wound sepsis. Flt3L treatment significantly increased CD28 expression on CD4⁺ and CD8⁺ T cells in the spleen and lymph nodes of septic mice compared to vehicle-treated mice. Flt3L thereby restored co-stimulatory signals to T cells and potentially preserved optimal T cell functioning.

Activated CD4⁺ and CD8⁺ T cells, along with NK cells, are known to be the predominant producers of IFN γ (32). IFN γ is a key cytokine responsible for activation of monocytes and macrophages which are required for microbial elimination during sepsis (33). Sepsis is known to produce a defect in T cell IFN γ production and previous studies using non-burn murine models of sepsis show that CD4⁺ and CD8⁺ splenocytes produce low levels of IFN γ as compared to non-septic controls (28). Therapies that restore T cell IFN γ production have been shown to improve survival in animal models of sepsis (28). Our results show that burn wound sepsis causes a marked decline in IFN γ production by CD8⁺ T cells in both the spleen and lymph nodes, implying functional impairment of CD8⁺ T cells. Flt3L treatment significantly restored the CD8⁺ T cell IFN γ production in the spleen and lymph nodes, and boosted the basal IFN γ production in CD4⁺ T cells. We hypothesize that preservation of

IFN γ production in Flt3L-treated mice is a potential mechanisms of Flt3L-induced protection of T cell function during burn wound sepsis.

Dendritic cells, macrophages and monocytes form the first line of defense against infections. Dendritic cells play a critical role in antigen presentation to T cells. Immature dendritic cells recognize invading microorganisms through toll-like receptors and other pattern recognition receptors, followed by migration into lymphoid organs such as lymph nodes where they present the captured antigens to T cells and facilitate effective T cell function (34). Our results show that burn injury and sepsis cause a significant decline in dendritic cell counts in the wound draining lymph nodes, with no change in the spleen. Importantly, Flt3L treatment prevented the decline in dendritic cell numbers in the lymph nodes and significantly increased dendritic cell numbers in the spleen during burn wound sepsis. These results are in agreement with previous studies showing that Flt3L treatment expands dendritic cell populations (13). Flt3L treatment also augmented macrophage and monocyte counts in the spleen post burn wound infection. The combined expansion of these myeloid populations may contribute to the improved antimicrobial immunity observed in mice treated with Flt3L.

Optimal antigen processing and presentation by dendritic cells requires dendritic cell activation and maturation characterized by increased levels of expression of major histocompatibility complex (MHC) molecules and T cell co-stimulatory molecules such as CD80/CD86 (34). MHC II molecules are required for effective presentation of foreign pathogens to CD4⁺ and CD8⁺ T cells for effective immune response against infection. Dendritic cell MHC II expression has been shown to be decreased in non-burn related sepsis (29). Results from the present study show that burn wound sepsis causes a significant decline in MHC II expression on splenic dendritic cells, which was partially, but significantly attenuated by Flt3L treatment. Although, MHC II expression was decreased on splenic dendritic cells post burn wound sepsis, the expression of CD80 was increased following burn wound sepsis and Flt3L treatment tended to decrease it, although CD80 remained significantly higher than the sham control group in both the spleen and lymph nodes. One possible explanation for this observation is that Flt3L caused a significant expansion of immature dendritic cells and was unable to completely restore MHC II and CD80 expression. We did not observe any change in CD86 expression in spleen and lymph nodes post burn wound sepsis with or without Flt3L. Nevertheless, as discussed above, expression of CD28, the T cell co-stimulatory binding partner of CD80/86, was significantly lower on CD4⁺ and CD8⁺ T cells post burn wound sepsis. Therefore, it is possible that the uneven expression levels of CD28 on T cells and CD80 on dendritic cells ultimately negatively impact the T cell functions during burn wound sepsis.

The interaction between Programmed Death Ligand 1 (PD-L1) on innate immune cells and Programmed Death Receptor 1 (PD-1) on T cells is another mechanism known to downregulate T cell functions (31). Both animal and human studies have shown increased expression of PD-1 and PD-L1 during sepsis and associated T cell dysfunction (23, 35). Previous studies have indicated that increased PD-L1 expression is associated with monocyte dysfunction, lymphopenia, increased T cell apoptosis and T cell dysfunction. Blockade of PD-L1 was able to reverse these effects to varying degrees (24). Our results show that burn wound sepsis causes a significant upregulation of PD-L1 expression on

splenic dendritic cells and macrophages and a trend towards increased expression on inflammatory monocytes. Therefore, augmented PD-L1 expression on antigen presenting cells might be another mechanism contributing to T cell dysfunction during burn wound sepsis.

In conclusion, our results demonstrate that burn injury and associated wound infection caused T cell depletion and dysfunction. As discussed above, the potential mechanisms include reduced co-stimulatory CD28 expression on T cells, impaired cytokine (IFN γ) secretion by T cells and increased T cell inhibitory PD-L1 expression on antigen presenting cells such as dendritic cells and macrophages. Burn wound sepsis also causes a significant depletion of dendritic cells and macrophages in the spleen and reduced expression of MHC II on dendritic cells in the spleen, which might negatively impacts T cell activation. Therefore, burn wound sepsis impairs T cell functions both directly and indirectly leading to impaired adaptive immune responses and increased susceptibility to infection. Flt3 ligand treatment not only attenuated T cell loss and dysfunction in the spleen and wound draining lymph nodes, but also expanded dendritic cells and macrophages in the spleen, prevented dendritic cell depletion in the lymph nodes and significantly decreased PD-L1 expression on dendritic cells and macrophages. Furthermore, Flt3L treatment significantly improved survival post burn wound sepsis. Therefore, Flt3L not only acts to improve T cell function but also facilitates an effective antigen presenting cell phenotype. Our results also indicate that coordinated actions of innate and adaptive immune system are required to decrease mortality in burn-injured patients. Flt3L certainly holds significant therapeutic potential. CellDex Therapeutics (a pharmaceutical company which holds the proprietary rights to this compound), is already testing Flt3 Ligand in clinical trials for potential applications in combination immunotherapy for lymphoma, and autologous and allogeneic hematopoietic stem cell transplantation (related abstracts were presented by CellDex Therapeutics scientists and collaborators at the American Association for Cancer Research Conference 2016, and the American Society for Blood and Marrow Transplant Conference 2016). CellDex Phase 1 clinical trial studies have also shown safety profile for Flt3 Ligand (data presented at the American Society of Hematology Conference 2012). Therefore, the therapeutic potential of Flt3L to improve T cell function and survival during burn wound sepsis merits further investigation.

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Figure 1. Flt3L treatment protects against burn wound infection induced organ damage and improves survival

(The X axis symbols indicate the following: S = Sham (no burn), Veh = Vehicle treatment, Flt3 = Flt3 Ligand treatment). (A) Time line depicting the burn injury and wound infection model along with Flt3L treatment (10 µg via i.p route starting a day after burn injury). To evaluate the systemic spread of infection, *Pseudomonas aeruginosa* colony counts were measured on day 2 post wound infection and the graphs depict colony forming units of the bacteria in the blood (B) and the lungs (C). Following parameters were measured on day 4 post burn injury and day 2 post burn wound infection – (D) serum blood urea nitrogen, BUN; (E) serum alanine aminotransferase, ALT; and (F) serum aspartate amino transferase, AST. n=8-10 in each group and P<0.05. (G) Survival study: Three groups of mice including - burn injured alone (no infection, black line); burn injured and vehicle treated wound infected mice (blue line); and burn injured and Flt3L treated wound infected mice (red line) were monitored for survival for seven days post *Pseudomonas aeruginosa* wound inoculation. n=10 in each group. *significantly different from sham (S) group; # significantly different from vehicle treated burn group (no infection); \$ significantly

different from vehicle treated burn wound infection group. For survival curve, ** represents significantly different as compared to vehicle treated group.

Figure 2. Burn wound infection caused a significant decline in the blood absolute lymphocyte counts

(The X axis symbols indicate the following: S = Sham (no burn), Veh = Vehicle treatment, Flt3 = Flt3 Ligand treatment). (A) Total white blood cell counts. (B) Blood lymphocyte counts. (C) Blood neutrophil counts. n=8-10 in each group. *significantly different from sham (S) group; # significantly different from vehicle treated burn group (no infection); \\$ significantly different from vehicle treated burn wound infection group.

Figure 3. Effect of burn injury and wound infection with or without Flt3L treatment on CD4⁺ and CD8⁺ T lymphocyte cell counts
 (The X axis symbols indicate the following: S = Sham (no burn), Veh = Vehicle treatment, Flt3 = Flt3 Ligand treatment). Upper panel depicts cell counts in the spleen and lower panel depicts cell counts in the wound draining lymph nodes. Flow cytometry was used to measure the individual CD4⁺ (A, C) and CD8⁺ T (B, D) cell counts. n=10-15 in each group and P<0.05. *significantly different from sham (S) group; # significantly different from vehicle treated burn group (no infection); \$ significantly different from vehicle treated burn wound infection group.

Figure 4. Effect of burn injury and wound infection with or without Flt3L treatment on IFN γ production by CD4⁺ and CD8⁺ T cells

(The X axis symbols indicate the following: S = Sham (no burn), Veh = Vehicle treatment, Flt3 = Flt3 Ligand treatment). IFN γ production was used as a marker for T cell function. Intracellular IFN γ was measured after T cell stimulation with PMA/ionomycin. Upper panel depicts data for spleen cells and lower panel depicts data for wound draining lymph nodes. Percentage of intracellular IFN γ expressing CD4⁺ (A and C) and CD8⁺ T cells (B and D) was measured in the spleen and lymph nodes. n=7-10 in each group and P<0.05.

*significantly different from sham (S) group; # significantly different from vehicle treated burn group (no infection); \$ significantly different from vehicle treated burn wound infection group.

Figure 5. Effect of burn injury and wound infection with or without Flt3L treatment on CD28 expression by CD4⁺ and CD8⁺ T cells

(The X axis symbols indicate the following: S = Sham (no burn), Veh = Vehicle treatment, Flt3 = Flt3 Ligand treatment). Upper and lower panel show the percentage of CD28 expression on CD4⁺ and CD8⁺ T cells in the spleen and lymph nodes respectively. The graphs **A** and **C** depict CD28 expression on CD4⁺ T cells in the spleen and lymph nodes respectively; and the graphs **B** and **D** depict CD28 expression on CD8⁺ T cells in the spleen and lymph nodes respectively. n=10-15 in each group and P<0.05. *significantly different from sham (S) group; # significantly different from vehicle treated burn group (no infection); \$ significantly different from vehicle treated burn wound infection group.

Spleen

Lymph node

Figure 6. PD-1 expression levels on T cells following burn injury and wound infection with or without Flt3L treatment

(The X axis symbols indicate the following: S = Sham (no burn), Veh = Vehicle treatment, Flt3 = Flt3 Ligand treatment). Upper and lower panel show the percentage of PD-1 expression on CD4⁺ and CD8⁺ T cells in the spleen and lymph nodes respectively. **A** and **C** show the percentage of PD-1 expression on CD4⁺ T cells in the spleen and lymph nodes respectively; and the graphs **B** and **D** depict the percentage of PD-1 expression on CD8⁺ T cells in the spleen and lymph nodes respectively. n=8-12 in each group and P<0.05.

*significantly different from sham (S) group; # significantly different from vehicle treated burn group (no infection); \$ significantly different from vehicle treated burn wound infection group.

Spleen

Lymph node

Figure 7. Effect of burn injury and wound infection with or without Flt3L treatment on CD11c⁺ dendritic cell counts and phenotype
 (The X axis symbols indicate the following: S = Sham (no burn), Veh = Vehicle treatment, Flt3 = Flt3 Ligand treatment). Figures **A** and **D** show the CD11c⁺ dendritic counts in spleen and lymph nodes respectively; Figures **B** and **E** show the percentage of PD-L1 expression on CD11c⁺ dendritic counts in spleen and lymph nodes respectively; and **C** and **F** depict the percentage of MHCII expression on CD11c⁺ dendritic counts in spleen and lymph nodes respectively. n=8-10 in each group and P<0.05. *significantly different from sham (S) group; # significantly different from vehicle treated burn group (no infection); \$ significantly different from vehicle treated burn wound infection group.

Spleen

Lymph node

Figure 8. CD80 and CD86 expression levels on CD11c⁺ dendritic cells following burn injury and wound infection with or without Flt3L treatment

(The X axis symbols indicate the following: S = Sham (no burn), Veh = Vehicle treatment, Flt3 = Flt3 Ligand treatment). Figures **A** and **C** show the percentage of CD80 expression on CD11c⁺ dendritic cells in the spleen and lymph nodes respectively; and the figures **B** and **D** depict the percentage of CD86 expression on CD11c⁺ dendritic cells in the spleen and lymph nodes respectively. n=8-12 in each group and P<0.05. *significantly different from sham (S) group; # significantly different from vehicle treated burn group (no infection); \$ significantly different from vehicle treated burn wound infection group.

Figure 9. PD-L1 expression levels on F4/80⁺ macrophages and inflammatory monocytes following burn injury and wound infection with or without Flt3L treatment

(The X axis symbols indicate the following: S = Sham (no burn), Veh = Vehicle treatment, Flt3 = Flt3 Ligand treatment). The bar graphs **A** and **C** show the F4/80⁺ macrophage cell counts and Ly6C⁺ inflammatory monocyte cell counts in the spleen respectively; and the graphs **B** and **D** depict the percentage of PD-L1 expression on F4/80⁺ macrophages and Ly6C⁺ inflammatory monocytes in the spleen respectively. n=8-12 in each group and P<0.05. *significantly different from sham (S) group; # significantly different from vehicle treated burn group (no infection); \$ significantly different from vehicle treated burn wound infection group.