Stimulation of Hematopoiesis by the Fms-Like Tyrosine Kinase 3 Ligand Restores Bacterial Induction of Th1 Cytokines in Thermally Injured Mice

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Patients with large burn injuries are susceptible to opportunistic infections due to impaired functions of multiple effector cells of innate immunity and acquired immunity, including macrophages, dendritic cells (DC), natural killer (NK) cells, and T cells. The ability of a host to produce Th1 cytokines, such as gamma interferon (IFN- γ) and interleukin-12 (IL-12), upon infectious challenge is also impaired after burn injury. Stimulation of hematopoiesis, to regenerate new immune cells, may be an effective strategy for improving resistance to infections after severe burn trauma. Fms-like tyrosine kinase 3 ligand (Flt3L) is a hematopoietic cytokine that stimulates the expansion and differentiation of NK cells and DC. Using a mouse model, we tested the hypothesis that Flt3L treatments after burn injury stimulate the production of functional effector cells of innate immunity and restore appropriate Th1 cytokine responses to *Pseudomonas aeruginosa*, a common source of pneumonia and wound infections in burn victims. Flt3L increased splenic cellularity in sham (uninjured) and burned mice and increased the numbers of NK cells (DX5⁺) and DC (CD11c⁺). In response to *P. aeruginosa*, significant increases in the serum IFN- γ levels and the numbers of splenic IFN- γ -producing DC, NK cells, and T cells were observed in Flt3L-treated burned mice compared to the values obtained for untreated burned mice. The splenic levels of IL-12 and IL-15 mRNAs and the IL-12 and IL-15 receptors were also increased. In addition, Flt3L treatment restored the ability of splenic cultures prepared from burned mice to produce IFN- γ and IL-12 after in vitro challenge with *P. aeruginosa*. Flt3L may have potential for restoring NK cell and DC functions and improving immunity after burn injury.

Fms-like tyrosine kinase 3 ligand (Flt3L) is a cytokine that stimulates the expansion and differentiation of hematopoietic progenitor cells to generate mature immune cells of both myeloid and lymphoid lineages. Flt3L interacts with a tyrosine kinase receptor, referred to as Fms-like tyrosine kinase 3 (Flt3) or fetal liver kinase 2 (flk-2). Although Flt3L is ubiquitously expressed in human and mouse tissues, its receptor is expressed almost exclusively in progenitor and stem cells in the bone marrow, thymus, and spleen, and it is not present on mature cells, mast cells, or erythroid progenitors (16). Flt3L has been shown to promote the expansion of dendritic cells (DC), natural killer (NK) cells, and (to a lesser extent) B and T lymphocytes. Transgenic mice lacking Flt3L have reduced numbers of bone marrow progenitor cells, NK cells, and myeloid- and lymphoid-related DC (28). In vivo administration of Flt3L to normal mice for 5 days causes a dramatic increase in the number of progenitor cells in the bone marrow, spleens, and peripheral blood of the mice. By 10 days of Flt3L administration, there are significantly greater numbers of progenitors, as well as mature DC, NK cells, NK/T cells, and T cells in the spleens, liver, and peripheral blood. These expanded populations are functional and responsive to appropriate stimuli (3, 44). Flt3L treatment has been demonstrated to significantly increase the resistance of mice to lethal infections with herpes

simplex virus, with decreased amounts of latent virus in neurons and elevated numbers of hepatic and splenic NK lymphocytes and DC (46). The effects of Flt3L are transient, and hematopoietic parameters begin to return to baseline levels within 1 week of withdrawal (6). Administration of Flt3L to human volunteers results in dramatic increases in the number of DC in peripheral blood, and no toxicity is observed (27). These characteristics suggest that Flt3L may have the potential to enhance immune function in patients with inadequate immunity. Clinical trials are being used to investigate the potential of Flt3L as an agent to treat immunologically responsive cancers (12, 39). Flt3L may also be beneficial to burn patients who need enhanced immunity against infectious organisms.

Thermal injury has effects on the immune system that leave burn victims susceptible to infection with opportunistic organisms (41). A multitude of immunological alterations have been reported to occur both in burned patients and in animal models of burn injury. These alterations include depressed NK cell activity (5, 23, 45), decreased T-cell proliferation, and altered cytotoxic T-lymphocyte activities (20, 21, 47). Additionally, impairments in macrophage and neutrophil functions have been reported (9, 25, 40). Burn injury also causes significant alterations in cytokine profiles. Specifically, induction of the Th1-promoting cytokine interleukin-12 (IL-12) and the Th1 cytokines gamma interferon (IFN- γ) and IL-2 is suppressed (33, 34). IFN- γ and IL-12 are critical for producing adequate antimicrobial responses during innate immunity. Specifically, NK cell-derived IFN- γ activates neutrophils and macrophages, induces production of phagocytic reactive oxygen species, and is

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involved in activation of the alternative complement cascade (19, 42, 52). The critical role of NK cell-derived IFN- γ has been demonstrated in mice that have been either genetically or transiently depleted of NK cells or IFN- γ (11). NK cell or IFN- γ loss results in rapid bacterial growth and spread of infection at early times after infection (13). NK cell-derived IFN- γ not only activates macrophages for enhanced killing activity but also stimulates IL-12 production (4). Macrophage- and DC-derived IL-12 further enhances IFN- γ production by NK cells (8, 15). This feedback loop amplifies innate immunity to prevent the spread of infection and also stimulates acquired immune responses (1). Therefore, early induction of IFN- γ and IL-12 in vivo is a useful marker of host innate immune responses to microbial infection.

We tested the hypothesis that Flt3L treatments after burn injury expand populations of effector cells of innate immunity and restore early IFN- γ and IL-12 production upon bacterial challenge. We report that Flt3L treatment of burned mice expands NK cell and DC populations in the spleen. Additionally, IFN- γ production and IL-12 production after bacterial challenge are enhanced in Flt3L-treated mice with burn trauma.

MATERIALS AND METHODS

Burn model. All protocols were approved by the Institutional Animal Care and Use Committee and met National Institutes of Health guidelines for the care and use of experimental animals. Female BALB/c mice that were 6 to 8 weeks old (20 to 22 g) were housed in an institutional animal care facility and were allowed to acclimate for 1 week prior to use. Prior to burn injury, mice were anesthetized with 2.5% isoflurane, and the backs and sides were shaved and covered with a Zetex cloth containing a rectangular opening corresponding to 40% of the mouse total body surface area (TBSA). The body surface area was calculated based on weight by using a constant for mice as described previously (49). A full-thickness flame burn was induced by using a Bunsen burner applied to the exposed skin. The full thickness of the injury was confirmed by loss of coloration and lack of bleeding upon incision (49). Additionally, histological analysis of burn wound sections showed a disorganized epithelium, loss of hair follicles, and coagulation of proteins and inflammatory infiltrate deep in the skin in the panniculus carnosus (data not shown). Fluid resuscitation was administered immediately by intraperitoneal injection of 3 ml of lactated Ringer's solution (LR). Buprenorphine (2 mg/kg) was given for analgesia. As controls, sham mice were anesthetized, shaved, and received buprenorphine and 1 ml of resuscitation fluid. Mice were allowed to recover and were provided food and water ad libitum.

Flt3L treatments. Recombinant human Flt3L was provided by Immunex Corporation (Seattle, Wash.). Flt3L (10 μ g in 1 ml of LR) was given by intraperitoneal injection once daily for 5 or 10 days. It has been established that 10 μ g/mouse/day for 10 days is the optimal dose for maximum stimulation of NK cell and DC population expansion and differentiation (26, 44). Mice that received control treatments were injected with 1 ml of LR once daily for 5 or 10 days. Burned mice were started on the treatment regimen within 2 h after injury.

Bacterial challenge. *Pseudomonas aeruginosa* strain 1244 was grown to the log phase in tryptic soy broth, and numbers of CFU were determined by plating serial dilutions on tryptic soy agar. Heat inactivation was performed by incubation at 56°C for 1 h. Bacterial killing was confirmed by lack of growth upon plating of heat-killed organisms on tryptic soy agar. For immunological challenge, mice received an intraperitoneal injection of 10^9 CFU of heat-killed *P. aeruginosa* (HKPA). Mice were euthanized by cervical dislocation within 6 h of bacterial challenge.

Splenocyte preparation. Spleens were aseptically harvested and placed in RPMI 1640 supplemented with 10% fetal bovine serum, and the cells were dispersed with the plunger of a 1-ml syringe. The homogenate was passed through a nylon mesh strainer, and red blood cells were lysed (erythrocyte lysis kit; R&D Systems, Minneapolis, Minn.). Splenocytes were pelleted by centrifugation at $1,000 \times g$ and 4°C for 5 min and counted in phosphate-buffered saline with a hemocytometer. The concentration of splenocytes was adjusted to 10^7 cells/ml in phosphate-buffered saline for antibody staining or in RPMI 1640 for cell culturing.

Flow cytometry. Splenocytes (1×10^6 cells in 0.1 ml of phosphate-buffered saline) were incubated with 0.5 μ g of fluorescein isothiocyanate-conjugated antibodies against specific cell surface markers for 30 min at 4°C, washed in phosphate-buffered saline, and then fixed and permeabilized for intracellular cytokine staining by incubation in Cytofix/Cytoperm solution (BD Pharmingen, San Diego, Calif.) for 10 min at 4°C. Cells were washed twice in PermWash (BD Pharmingen) and then incubated with phycoerythrin-conjugated antibodies (0.5 μ g) specific for IFN- γ for 30 min at 4°C, washed in phosphate-buffered saline, and fixed in 1% paraformaldehyde. Cells were analyzed with a FACSort flow cytometer (Becton Dickinson), and specific staining was determined by comparison with appropriate antibody isotype controls. Antibodies to DX5, IFN- γ , and CD11c were purchased from BD Pharmingen, and CD3 antibodies were obtained from Caltag Laboratories (Burlingame, Calif.). Absolute sizes of specific cell populations were determined by multiplying the total number of cells per spleen by the percentage of splenocytes detected by positive staining with specific antibodies. DC were analyzed by gating on cells with high forward light scatter (see Fig. 5B) and high CD11c expression, as described previously (15, 51).

Cell culture. For overnight stimulation, 1×10^6 cells were cultured in 96-well plates in RPMI 1640 supplemented with 10% fetal calf serum in the presence of HKPA (2×10^6 CFU), IL-15 (1 ng/ml), IL-18 (1 ng/ml), or IL-12 (10 ng/ml). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. IFN- γ levels in media and mouse sera were measured by an enzyme-linked immunosorbent assay (ELISA) (eBiosciences, San Diego, Calif.). IL-12 ELISA kits and recombinant cytokines were purchased from R&D Systems.

RPA. Spleens were harvested and flash frozen in liquid nitrogen, and total RNAs were later isolated by using Tri-Reagent (Molecular Research Center, Cincinnati, Ohio). Specific mRNAs were detected by using the Riboquant system (BD Pharmingen). Briefly, 15 μ g of RNA was hybridized with radiolabeled RNA probes generated from template sets mCK-1, mCK-2b, mCR-1, and mCR-3, and RNase protection assay (RPA) reactions were performed according to the manufacturer's instructions. Protected RNA species were separated by electrophoresis at 50 W on 5% polyacrylamide denaturing gels in 0.5 $\times$ Tris-borate-EDTA buffer. The gels were dried and exposed to autoradiography film. The presence of equivalent amounts of RNA in all samples was verified by using the L32 housekeeping gene transcript.

Statistical analyses. An unpaired, two-tailed Student's *t* test was used to compare two groups, and Welch's correction was applied when unequal variances were detected by an *F* test. Multiple groups were analyzed by analysis of variance, followed by a Tukey-Kramer multiple-comparison test. A *P* value of 0.05 or less was considered significant. All statistical analyses were performed by using the GraphPad Instat software package.

RESULTS

Flt3L stimulates hematopoiesis and expansion of NK cell and DC populations similarly in burned mice and in normal mice. Initially, we examined the ability of Flt3L treatments to increase DC and NK cell numbers and overall splenic cellularity in mice that were subjected to burn injury. Both uninjured (sham) and burned mice were injected with 10 μ g of Flt3L each day for 10 days, a dose that has been shown to induce maximum effects under normal conditions (26, 44). Control mice received LR according to the same regimen. Figure 1 shows that splenic cellularity was significantly increased in sham mice following 10 days of Flt3L treatment. Specifically, there were approximately 2.8-fold more splenocytes in Flt3L-treated mice than in LR-treated mice. Similarly, splenic cellularity was significantly increased in burned mice that received Flt3L treatments for 5 days (1.7-fold-higher splenic cellularity) or 10 days (2.2-fold-higher splenic cellularity) after injury.

Fluorescence-activated cell sorter analysis was performed following staining of splenocytes with antibodies to DX5, CD3, and CD11c in order to identify NK cells, T cells, and DC, respectively. Flt3L-treated sham mice contained significantly higher numbers of DC (10.6-fold more DC) and NK cells (3-fold more NK cells) than LR-treated mice (Fig. 2). The

FIG. 1. Flt3L treatment increases splenic cellularity in normal and burned mice. Sham mice (four mice per group) were treated with either Flt3L or an equivalent volume of LR for 10 days. Mice with 40% TBSA burns were treated with either Flt3L or LR for 5 days ($n = 8$ or 9) or 10 days ($n = 3$). Cell counting was performed with splenocyte preparations. The data are expressed as means $\pm$ standard errors of the means. An asterisk indicates that a value is significantly higher than the value for the corresponding LR-treated mice; a number sign indicates that a value is significantly lower than the value for burned mice treated with Flt3L for 10 days ($P < 0.05$).

absolute numbers of CD3⁺ cells per spleen were not significantly different in Flt3L-treated mice and LR-treated mice. There was also a significant increase in the absolute number of NK cells per spleen in burned mice after 5 days of Flt3L treatment (Fig. 2A). There were approximately threefold more DX5⁺ cells in the spleens of burned mice treated with Flt3L for 5 days than in the spleens of burned mice treated with LR for 5 days. After 10 days of Flt3L treatment the number of DX5⁺ cells was not significantly different from the number of DX5⁺ cells in LR-treated mice or in burned mice that were treated with Flt3L for 5 days. Flt3L treatment induced a significant increase in the absolute numbers of DC in spleens of burned mice (Fig. 2B). Specifically, there were approximately 5- and 3.8-fold more CD11c⁺ splenocytes in mice after 5 and 10 days of Flt3L treatment, respectively, than in mice in the corresponding LR-treated groups. Flt3L treatment of burned mice for 10 days further increased the number of CD11c⁺ cells beyond the number after 5 days of treatment (1.6-fold more cells). Flt3L treatment of burned mice had no effect on the number of CD3⁺ cells in the spleen (Fig. 2C). Burn injury alone (no Flt3L) did not significantly alter the numbers of splenic DX5⁺, CD11c⁺, or CD3⁺ cells compared to the numbers of these cells in the sham, uninjured mice.

Flt3L treatments prevent burn-induced suppression of IFN- γ induction by HKPA. IFN- γ induction has been shown to be severely impaired following thermal injury (34, 48). We examined production of IFN- γ by cultured splenocytes from Flt3L-treated burned mice after challenge with HKPA in vitro. Splenocytes from burned mice, harvested 5 days after injury, produced significantly lower levels of IFN- γ (17.6-fold-lower levels) upon bacterial challenge than splenocytes harvested from sham, uninjured mice produced (Fig. 3). Splenocytes from burned mice treated with Flt3L for 5 days produced significantly higher levels of IFN- γ (31-fold-higher levels) than splenocytes from burned mice that received control LR injections produced. There was not a significant difference between the IFN- γ levels produced by splenocytes from Flt3L-treated burned mice and by splenocytes from sham, uninjured mice.

Since Flt3L treatment of burned mice restored in vitro IFN- γ production to sham levels, we examined splenic IFN- γ production after 4 h of in vivo HKPA challenge in burned

FIG. 2. Flt3L treatment increases the numbers of NK cells and DC in spleens of normal and burned mice. Sham mice were treated with Flt3L or LR for 10 days ($n = 4$). Mice with 40% TBSA burns were treated with Flt3L or LR for 5 days ($n = 4$ or 5) or 10 days ($n = 3$). Flow cytometric analysis was performed with splenocytes stained with antibodies to surface markers for NK cells (DX5⁺) (A), DC (CD11c⁺) (B), and T cells (CD3⁺) (C). The data are expressed as means $\pm$ standard errors of the means. An asterisk indicates that a value is significantly higher than the value for the corresponding LR-treated mice; a number sign indicates that a value is significantly higher than the value for mice treated with Flt3L for 5 days ($P < 0.05$).

FIG. 3. Flt3L treatment of burned mice restores IFN- γ induction by HKPA challenge of cultured splenocytes. Mice with 40% TBSA burns were treated with Flt3L or LR for 5 days and sacrificed 5 days after injury. The sham mice were uninjured and treated with LR. Splenocytes (10^6 cells/well) were cultured overnight in the presence of 2×10^6 CFU of HKPA. IFN- γ levels in media were measured by the ELISA. The data are expressed as means $\pm$ standard errors of the means (four mice/group), with in vitro stimulation performed in duplicate. An asterisk indicates that a value is significantly higher than the value for the burned mice treated with LR ($P < 0.05$).

mice. There was a higher proportion of IFN- γ -producing cells in the spleens of Flt3L-treated mice. In the burned mice represented by histograms in Fig. 4, approximately 6.5% of the splenocytes of untreated (LR) mice were producing IFN- γ 4 h after HKPA injection in vivo, whereas approximately 33% of the splenocytes from Flt3L-treated mice were producing IFN- γ (Fig. 4A). Compared to the absolute numbers of splenic IFN- γ -producing cells in burned mice that received control LR injections for 5 or 10 days, Flt3L treatment of burned mice for 5 or 10 days significantly increased the absolute numbers of splenic IFN- γ -producing cells following bacterial challenge (Fig. 4B). Specifically, there were 5.5-fold more IFN- γ -producing cells in the spleens of burned mice treated with Flt3L for 5 days and 3-fold more IFN- γ -producing cells in the spleens of burned mice treated with Flt3L for 10 days than in the spleens of burned mice that received control LR injections for 5 and 10 days, respectively. There were significantly more IFN- γ -producing cells (2.3-fold more cells) in the spleens of burned mice treated with Flt3L for 10 days than in the spleens of burned mice treated with Flt3L for 5 days. Without Flt3L treatment, the number of IFN- γ producers was significantly higher (4.4-fold higher) at 10 days after injury than at 5 days after burn injury, which is consistent with the gradual recovery of immune function that occurs over time.

We next examined the cellular sources of IFN- γ in spleens of burned mice challenged with HKPA. NK cells and T lymphocytes are the major producers of IFN- γ after bacterial challenge. However, another source of IFN- γ in the spleen is the CD11c⁺-CD11b⁻-CD8⁺ subset of DC, also referred to as DC1 or lymphoid-related DC (32, 53). After 5 days of Flt3L treatment, there were significantly more NK cells, T lymphocytes, and DC producing IFN- γ in the spleen upon bacterial challenge (Fig. 5). Specifically, there were 2.7-fold more IFN- γ ⁺ DX5⁺ cells and 3.2-fold more IFN- γ ⁺ CD3⁺ cells in spleens of burned mice treated with Flt3L than in the spleens of LR-treated burned mice (Fig. 5A). To assess the numbers of IFN- γ -producing DC, CD11c⁺ IFN- γ ⁺ cells were analyzed by using an acquisition gate set on high forward light scatter (Fig. 5B) and high CD11c expression. This gating technique is used to select DC and exclude lymphocytes that express low levels of CD11c (15, 51). There were approximately 9.8-fold more IFN- γ ⁺ CD11c^{high} cells in this region. Although the absolute

numbers of IFN- γ -producing DC and NK cells increased, Flt3L treatment of burned mice did not significantly affect the percentages of DC and NK cells making IFN- γ , but it significantly increased (threefold increase) the percentage of T cells producing IFN- γ (Fig. 5A).

To determine if circulating levels of IFN- γ are also increased by Flt3L treatment, we measured IFN- γ in sera of burned mice after bacterial challenge. HKPA induced significantly greater serum levels of IFN- γ in burned mice treated with Flt3L for 5 days than in mice that received 5 days of LR treatment (Fig. 6). Specifically, Flt3L-treated, burned mice had 14.7-fold-higher serum levels of IFN- γ after 4 h of stimulation.

Flt3L treatments prevent burn-induced suppression of the IFN- γ -inducing cytokines IL-12 and IL-15. The production of IFN- γ by NK cells during early responses to infection is largely dependent upon macrophage- and DC-derived IL-12, and IL-12 production is impaired by burn injury. We examined the ability of Flt3L treatment of burned mice to restore IL-12 induction by HKPA challenge in vitro. Splenocytes from burned mice, harvested 5 days after injury, produced significantly lower levels of IL-12 (21.4-fold-lower levels) upon bacterial challenge than splenocytes harvested from sham, uninjured mice produced (Fig. 7). Splenocytes from burned mice treated with Flt3L for 5 days produced significantly higher levels of IL-12 (14.6-fold-higher levels) than splenocytes from burned mice that received control LR injections produced. There was not a significant difference between the IL-12 levels produced by splenocytes from Flt3L-treated burned mice and the IL-12 levels produced by splenocytes from sham, uninjured mice. We also examined IL-12 mRNA levels after in vivo HKPA challenge in spleens of burned mice treated with Flt3L or LR for 5 days. IL-12 is a heterodimeric protein composed of the p35 and p40 proteins (7). There were increases in the relative levels of the IL-12 p35 and IL-12 p40 mRNAs in the mice treated with Flt3L (Fig. 8). IL-12 also works synergistically with IL-15 and IL-18 to induce IFN- γ production (14, 24). We examined the mRNAs of these cytokines in spleens after in vivo challenge with HKPA. The IL-15 mRNA levels in the burned mice treated with Flt3L were higher than the levels in LR-treated burned mice, but the IL-18 mRNA levels were the same in the two groups (Fig. 8).

Flt3L treatment of burned mice restores splenic responsiveness to IFN- γ -inducing cytokines. Major burn injury causes decreased responsiveness of splenocytes to IFN- γ -inducing cytokines (48). We next examined the ability of isolated splenocytes from burned mice treated with Flt3L or LR to respond to cytokine stimulation. Incubation of splenocytes with IL-12 alone, IL-12 plus IL-15, or IL-12 plus IL-18 resulted in IFN- γ levels that were significantly higher in the Flt3L-treated group than in the LR control treatment group (Fig. 9). Specifically, splenocytes harvested from burned mice after 5 days of Flt3L treatment produced 3.3-, 2.1-, and 3-fold more IFN- γ after stimulation with IL-12 (Fig. 9A), IL-12 plus IL-15 (Fig. 9B), and IL-12 plus IL-18 (Fig. 9C), respectively, than splenocytes from LR-treated burned mice produced.

Since decreased responsiveness of splenocytes to IFN- γ -inducing cytokines after burn injury is associated with decreased expression of the corresponding cytokine receptors (48), we examined the relative expression levels of these cytokine receptors in spleens of burned mice treated with Flt3L or

FIG. 4. Flt3L treatment restores IFN- γ -producing cells to spleens of burned mice. Mice with 40% TBSA burns were treated with Flt3L or LR for 5 and 10 days and then injected with HKPA (10^9 CFU) on days 5 and 10, respectively, after injury. Splenocytes were harvested after 4 h of bacterial challenge and analyzed by flow cytometry following intracellular staining with antibodies to IFN- γ . (A) Representative data for individual mice (5 days after injury; with 5 days of treatment) are presented as histograms showing 10,000 events. The regions containing IFN- γ ⁺ cells are indicated by an arrow and were determined by comparisons with isotype controls. The number of events (y axis) is on a scale from 0 to 64. (B) Data for four or five mice per group (5 days after burn injury) and for three mice per group (10 days after burn injury) are expressed as means $\pm$ standard errors of the means. An asterisk indicates that a value is significantly higher than the value for LR-treated mice 5 days after burn injury; a number sign indicates that a value is significantly lower than the value for Flt3L-treated mice 10 days after burn injury ($P < 0.05$).

LR. Figure 10 shows that Flt3L treatment of burned mice resulted in a relative increase in the splenic mRNA levels of the IL-15 receptors IL-15R α and IL-2R β , the IL-12 receptor IL-12R β 1, and the IL-2 receptors IL-2R β and IL-2R α .

DISCUSSION

Using an in vivo bacterial challenge model, we showed that burns result in significant impairment of the early induction of IFN- γ by NK cells (48). This may be an important factor in the decreased resistance of burn victims to microbial infection. However, two clinical trials, in which the treatment of burn or trauma patients with IFN- γ was evaluated, showed no benefit of exogenous IFN- γ with respect to the incidence of infections or infection-related mortality (36, 55). Although a decreased IFN- γ level is a useful marker of burn-induced immunosuppression, it is only one of many alterations that occur in the immune system after burn injury. Within the IFN- γ -regulating network alone there are impairments in multiple cell types that include decreased production of the inducing cytokines IL-12, IL-2, and IL-15, as well as decreases in expression of the corresponding receptors (34, 48). Therefore, it is unlikely that treatments with single cytokines, such as IFN- γ , are sufficient to restore the functions and preinjury conditions of so many different immune cells that interact to destroy invading microbes. We therefore propose that stimulation of hematopoiesis to provide burn victims with new effector cells of the immune system has potential for treatment of burn-induced suppression of host defense mechanisms. This approach has been proposed previously. Specifically, treatment with granulocyte colony-stimulating factor or granulocyte-macrophage colony-stimulating factor (GM-CSF) after burn injury has been shown to have positive effects on survival and immune cell function (10, 17), including improved T-cell proliferation and IL-2 production in a rat burn model (29).

We examined the cytokine Flt3L as a stimulator of hematopoiesis in burned mice. Due to the properties of cell populations that are expanded and differentiated in normal mice by Flt3L, this cytokine appears to have appropriate therapeutic potential for treating burn-induced susceptibility to infections. Parajuli et al. compared the properties of cell populations expanded by in vivo treatment of normal mice with Flt3L or GM-CSF and reported that Flt3L expanded the populations of both the CD4⁺ and CD8⁺ T-cell subsets, while GM-CSF expanded only the population of the CD4⁺ subset (35). This may be an important property since burn patients have deficiencies in the CD8⁺ population (37, 56). Flt3L also promoted the generation of both type 1 DC (Th1 promoting) and type 2 DC (Th2 promoting), while GM-CSF expanded only the type 2 DC population. Both GM-CSF and Flt3L promoted the induction of splenic IL-12 and IFN- γ , but Flt3L induced approximately twofold-higher levels of these Th1 cytokines. GM-CSF promoted the induction of splenic IL-10 and IL-4, but Flt3L did not affect splenic levels of these Th2 cytokines (35). Considering that burns induce a Th1-Th2 balance shift towards Th2 predominance and the properties of Flt3L mentioned above, Flt3L may stimulate the expansion and differentiation of populations of immune cells that are more appropriate for restoring normal parameters of immune function to burn patients.

A **B**

FIG. 5. Flt3L increases the number of IFN- γ -producing NK cells, DC, and T cells in spleens of burned mice. Mice with 40% TBSA burns were treated with Flt3L or LR for 5 days and then injected with HKPA (10^9 CFU) 5 days after injury. Splenocytes were harvested after 4 h of bacterial challenge and analyzed by flow cytometry after surface staining with antibodies to DX5 to identify NK cells, with antibodies to CD11c to identify DC, or with antibodies to CD3 to identify T cells, followed by intracellular staining with antibodies to IFN- γ . Only CD11c^{high} cells within region 2 (R2) were included in the analysis of DC. (A) Total numbers of IFN- γ -producing DX5⁺, CD11c⁺, CD3⁺ cells per spleen. The percentage of cells in each population that were producing IFN- γ is indicated. The data are expressed as means $\pm$ standard errors of the means for four or five mice per group. For each population, an asterisk indicates significantly higher absolute numbers than in the LR group; the number sign indicates a significantly higher percentage than in the LR group. (B) Forward scatter (FSC)-versus-side scatter (SSC) scattergram obtained with splenocytes of an Flt3L-treated burned mouse, showing the monocyte-DC analysis region (R2) used for determination of CD11c^{high}/IFN- γ ⁺ DC, to exclude IFN- γ ⁺/CD11c⁺ lymphocytes, contained predominantly in region 1 (R1).

There have been reports that thermal injury affects bone marrow progenitor cells, inducing shifts in the commitment of myeloid progenitor cells toward a monocytic lineage (31, 43). In order to determine if Flt3L is able to induce hematopoietic effects in burned mice similar to those induced in uninjured

mice, we treated burned mice with Flt3L for 5 or 10 days. Although 10 days of treatment produced the maximum effects in uninjured mice, we included a shorter treatment course to determine if effects could be achieved sooner, since the susceptibility of patients to infections is greater at earlier times

FIG. 6. Flt3L treatment of burned mice increases serum levels of IFN- γ upon bacterial challenge. Mice with 40% TBSA burns were treated with Flt3L or LR for 5 days and then injected with HKPA (10^9 CFU) 5 days after injury. Blood was harvested after 4 h of bacterial challenge, and IFN- γ levels in sera were measured by the ELISA. The data are expressed as means $\pm$ standard errors of the means (three mice per group). The asterisk indicates that the value is significantly higher than the value for burned mice treated with LR for 5 days ($P < 0.05$).

after injury. Additionally, burn patients may not need maximum expansion and differentiation of progenitor cell populations in order to increase their resistance to microbial infections. We show that burned mice respond to Flt3L like normal, uninjured mice respond. In both sham and burned mice, there are significant increases in splenic cellularity after Flt3L treatments, associated with enhanced numbers of splenic DC and NK cells. Although previous studies with normal mice have shown that Flt3L treatment for 10 days results in the maximum effects (26, 44), we found that a shorter course of treatment (5 days) induces a similar expansion of NK and DC populations in burned mice.

Since NK cell-derived IFN- γ induction is impaired after burn injury and is a useful marker of innate immune responses to bacterial infection, we examined the ability of Flt3L treatments to restore early induction of IFN- γ to burned mice. We used HKPA as a stimulus of IFN- γ production in this study. Heat-killed organisms are used experimentally as stimuli to assess early cytokine responses to bacterial products (18, 54), and the effects of burns on IFN- γ regulation after HKPA challenge have been characterized previously (48). Splenocytes from burned mice, harvested 5 days after injury and cultured

FIG. 7. Flt3L treatment of burned mice restores IL-12 induction by HKPA challenge of cultured splenocytes. Mice with 40% TBSA burns were treated with Flt3L or LR for 5 days and sacrificed 5 days after injury. Sham mice were uninjured and treated with LR. Splenocytes (10^6 cells/well) were cultured overnight in the presence of 2×10^6 CFU of HKPA. IL-12 levels in media were measured by the ELISA. The data are expressed as means $\pm$ standard errors of the means (four mice per group), with in vitro stimulation performed in duplicate. An asterisk indicates that a value is significantly higher than the value for the burned mice that received LR treatments ($P < 0.05$).

FIG. 8. Flt3L treatment of burned mice increases the relative levels of splenic mRNAs for the IFN- γ -inducing cytokines IL-12 and IL-15. Mice with 40% TBSA burns were treated with Flt3L or LR for 5 days and then injected with HKPA (10^9 CFU) 5 days after injury. Spleens from three mice in each group were harvested, and the mRNA was analyzed by the RPA by using 15 μ g of RNA per hybridization mixture. The L32 housekeeping gene is an internal control for overall mRNA quantity.

overnight in the presence of HKPA, produced significantly lower levels of IFN- γ than splenocytes from sham, uninjured mice produced. Treatment of burned mice with Flt3L for 5 days restored IFN- γ induction to levels that were not statistically different from those exhibited by the sham group. In vivo induction of IFN- γ in spleens of burned mice was also restored by Flt3L treatment, and there was a corresponding increase in serum levels. Five days of treatment was sufficient to significantly increase the numbers of splenocytes producing IFN- γ in response to HKPA, yet 10 days of treatment further enhanced the number of IFN- γ producers. It is not known what levels of IFN- γ and/or functional effector cell restoration are necessary to restore natural resistance to infections, and future studies should address this issue. However, the restoration of IFN- γ -producing cells to burned mice treated with Flt3L suggests that this treatment may improve the ability of innate immune responses to control early stages of bacterial infection. NK cells and DC are important effector cells of innate immunity, and Flt3L treatment of burned mice increased the numbers of splenic IFN- γ -producing DC and NK cells during the early responses to bacterial challenge. Although there were dramatic increases in the absolute numbers of splenic CD11c⁺ DC and DX5⁺ NK cells, the percentages of these cell types producing IFN- γ were not increased, indicating that Flt3L restores IFN- γ -producing NK cells and DC by increasing the numbers of functional cells in the spleens of burned mice. The absolute number of IFN- γ -producing T cells during the early response to HKPA was also increased by Flt3L. Interestingly, the absolute number of CD3⁺ T cells was not significantly increased, indicating that there was an increase in the percentage of T cells making IFN- γ . This suggests that Flt3L may have some stimulatory effects on the ability of T cells to produce IFN- γ in response to HKPA. This may be due in part to the restoration of IL-12 and IL-15 induction by Flt3L treatment of burned mice. IL-12 and IL-15 are potent inducers of IFN- γ , and IL-15 is an activator of both NK and cytotoxic T-cell responses to bacteria (50). However, the specific effects of Flt3L on T-cell regulation of IFN- γ were not determined in these studies. Although the IL-12 and IL-15 mRNA levels were

FIG. 9. Flt3L treatment of burned mice increases responsiveness of splenocytes to IFN- γ -inducing cytokines. Mice with 40% TBSA burns were treated with Flt3L or LR for 5 days and sacrificed 5 days after injury. Splenocytes (10^6 cells/well) were cultured overnight in the presence of IL-12 (A), IL-12 plus IL-15 (B), or IL-12 plus IL-18 (C). IL-12 was added at a concentration of 10 ng/ml, and IL-15 and IL-18 were each added at a concentration of 1 ng/ml. IFN- γ levels in media were measured by the ELISA. The data are expressed as means $\pm$ standard errors of the means (four mice per group), with in vitro stimulation performed in duplicate. An asterisk indicates that a value is significantly higher than the value for the burned mice that received LR treatments ($P < 0.05$).

higher in Flt3L-treated mice, Flt3L did not cause an increase in IL-18 mRNA levels in spleens of burned mice. However, IL-18 production is not impaired by burn injury, whereas production of IL-12 and IL-15 is significantly suppressed after burn injury (2, 34, 48). This suggests that Flt3L may restore burn-induced deficits in cytokine production without inducing overproduction of cytokines that are not affected by the injury.

It was recently reported that immature, Flt3L-mobilized DC from cancer patients do not produce IL-12 after in vitro stimulation with CD40L or IFN- γ alone but require multiple signals for complete maturation (30). However, we show here that Flt3L treatment of burned mice restored IL-12 production by total splenocytes stimulated in vitro with HKPA. Additionally, the relative expression of IL-12 p35 and p40 mRNAs was higher in Flt3L-treated mice. Future studies should include

FIG. 10. Flt3L treatment of burned mice increases the relative levels of splenic mRNAs for IFN- γ -inducing cytokine receptors. Mice with 40% TBSA burns were treated with Flt3L or LR for 5 days and then injected with HKPA (10^9 CFU) 5 days after injury. Spleens from three mice in each group were harvested, and the mRNA was analyzed by the RPA by using 15 μ g of RNA per hybridization mixture. The L32 housekeeping gene is an internal control for overall mRNA quantity.

analysis of the phenotypes of IL-12-producing cells in spleens of Flt3L-treated, HKPA-stimulated mice to determine if IL-12 production is derived from macrophages or specific DC populations.

In addition to bacterial stimuli, the cytokines IL-12, IL-15, and IL-18 are potent inducers of IFN- γ expression. Specifically, IL-12 works alone or synergistically with IL-15 or IL-18 to induce IFN- γ production (14, 24). We have shown that burn injury suppresses the responsiveness of splenocytes to stimulation with these IFN- γ -inducing cytokines. Additionally, there is a loss of expression of the corresponding receptors for these cytokines, which may be responsible for decreased induction of IFN- γ after stimulation with the cytokines (48). Flt3L treatment of burned mice for 5 days restored responsiveness of splenocytes to in vitro stimulation with IL-12 alone, IL-12 plus IL-15, or IL-12 plus IL-18. Since equal numbers of cells were plated in all wells in the in vitro assays, the increased responsiveness to these cytokines was likely due to a greater proportion of newly generated IFN- γ -producing cells expressing receptors for the cytokines. Although the levels of cytokine receptors on Flt3L-generated cells were not directly measured in this study, examination of mRNA levels in spleens of Flt3L-treated mice revealed relatively higher expression of the IL-15, IL-12, and IL-2 receptors.

In our model early responses to infection are examined with a focus on Th1 cytokine production by DC and NK cells. We have not yet examined the effects of Flt3L on microbicidal activities of these cell types or on later stages of acquired immunity that are also known to be altered after burn injury, including T-cell proliferation, cytotoxic T-lymphocyte activity, and antigen-specific antibody isotype profiles. However, other studies with uninjured mice have suggested that Flt3L may also improve acquired immune responses in burned mice. For example, in response to soluble ovalbumin, Flt3L-treated mice produce large amounts of antigen-specific immunoglobulin G2a (IgG2a) and there is a comparatively smaller increase in the amount of IgG1, whereas GM-CSF-treated mice produce mostly IgG1 and little IgG2a (38). Considering that burns cause a decrease in antigen-specific IgG2a isotype production

(22), Flt3L may help to restore normal antibody isotype production profiles after burn injury. Additionally, Flt3L may improve acquired immunity by enhancing expansion of the antigen-specific T-cell population and/or the lifetime of antigen-specific T cells (38).

In conclusion, Flt3L is able to promote the expansion of DC and NK cell populations in burned mice. These expanded populations are capable of producing IFN- γ during the early response to bacterial challenge and in response to cytokine stimulation, and Flt3L increases overall IFN- γ induction in burned mice. These data suggest that Flt3L may have effects that could improve innate immune responses to bacterial infection after burn injury and that studies to investigate other aspects of immunity, bacterial clearance, and resistance to infection-associated mortality after burn injury are warranted.

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