Innate Lymphocyte Subsets and Their Immunoregulatory Roles in Burn Injury and Sepsis

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The vast majority of clinical and basic science research on the immune consequences of burn injury and sepsis conducted during the last three decades has focused mainly on the roles of macrophages, neutrophils and, to a lesser extent, conventional T lymphocytes. During recent years, however, it has become increasingly clear that minor subsets of innate immune cells, innate regulatory lymphocytes in particular, are central to processes involved in both protective immunity and immunopathology. Recent reports by our laboratory and others have just begun to shed light on the critical roles of innate lymphocyte subsets, including natural killer T cells, natural killer cells, gamma-delta T cells, and naturally occurring CD4+CD25+ regulatory T cells during the immune response to burn injury and sepsis. Given their emerging importance and documented upstream regulatory capacities over macrophage, dendritic cell, and T lymphocyte functions, innate regulatory lymphocytes represent attractive new targets for therapeutic intervention for the overall immune paralysis that occurs with injury and sepsis. Here, we provide an overview of the current state of knowledge of these particular cell subsets in the immune response to burn injury and sepsis.


Injury, infection, and sepsis continue to be significant causes of death in the United States today, despite recent advances in critical care medicine, the development of more effective antibiotics, and a greater understanding of the immune consequences of burn trauma and sepsis. Although most research during recent years has focused on the roles of conventional phagocytes of the innate immune system, including neutrophils and macrophages, and conventional lymphocytes of the adaptive immune system, including T and B lymphocytes, less frequent populations of regulatory lymphocytes are significantly understudied for their roles in injury and infection. In this review, we will consider the immunoregulatory roles of four cell types, including natural killer (NK) cells, natural killer T (NKT) cells, gamma-delta (γδ) T cells, and naturally occurring regulatory T cells (Treg) cells in the context of injury, with an emphasis on burn injury and the development of sepsis thereafter. Given their emerging roles as powerful regulators of both innate and adaptive components of immunity, these regulatory cell populations warrant further exploration for their roles in the basic biology of immunity after burn injury and during sepsis and, moreover, offer potential new avenues of investigation for the development of therapeutic approaches to injury-induced immune paralysis and sepsis.

NATURAL KILLER CELLS

NK cells originally were described for their ability to lyse tumor cells without previous antigen exposure or stimulation. Although they arise from common lymphoid progenitors, they do not express clonally derived antigen receptors such as those found on B and T cells. NK cells are widely distributed amongst the peripheral blood, spleen, and bone marrow under normal conditions but migrate to sites of inflammation in response to various chemokines. Upon activation, they rapidly release proinflammatory cytokines,
including interferon (IFN)-γ and tumor necrosis factor (TNF-α)2. Upon arrival to inflamed or infected sites, NK cells lyse dangerous self or nonself cells depending on the combination of inhibitory and activating signals conveyed by the target, including major histocompatibility complex (MHC) class I molecules and killer immunoglobulin-like receptors. Primary mechanisms of killing by NK cells include perforin, granzymes, TNF-α, and Fas/FasL. The known roles of NK cells in injury and sepsis are described below and are summarized in Table 1.

Natural Killer Cells in Injury

NK cells rapidly produce IFN-γ in response to microbial threats, and one might expect to see the same type of NK activation in the inflammatory reaction associated with trauma or burns, but this does not appear the case. In fact, injury suppresses the innate cellular immune system, a phenomenon termed “clinical immune paralysis.”3–5 First described in the late 1980s, clinical immune paralysis was associated with defective macrophage function, decreased histocompatibility leucocyte antigen expression, and depressed mitogen-induced responsiveness of peripheral blood mononuclear cells.3 NK-derived IFN-γ primes or augments each of the innate cellular immune functions listed previously, and transient IFN-γ deficiency is a key feature of clinical immune paralysis. In the case of burn injury, it is reasonable to suggest that decreased IFN-γ within the immune compartment (vs elevations in the circulation) renders the host more susceptible to infectious complications. Potential causes for decreased IFN-γ production by NK cells may include decreased NK cell numbers overall, inhibition of NK function by burn-associated soluble mediators, or by other suppressive immune cell populations.3,5,6

The question of NK cell numbers after injury has not been definitively answered. Studying both burn and trauma patients, Blazar et al7 found the percentage of NK cells (in blood) was either normal or increased. Burned patients had significantly decreased NK cytotoxicity compared with age-matched controls. Trauma patients exhibited similarly depressed NK cell function but over a shorter period than the burn patients (3 to 6 days after injury vs up to 40 days after burn). To investigate the mechanism for this

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<td>CLP-associated acute lung injury was associated with dysfunctional NK cells found in BAL fluid.</td>
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**BAL**, bronchoalveolar lavage; **CLP**, cecal ligation and puncture; **IL**, interleukin; **NK**, natural killer.
NK-cell defect, these investigators infused cortisol, epinephrine, and glucagon into healthy volunteers and repeated the cytotoxicity assay, revealing a 66% reduction in NK cytotoxicity in the experimental group. Hence, it seemed that stress hormones found after injury, and not decreased NK cell number, accounted for the NK-cell defect. However, in another group of trauma patients (including those with burns), NK-cell counts were reduced from days 6 to 14 after injury among patients who went on to develop severe sepsis compared with control patients with similar injury severity scores who did not become septic. Taken together, one cannot draw too many conclusions on what happens to NK-cell numbers after burns or trauma, given the varied patient populations, NK-cell antibodies used, and time points of measurement. However, it seems that alterations of NK functionality do not correlate with overall NK-cell numbers. Moreover, because granulocyte and lymphocyte absolute numbers change in the circulation, it is possible that altered NK-cell frequencies are not the result of changes in absolute number of NK cells themselves but are instead reflective of the absence of other leukocyte populations. Alternatively, a propensity for greater cortisol or epinephrine levels after injury might also render NK cells more defective.

Although the question of NK-cell numbers after injury remains unanswered, several investigators have more systematically evaluated injury-associated soluble mediators for potentially suppressive effects on NK cells after trauma or burn injury. Hauser et al incubated plasma from trauma patients withuffy coats (contains NK cells among other lymphocytes) from healthy age-matched volunteers. Plasma from trauma patients, but not healthy controls, suppressed NK function after 40 hours of incubation as measured by K-562 cytotoxicity assay. To determine whether soluble factors, such as interleukin (IL)-4, IL-10, or transforming growth factor (TGF)-β suppressed NK function in injured patients, neutralizing anti-IL-4, anti-IL-10, and anti-TGF-β1 were added to the cell cultures; however, no reversal of NK suppression by any of these antibodies was observed. However, catalase, a respiratory burst byproduct, did reverse NK-cell suppression.

Because reactive oxygen metabolites elaborated during the respiratory burst of monocytes suppress NK cytolytic function, Joshi et al further showed that monocytes from the buffy coat reversed NK cell suppression when incubated with plasma of trauma patients suggesting that NK suppression in trauma patients was monocyte-dependent. Even without the monocytes, the buffy coat contains other cells, and these results may not reflect a purely NK-cell phenomenon. These same authors conducted similar studies in patients with fracture/soft-tissue injury. In addition to using plasma, they also evaluated the fracture site itself for suppressive cytokines. Fracture/soft-tissue injury hematoma samples obtained during open fracture fixation were centrifuged to obtain a “fracture supernatant.” Peripheral blood plasma samples were obtained simultaneously. Fracture supernatant suppressed NK cell function at an earlier time point (16 hours) than peripheral blood plasma. Surprisingly, antibodies to IL-4 and IL-10 further suppressed lysis by 7% and 4%, respectively. Antibodies to TGF-β1 had no effect. Adding IL-12 to the fracture supernatant samples restored lysis, but adding IFN-γ failed to do so.

Unlike the first experiment, adding catalase had no effect. Because the fracture supernatant suppresses innate cellular immunity at very early time points, this fluid may be a source for circulating immunosuppressive mediators. One might imagine that fracture manipulation would release these mediators into systemic circulation. Therefore, these studies may have implications for the optimal timing of such interventions (ie, within 24 hours of injury). The authors explain the unexpected increase in NK suppression with IL-4 and IL-10 blockade by noting that IL-10 may suppress other accessory cells with a suppressive phenotype. Both studies from this group use buffy coat as a source of NK cells, but this is not a pure NK-cell population as it contains other leukocytes such as cytotoxic T cells. Nevertheless, NK cytotoxicity was suppressed by factors in both serum and fracture hematoma in these injured patients.

Serum from burn patients contains abnormally elevated IL-6 and soluble IL-2 receptor alpha (sIL-2Ra), the extracellular domain of the IL-2 receptor. Recombinant sIL-2Ra inhibited NK-cell activity by 50% in vitro. Further, the concentration of sIL-2Ra in burn patients correlated with the amount of dietary fat intake, suggesting a beneficial role for low-fat diets after burn. Many others have investigated modifying the dietary lipid content and composition to improve outcomes in burn patients and trauma patients. In their observational study, Pratt et al describe how burn injury affected fatty acid composition of immune cell membranes so that an optimal fat composition formula could potentially modulate immune function after burn injury. Specifically, lymphocyte phospholipid 20:4n-6 (a specific fatty acid chain length and configuration) content was 30% to 60% lower in the first 35 days after burn compared with later time points (days 36-50). They observed lower IFN-β and depressed NK cytotoxicity in this same early period, which increased in the later phase. Aside from soluble factors, these authors suggest that...
impaired synthesis of 20:4n-6 fatty acid within lymphocytes themselves contributed to clinical immune paralysis seen after burn injury. How modulating dietary fat intake could alter leukocyte phospholipid content in a beneficial manner remains unknown. Whether altered NK-cell membrane phospholipid content depresses their cytotoxic function also remains unstudied.

Clinical immune paralysis predisposes the burn population to septic complications. A laboratory model for this is to follow a scald burn with cecal ligation and puncture (CLP) to make previously burned mice septic. Because IL-12 induces the production of IFN-γ by NK cells (and T cells), O’Suilleabhan et al35 explored the efficacy of IL-12 therapy in postburn sepsis using the burn + CLP mouse model. IL-12 administered beginning 3 days after burn injury for 5 days increased survival after CLP (performed 10 days after burn injury) compared with that of a nonburned group. Administering anti-IFN-γ antibodies to the IL-12-treated group reduced their survival to that of the saline-treated burn group, proving that the IFN-γ, and not the IL-12 levels, was a key determinant of survival. Administration of IFN-γ instead of IL-12, however, had no effect on survival. IL-12 therapy also decreased the splenocytes production of IL-4 (mRNA and protein). Although this study did not specifically evaluate NK-derived IFN-γ, studies by Ami et al16 examined IL-18 as a therapy for postburn sepsis. Macrophages and Kupffer cells produce IL-18, which stimulates NK cells to produce IFN-γ in the presence of IL-12. Here, they assayed IFN-γ produced by liver mononuclear cells (containing large amounts of NK cells). IL-18 therapy restored IFN-γ production by these cells after a contact burn and subsequent CLP or lipopolysaccharide (LPS) shock. Like IL-12 therapy, IL-18 significantly prevented burn-induced mortality, perhaps via inhibition of IL-10. Together, these two studies provide an experimental model for the “second hit” after burn injury and suggest cytokine therapies to modify the IFN-γ deficiency seen in clinical immune paralysis. Neither study definitively links the IFN-γ deficiency to NK cells, but one can assume their IFN-γ production changes based on what is known about NK-derived IFN-γ production in sepsis alone.

NK Cells in Infection and Sepsis
In the setting of sepsis, antigen-presenting cells (APCs; macrophages and dendritic cells) recognize bacterial antigens or products such as endotoxin and secrete IL-12, the primary activator of NK cells, causing them to secrete large amounts of IFN-γ and TNF-α. These two cytokines contribute to the clinical picture of septic shock and multisystem organ failure. Even without bacterial products, combinations of either IL-2 or IL-15 (IL-15 signals via part of the IL-12 receptor) with IL-12 induce a lethal inflammatory response in mice that is not seen when NK cells are depleted.18 Macrophages and NK cells are interdependent: monocytes depend on NK-derived IFN-γ for their maturation, and they, in turn, secrete IL-12 which further stimulates NK cells.3 When stimulated, NK cells produce copious IFN-γ, IL-10 and IL-4, on the other hand, generally suppress NK cell activity.19

Recently, Scott et al further characterized the NK-macrophage interaction as contact dependent via CD40-CD154 cell surface receptors on macrophages and NK cells, respectively. In an in-vitro model of LPS shock, these authors conducted a series of experiments co-culturing mouse peritoneal macrophages and splenic NK cells. They demonstrated that effective macrophage phagocytosis depends on NK cell contact via CD40-CD154 interaction.20 Other investigators have confirmed that macrophages need NK cells for successful activation and phagocytosis; this requirement is not just limited to LPS shock. For example, Goodshall et al demonstrated that NK-depleted mice had macrophages incapable of ingesting the same number of E. coli as controls in the setting of CLP, a model of septic peritonitis. Interestingly, the macrophages from NK-depleted mice captured higher numbers of bacteria on their outer surface, but could not ingest them, suggesting that NK cells may promote internalization of pathogens by macrophages.21 In similar CLP sepsis experiments, Scott et al showed that depletion of NK cells either augmented or hindered macrophage function depending on how the NK cells were depleted.22 Using the myeloperoxidase assay to measure neutrophil accumulation in lung and liver tissues four hours after CLP, these investigators found no difference among the groups, thereby suggesting defects in macrophage but not neutrophil function during the early (4 hours) stages of bacterial clearance in NK-depleted mice.22 In yet another model of sepsis, Goldmann et al used intravenous infection with Streptococcus pyogenes to show that NK cell-derived IFNγ correlated with decreased survival.23 Despite using different models of sepsis and different methods, these three groups of investigators demonstrated that NK cells are important for the augmentation of macrophage activation and phagocytosis in a variety of pathologic conditions. NK-mediated macrophage activation represents a critical mode of clearing bacteria early in the infection prior to the arrival of neutrophils and certainly before adaptive responses.
Aside from examining macrophage function, studies of sepsis in NK-deficient mice have also led to the notion that NK cells either directly or indirectly, contribute to serum cytokine levels as well. The most consistent finding was a decreased level of sepsis-related IFN-γ within 24 to 48 hours post-infection when NK cells were absent. For example, Goldmann et al. depleted mice of NK cells using rat anti-asialo GM1 antibodies before infecting them with S. pyogenes and observed that IFN-γ was significantly lower in NK-depleted mice compared with control mice. The NK-deficient mice survived an average of 3 days longer than controls, but mortality eventually reached 100% in both groups. Certainly, one cannot attribute prolonged survival to lower IFN-γ alone, because sepsis is multifactorial. Yet, NK cells prove the major source of serum IFN-γ, a cytokine that, when elevated in the circulation, is associated with decreased survival in both gram-positive and gram-negative sepsis syndromes in humans and mice. Because NK cells remain the primary IFN-γ producers in sepsis, it is constructive to examine the downstream effects of this cytokine. IFN-γ activates signal transducer and activator of transcription (STAT), a transcription factor common among peripheral mononuclear cells that then transcribes many IFN-γ-sensitive genes. In one study, splenocytes from NK-deficient mice infected (intraperitoneal) with E. coli exhibited significantly less phosphorylated STAT1 compared with splenocytes harvested from control mice. NK cells may interact with multiple cellular compartments including macrophages by secreting IFN-γ, activating the STAT signaling mechanism.

Under ideal conditions, the aforementioned cellular and molecular events described serve to activate the immune system to clear bacteria. However, when uncontrolled, they also destroy host organs, and NK cell activation most likely contributes to multiorgan failure. In the model of E. coli peritonitis discussed previously, histologic analysis of mouse organs 4 hours after infection revealed increased hemorrhage in the lungs and kidneys of control mice compared to NK-depleted mice. Hirsh et al. examined the clinically relevant scenario of NK cell-mediated acute lung injury in response to CLP (intra-abdominal sepsis). As in Badgwell’s study, these authors found inflammatory lung damage as measured by histologic scoring and higher protein and PMN content of bronchoalveolar lavage fluid; these changes peaked at 4 days. At this same time point, the percentage of perforin-positive NK cells in the CLP group was significantly less than sham-operated mice (35% vs 46%). When assayed for their cytolytic capability, the NK cells from CLP mice killed fewer targets than their sham-operated targets 4 days postoperatively. Unlike their systemic counterparts, the NK cells isolated from the lung tissue displayed decreased IFN-γ but increased TNF-α expression. Therefore, peak sepsis-induced inflammatory lung changes correlated with dysfunctional NK cytolytic activity and cytokine expression. Hence, NK cells play a salutary role in sepsis via their interactions with macrophages, but their secreted IFN-γ and altered function in tissues distant from the septic source may contribute to the pathology of multiorgan failure.

**NATURAL KILLER T CELLS**

NKT cells are a rare subset of innate lymphocytes that regulate both the innate and adaptive immune responses. They comprise 1% to 2% of all lymphocytes in the spleen, lymph nodes, and peripheral circulation; however, they make up the vast majority of lymphocytes found within the liver. These cells recognize glycolipid antigens presented by the CD1d antigen presentation molecules, which, like MHC class I molecules, associate with β2 microglobulin to form a complex on the surfaces of APCs. Humans possess five distinct CD1 genes (a through e) whereas mice only express CD1d. Because of a remarkable degree of conservation, mouse CD1d-restricted NKT cells recognize human CD1d and vice versa, making mice an excellent model to study the roles of these cells in immune function and immunopathology. NKT cells express an αβ T-cell receptor and CD3-like conventional T cells but also express NK-cell markers such as CD56 and NK1.1. Originally, investigators defined NKT cells as those expressing an invariant TCRα chain (Vα14/Jα281) that associated with a variety of β chains (Vβ2, 7, or 8). The majority (roughly 85%) of NKT cells express this invariant chain, whereas a less-frequent subset of NKT cells uses a non-CD1d restricted diverse T-cell receptor (TCR) repertoire. Therefore, NKT cells expressing the invariant TCR are referred to as “invariant NKT cells,” and this review will focus on this subset.

Antigen-presenting cells that express CD1d include macrophages, dendritic cells, and marginal zone B cells. NKT cells recognize both self and foreign glycolipid antigens in the context of CD1d. For example, alpha-galactosylceramide (α-GalCer) is a glycolipid antigen isolated from a marine sponge and, more recently, isoglobotrihexosylceramide (iGb3) was discovered as an endogenous antigen recognized by NKT cells. Stimulation with α-GalCer causes NKT cells to rapidly (within 30 to 60 minutes) release large amounts of cytokines (IFN-γ, IL-4, IL-10, IL-13, and TGF-β), and their rapid response makes them a

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key component of the innate response. The type of NKT cell response to antigen stimulation varies depending on the nature of the antigen and the cytokine background in which it is presented. NKT cells acquire either an IL-4 or IFN-γ producing phenotype depending on their environment. In the presence of APC-derived IL-12s, NKT cells secrete primarily IFN-γ (and IL-4 to a much lesser degree), whereas they secrete predominantly IL-4 in the absence of IL-12. Similarly, when exposed to IL-10 or TGF-β-producing APCs, NKT cells assume an IL-4/IL-10-producing phenotype. Whether the NKT cells secrete IFN-γ or IL-4 during the early phase of the immune response influences the progression of Th1 vs Th2 differentiation, respectively. Not only do NKT cells themselves secrete IFN-γ, but they also induce NK cells to do the same. In addition to their regulatory role, NKT cells also possess effector capabilities that involve IL-12-mediated perforin and Fas ligand mechanisms. Although the contribution of NKT cells to immunopathology associated with injury and sepsis has not been extensively studied, their known involvement is described herein and in Table 2.

**Natural Killer T Cells in Injury**

As discussed previously, many attribute the clinical immune paralysis after trauma or burn injury to a dysfunctional innate cellular immune system. Most of this work, however, does not specifically involve NKT cells. Recently, our laboratory has defined how NKT cells participate in burn-induced T cell immunity in a murine dorsal scald injury model. Our studies demonstrated that suppression of T-cell immunity after burn occurs in part, from an active suppression of T-cell function by CD1d-restricted, invariant (Vα14-Jα18) NKT cells. We also observed that the suppression of T-cell immunity correlated with increased overall production of IL-4 that is produced almost exclusively by the NKT cell population. Blocking the activation of NKT cells via an anti-CD1d antibody prevented this immune suppression. As in other models of injury and sepsis, increased IL-4 directs the immune response in a Th2 direction and actively suppresses the effector functions of both lymphocytes and APCs.

Subsequent studies by Palmer et al revealed that this suppression is antigen-specific and requires APCs that express CD1d. Adoptive transfer of purified CD1d-positive but not CD1d-negative APCs from burned mice could suppress a primed T cell response in an uninjured recipient. Even at 28 days after adoptive transfer, these antigen-primed APCs could suppress DTH response in unburned recipients. CD1d-positive APCs from burn-injured mice could not, however, induce immune suppression in unburned, NKT cell-deficient re-

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**Table 2. Natural killer T (NKT) cells**

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<td>Burn-induced suppression of T-cell immunity stems from NKT cell-derived IL-4. Blockade of CD1d-NKT cell interactions prevents injury-induced suppression of T-cell immunity.</td>
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<td>Palmer et al 44</td>
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<td>Mouse intratracheal Streptococcal infection</td>
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cipients. Taken together, these studies indicate that burn-induced T-cell suppression arises from direct interaction between CD1d-expressing APCs and NKT cells that leads to the production of immune suppressive cytokines by the NKT cells. Activation of NKT cells must occur early (within 24 hours) of injury, but the effects of NKT-cell mediated immune suppression last up to a month after injury. Our laboratory is currently investigating the molecular mechanism by which burn injury converts the phenotype of the NKT cell toward one that is immunosuppressive rather than immunoprotective. Preliminary results support the notion that when NKT cells are activated in the presence of high levels of proinflammatory cytokines, they acquire a phenotype that actively suppresses both APC and CD4+ T cell effector functions. A working model for the involvement of NKT cells in burn-induced immune suppression is represented in Figure 1. With such a prompt and durable immune suppression, it is no surprise that 30% of burn patients succumb to infectious complications,\(^4\) and the unique mechanisms involved in NKT cell activation offer attractive targets for immuno-therapeutic interventions for burn-induced immune suppression.

Natural Killer T Cells in Infection and Sepsis

Aside from α-GalCer, bacterial-derived antigens have also been shown to stimulate mouse and human NKT cells in vitro. For example, glycolipids from *Sphingomonas* induce NKT cells to secrete IL-4 and IFN-γ.\(^6\) Similarly, NKT cells may either directly or indirectly recognize certain mycobacterial phosphatidylinositol mannosides, because when injected subcutaneously into mice, these compounds induced granuloma formation that was NKT cell-dependent.\(^4\) Kawakami et al.\(^48\) have examined the role of NKT cells in host defense against another intracellular pathogen, the fungus *Cryptococcus neoformans*. Ja281ko (NKT-deficient) mice have impaired ability to clear cryptococcal infection as well as a weakened delayed-type hypersensitivity reaction to cryptococcal antigen. Clearly, NKT cells are well suited for immunity

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**Figure 1.** Regulation of T-cell immunity after burn injury by CD1d-restricted natural killer T (NKT) cells. Under normal conditions (ie, in the absence of injury), antigen presenting cells (APCs) can simultaneously present glycolipid antigens in the context of CD1d to NKT cells and peptide antigens in the context of major histocompatibility complex (MHC)-II to conventional CD4+ T cells. In the absence of injury, interleukin (IL)-12 produced by the APCs promotes the production of interferon (IFN)-γ and IL-2 by NKT cells, which facilitate protective immunity via promotion of APC and CD4+ T cell effector functions. However, in response to burn, APCs produce less IL-12 and instead produce copious amounts of IL-6 and TNF-α. In response to activation by CD1d in the presence of high levels of the proinflammatory cytokines, NKT cells lose their capacity to produce IFN-γ and secrete IL-4 and transforming growth factor (TGF)-β instead. The loss of IFN-γ production and increased production of immune suppressive IL-4 and TGF-β actively inhibit both APC and CD4+ T-cell effector functions.
against intracellular pathogens like Cryptococcus because of their ability to secrete IFN-γ and direct a Th1 response. Several investigators have described their role in immunity against other intracellular parasites, fungus, and viruses as well.49

Fewer studies have examined the involvement of NKT cells in defense against extracellular bacteria or bacterial products. An experimental model of systemic shock called the Shwartzman reaction can be induced in mice via two consecutive injections of LPS. The first injection causes an IL-12 induced IFN-γ production. Twenty-four hours later, the second LPS challenge results in a septic death.30,50 Remarkably, Dieli et al showed that NKT-deficient mice are resistant to the LPS-induced mortality (93–100% survival in NKT-deficient strains vs 0–3% survival in wild type). It was shown that their enhanced survival was accompanied by lower serum IFN-γ and TNF-α compared with the wild type.30 There was no difference in IL-12 levels between the two groups. In further experiments, Dieli et al51 proved that NKT cells play an important role in LPS-induced sepsis by providing IFN-γ at the priming stage. Nieuwenhuis et al52 similarly demonstrated that NKT cell-derived IFN-γ helps clear Pseudomonas aeruginosa from the lung. In a murine model of acute pneumonia, CD1d ko mice (also NKT-deficient) had impaired clearance of Pseudomonas from the lungs. Upon histologic examination, Nieuwenhuis et al observed that NKT-deficient mice had fewer neutrophils in the lung after infection and that decreased neutrophil content correlated with lower MIP-2 levels. MIP-2 is primarily a neutrophil chemotaxant attractant also known to recruit NKT cells as they constitutively express CXCR2.53,54 Of note, when administered before Pseudomonas inoculation, α-GalCer prevented infection-induced pneumonia at 24 hours in wild-type mice but not NKT-deficient mice.52 Assessing the in situ phagocytic activity of macrophages after α-GalCer treatment revealed significantly increased uptake of Pseudomonas by bronchoalveolar lavage-derived macrophages. A control lipid did not enhance their activity. These authors suggest that macrophage “priming” by NKT cell-derived IFN-γ led to increased macrophage activity and release of MIP-2, thereby protecting the mucosa by rapid ignition of phagocytosis while recruiting granulocytes.52 Similar results were seen by Kawakami et al,55 who used a Staphylococcus pneumoniae infection model to demonstrate impaired resistance to the pathogen in NKT-deficient animals. Instead of MIP-2, these authors examined a possible role for MCP-1, a monocyte chemotaxant, in recruitment of NKT cells to sites of infection. They found that MCP-1 levels correlated with the time course of NKT cell infiltration into the lungs.55 Hence, MCP-1 may play a role in recruiting NKT cells to sites of infection, but this has not been shown definitively. Nevertheless, NKT cells clearly do play a role in both gram positive and gram-negative pneumonia. These studies also suggest NKT cells assist mucosal immunity by regulating the local cytokine milieu.

GAMMA-DELTA T CELLS

Gamma-delta (γδ) T cells share many properties with traditional T cells, including cytokine production and cytotoxicity and variable TCRs. Like NKT cells, they have been shown to have diverse roles in control of viral, bacterial, and parasitic infections, as well as roles in tumor surveillance and wound repair and are thus, uniquely poised to regulate components of both innate and adaptive immunity.56,57 They comprise only 2% to 3% of the lymphocyte population in blood and lymph, although they can represent a much greater frequency among lymphocyte populations within tissue compartments, such as the gastrointestinal tract, gut, and skin.56,57 Each tissue compartment with γδ T cells has a limited number of distinct subsets, usually classified by their Vγ and Vδ chains. For example, resident lung γδ T cells express predominately Vγ4 TCR and resident dαl γδ T cells, also known as dendritic epidermal T cells express predominantly Vγ5-Vδ1 TCRs. There are few known antigens for γδ T cells, although mycobacterial cell wall products, heat shock protein 60 kDa, and alkylamines are among the candidates.58 Other γδ TCR ligands appear to include MHC class-I-like and MHC class I polypeptide-related proteins (MICA and MICB) and T10/22, all of which are induced by stress.59

Gamma Delta T Cells in Injury

As stated before, γδ T cells are relatively abundant in epithelial tissues and are implicated as having vital roles in wound repair, immune, and tumor surveillance in the skin.56,57,60,61 The method of substantial injury most studied in terms of γδ T cells is thermal injury. Thermal injury produces a profound inflammatory reaction that has both protective and immunopathologic effects. Our understanding of the role of γδ T cells in this process, although incomplete, gives the best insight into how γδ T cells affect the immune system after injury.

Early after burn injury, γδ T cells contribute to wound healing, inflammation, and overall survival.60–63 Schwacha et al62 subjected wild-type and γδ T cell-deficient mice to 25% TBSA third-degree burns and observed 48 hours later that mortality was 3-fold greater.
in γδ T cell-deficient animals compared with controls.\textsuperscript{62} Although no early changes were observed in immune function, splenic macrophages collected from γδ T cell-deficient mice 7 days after injury produced significantly less TNF-α and IL-6 compared with wild-type mice. This γδ-mediated alteration of the postburn proinflammatory state was not affected by IL-10, as IL-10 levels were consistent in both wild-type and γδ T cell-deficient groups.\textsuperscript{62}

One possibility for the differences in survival observed between wild-type vs γδ T cell-deficient mice after injury may be the result of the protective effects of γδ T cells on gut integrity. Loss of mucosal integrity can be an underlying cause of sepsis and death after massive injury, and γδ T cells are known to reside in great numbers in the gut, but the evidence is mixed as to their effects there after injury. Additionally, γδ T cells may contribute to neutrophil accumulation and local gut injury after remote burn injury by promoting production of TNF-α by neutrophils because studies have shown that anti-TNF-α antibodies decrease mucosal atrophy after injury.\textsuperscript{64–66} Mice that are TCR δ-deficient have decreased TNF-α levels after burn and a level of apoptosis and cell turnover that is less than injured wild-type animals.\textsuperscript{67,68} However, it is unclear at this point how the balance of immunoprotective vs the immunopathogenic effects of γδ T cells after injury relate to eventual clinical outcomes and whether they can become a potential clinical target in the future. The references discussed above are summarized in Table 3.

### Gamma-Delta T Cells in Infection and Sepsis

Recent studies of γδ T cells have shed light onto their roles in infection and sepsis, although sometimes with conflicting results.\textsuperscript{69,70} For example, compared with wild-type control mice, those deficient in γδ T cells have been shown to be more susceptible to Klebsiella pneumoniae sepsis. Enhanced susceptibility in the absence of γδ T cells correlated with lower circulating levels of IFN-γ and TNF-α. Meanwhile, other studies showed decreased morbidity in γδ T cell-deficient mice infected with Listeria monocytogenes.\textsuperscript{69,70} These results may represent a bimodal response for γδ T cells, where during early phases of infection and sepsis they act as proinflammatory cells that link the innate and adaptive immune responses and later act to down regulate the immune response altogether. Alternatively, these differences may be because of the roles of distinct subsets of γδ T cells and their individual roles in the immune response to various pathogens.\textsuperscript{56,70} Experimental infection with Listeria monocytogenes showed that during the early phase of infection, γδ T cells activate the adaptive immune response as well as NK cell function, possibly through the action of proinflammatory cytokines.\textsuperscript{71,72} The clearance of bacteria in this model was IFN-γ dependent, with most of the detected IFN-γ coming from NK and T cells, and in the absence of γδ T cells, there were reduced levels of IFN-γ. Signaling between γδ T cells, NK cells, and T cells may involve the expression of IFN-γ, TNF-α, or

### Table 3. γδ T cells

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<tr>
<th>Reviewed Article</th>
<th>Model System(s)</th>
<th>Major Findings</th>
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<tbody>
<tr>
<td>Schwacha et al\textsuperscript{62}</td>
<td>Mouse scald burn</td>
<td>At 48 hours after injury, mortality was three fold greater in γδ T cell-deficient mice. Splenic macrophages collected at seven days produced less tumor necrosis factor-α and interleukin-6.</td>
</tr>
<tr>
<td>Balazs et al\textsuperscript{64}</td>
<td>Mouse scald burn</td>
<td>γδ T cells contributed to neutrophil-mediated tissue destruction in lung and small intestines after burn.</td>
</tr>
<tr>
<td>Wu et al\textsuperscript{68}</td>
<td>Mouse scald burn</td>
<td>γδ T cells contributed, in part, to intestinal mucosal damage after burn; however, some intestinal damage still occurred in the absence of γδ T cells.</td>
</tr>
<tr>
<td>O’Brien et al\textsuperscript{70}</td>
<td>Mouse sepsis model with intravenous L. monocytogenes</td>
<td>Depletion of certain subsets of γδ T cells increased bacterial clearance, though depletion of all γδ T cell associated with lower bacterial clearance.</td>
</tr>
<tr>
<td>Hirsh et al\textsuperscript{76}</td>
<td>Mouse CLP</td>
<td>γδ T cells accumulated in lungs after CLP and their dysfunction appeared to contribute to acute lung injury.</td>
</tr>
<tr>
<td>Matsushima et al\textsuperscript{77}</td>
<td>Patients with systemic inflammatory distress syndrome</td>
<td>Systemic inflammatory distress syndrome correlated with loss of circulating γδ T cells.</td>
</tr>
<tr>
<td>Chung et al\textsuperscript{80}</td>
<td>Mouse CLP</td>
<td>Mice deficient in γδ T cells lacked inflammatory burst after CLP and had increased mortality.</td>
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CLP, cecal ligation and puncture.
other cytokines. Interestingly, in later stages of *L. monocytogenes* infection, γδ T cells act as a brake on the immune system, and in models of γδ T cell-deficient mice, there is an exaggerated inflammatory response leading to increased morbidity. A clinical correlate of the idea of early and late responses to pulmonary infection by γδ T cells was seen in patients with TB, in which loss of γδ T cells correlated with disease progression and increased inflammation and damage in the lung itself, while patients exposed to TB but without evidence of disease had higher levels of γδ T cells.

It has also been suggested that γδ T cells kill neutrophils during sepsis. Hirsh et al gave evidence that γδ T cells recognize HSP72 on neutrophils stimulated by LPS in a murine sepsis model, leading to direct killing of the neutrophils. Early after LPS-induced sepsis, γδ T cells showed signs of dysfunction, but in animals that survived, γδ T-cell function was not impaired, suggesting that loss of γδ T-cell function early lends to uncontrolled inflammatory responses. Related to this, defects in γδ T-cell number and function also were described in patients with systemic inflammatory response syndrome and sepsis. Matsushima et al noted that patients with SIRS had a decrease in number of circulating γδ T cells versus healthy controls and that the few γδ T cells that remained exhibited early signs of activation. Interestingly, the magnitude of γδ T cell loss and early activation marker expression correlated positively with the overall magnitude of SIRS.

Recently Chung et al explored the role of intraepithelial γδ T cells in sepsis after CLP. Although αβ T cells were reduced in gastrointestinal mucosa after CLP, γδ T cell numbers were increased. Additionally, although CLP increased plasma levels of TNF-α, IL-6, and IL-12 in wild-type mice, those deficient in γδ T cells had no such proinflammatory burst in cytokine levels and showed enhanced mortality. Therefore, it appears that the innate immune system requires γδ T cells in the gut and perhaps elsewhere, for effective bacterial clearance and that further investigation is warranted to understand their role in host defense during sepsis. A summary of studies that examine γδ T cells in infection and sepsis is provided in Table 3.

### CD4⁺CD25⁺ REGULATORY T CELLS (Treg)

Regulatory T cells are a heterogeneous class of lymphocytes that arise in the thymus and have an overall anti-inflammatory phenotype. Treg cells comprise 5% to 10% of CD4⁺ T cells in the peripheral circulation and lymphoid compartment but are relatively scarce in tissues and bone marrow. Broadly, they can be classified as either naturally occurring or inducible. Natural regulatory T cells are now classified as being CD4⁺CD25⁺ Treg cells. Inducible regulatory T cells include Tr1 cells, Th3 cells, and CD8⁺ regulatory T cells and are divided according to how they mature and the balance of anti-inflammatory cytokines they produce. For the purposes of this review, we will consider only the naturally occurring Treg cells and will refer to them hereafter as “Treg.”

Treg cells are defined by their constitutive expression of CD4, CD25, and the fork-head box P3 (FoxP3) transcription factor expression. Other markers seen in conjunction with these cells are CD38, CD62L, CD103, and glucocorticoid-induced tumor-necrosis factor receptor.

It is thought that their main role is to protect against autoimmunity by facilitating of tolerance to self-antigens. Treg cells require IL-2 for activation, and they have a TCR that allows recognition of a wide range of alloantigens on MHC class II. In response to MHC class II recognition, Treg cells can inhibit IL-2 release by both CD4⁺ and CD8⁺ T cells, mostly through inhibition mediated by TGF-β and IL-10. Treg cells also may be involved in regulation of NKT cells, as it has also been suggested recently that Treg cells inhibit NKT cell function while others suggest that likewise, NKT cells can augment Treg proliferation perhaps by NKT cell-derived IL-2. Further evidence for the role of Treg cells in tolerance comes from the observation that Treg cells are reduced both in number and function in certain types of autoimmune disease.

### Treg Cells in Injury

Because of the apparent role of Treg cells in regulation of the immune response after infection, investigators have examined whether Treg are involved in the suppression of the innate immune system after injury alone. Murphy et al examined whether Treg cells modulated the innate inflammatory process after burn injury. Seven days after burn, splenocytes depleted of T and B cells exhibited an exaggerated response to in vitro stimulation with either LPS or peptidoglycan (PGN) vs sham-injured animals. However, in Rag1-deficient mice, the inflammatory response was even greater, indicating that a T-cell population mitigated the response. Transfer of CD4⁺ T cells from wild-type mice to Rag1-deficient mice decreased the level of inflammation at 7 days. A series of transfer experiments revealed that transfer of CD4⁺CD25⁺ Treg cells to either Rag1-deficient mice or mice depleted of T and B cells at the time of injury reduced the magnitude of the inflammatory...
response to the level of burn burn-injured wild-type mice as indicated by levels of TNF-α, IL-1β, and IL-6. Because splenocytes in this study were stimulated with LPS or PGN, the findings indicate that the suppression involved signaling via the TLR4 (LPS) and TLR2 (PGN) receptors’ responses.

Additional studies by Choilean et al91 explored the mechanisms and timing of adaptive immunity suppression by Treg cells in a burn model. Proliferation assays revealed an increase of activity from draining lymph node Treg cells, but not spleen Tregs. They observed that conventional T cells produced high levels of the Th1 cytokine IFN-γ, whereas purified Treg cells produced low levels of Th1 cytokines and increased levels of IL-10 and TGF-β. Co-culture of Treg and CD4+ Treg cells resulted in decreased CD4+ T-cell proliferation only if direct cell-to-cell contact was allowed. Furthermore, co-cultures infused with anti-IL-10 or control IgG did not effect CD4+ T-cell proliferation, whereas infusion with anti-TGF-β revealed a dose-dependent inhibition of proliferation. Taken together, the inhibition caused by Treg cells on CD4+ T-cell proliferation in vitro was dependent on cell contact and TGF-β but not IL-10. Together, these results indicate a powerful role for the regulation of Th1 immunity after burn regulated by Treg cells, through direct contact and the secretion of TGF-β.

MacConmara et al92 examined the possible impact of Treg cells in trauma patients. Peripheral blood was collected from 19 patients and 5 control patients on days 1 and 7 after injury. The percentages of Treg cells and CD4+ T lymphocytes were determined by FACS analysis, and all CD4+ cells were collected by bead sorting. The percentage of both CD4+ T cells and Treg cells increased after trauma from days 1 to 7. Additionally, MacConmara examined the contribution of Treg cells to the regulation of Th1- and Th2-type cytokine production by CD4+ T cells collected from patients. Collections of all CD4+ T cells from trauma patients showed significant decreases in IFN-γ production upon in vitro stimulation. However, when Tregs were depleted, CD4+ T cells from trauma patients resumed their production of IFN-γ. The total patient Tregs showed increased production of IL-4, IL-5, and IL-10, compared with Tregs from control patients. Therefore, in in vitro assays, Treg cells from injured patients inhibited the Th1 (IFN-γ) response, and show increased expression of anti-inflammatory cytokines after trauma. Together, these results suggested involvement of Treg cells in the suppression of adaptive immunity subsequent to burn alone. Whether the Treg-mediated down-regulation of the adaptive immune system affects clinical outcomes remains to be determined. A summary of their known role in injury is provided in Table 4 and Table 5.

### Table 4. Treg cells

<table>
<thead>
<tr>
<th>Reviewed Article</th>
<th>Model System(s)</th>
<th>Major Findings</th>
</tr>
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<tbody>
<tr>
<td>Murphy et al90</td>
<td>Mouse burn + LPS or PGN stimulation</td>
<td>Treg cells decreased inflammatory cytokine release 7 days after burn and LPS or PGN stimulation.</td>
</tr>
<tr>
<td>Choilean et al91</td>
<td>Mouse burn model and in vitro co-cultures</td>
<td>Treg cells caused a decrease in CD4+ proliferation after burn dependent on cell-to-cell contact and transforming growth factor-β.</td>
</tr>
<tr>
<td>MacConmara et al92</td>
<td>In vitro assay with human trauma patient blood</td>
<td>Treg cells from injured patients inhibited Th1 response in vitro and increased anti-inflammatory cytokine production.</td>
</tr>
<tr>
<td>Caramalho et al93</td>
<td>Murine in vitro</td>
<td>Treg cells responded to LPS stimulation without presentation by antigen-presenting cells.</td>
</tr>
<tr>
<td>Heuer et al94</td>
<td>Mouse CLP with adoptive transfer</td>
<td>Mice receiving transfer of Treg cells had increased bacterial clearance and mast cell recruitment as long as the mice had an existing intact T-cell response.</td>
</tr>
<tr>
<td>Scumpia et al95</td>
<td>Mouse CLP</td>
<td>Mice depleted of Treg cells with CLP had no difference in mortality versus control mice with CLP.</td>
</tr>
<tr>
<td>Monneret et al98</td>
<td>Human septic patients</td>
<td>Decreased numbers of circulating Treg cells were seen early in sepsis, however their numbers eventually returned to normal.</td>
</tr>
</tbody>
</table>

CLP, cecal ligation and puncture; LPS, lipopolysaccharide; PGN, peptidoglycan.
the potential for these cells to have clinical effects in models of sepsis and infection. Caramalho et al\textsuperscript{93} reported that Treg cells respond to LPS stimulation without antigen presentation from APCs through recognition from Toll-like receptors followed by proliferation and activation, which represents a novel finding of a T lymphocyte carrying a receptor normally relegated to the innate immune system and offers a possible mechanism by which Treg cells may help bridge the gap between innate and adaptive immunity.\textsuperscript{93}

Heuer et al. recently showed that Treg cells have protective effects in a CLP model of polymicrobial sepsis.\textsuperscript{94} Before being given CLP, mice received either vehicle only, conventional CD4\textsuperscript{+} T cells, unstimulated Treg cells, or Treg cells previously stimulated with IL-2, anti-CD3, and anti-CD28. Two weeks after CLP, mice given either vehicle or CD4\textsuperscript{+} T cells had significantly greater mortality compared with mice given unstimulated Treg cells. However, mice given stimulated Treg cells had the lowest rates of mortality and overall best clinical outcome. This effect was dependent on the host having an intact T-cell population because no protective effect was observed when Treg cells were transferred to athymic mice given CLP. Mice given Treg cells had significant increase in bacterial clearance and mast cell recruitment to the peritoneum. In contrast, a recent study by Scumpia et al\textsuperscript{95} used CLP and mice depleted of Treg cells found no difference in mortality and small increase in Treg populations with intact mice with sepsis. Yet, other models of intestinal infection have presented evidence of decreased severity of disease with Treg cells but usually at the cost of higher bacterial loads.\textsuperscript{96,97} The presumption in these studies is that through the actions of IL-10 and TGF-β, Treg cells limit the inflammatory response to infection but inhibit clearance of disease.

Although animal models have generally suggested protective effects for Treg cells in systemic infection and sepsis, human data have not been as revealing. In patients with sepsis, an initial marked decreased in absolute numbers of both CD4\textsuperscript{+} cells and Treg cells is observed in the circulation.\textsuperscript{98} Over time, the percentage of Treg cells eventually increases to healthy control levels, which may be a result of a resistance to apoptosis in Treg populations.\textsuperscript{99} In patients with sepsis and immune paralysis, as evidenced by decreased histocompatibility leucocyte antigen DR on monocytes, nonsurvivors had the highest levels of Treg cells. Whether the increased Treg cells was symptom of advanced sepsis or contributed to it was not delineated.\textsuperscript{99}

## CONCLUSION

The idea that considerably rare populations of regulatory lymphocytes have such profound effects on immunity after burn injury and during sepsis is a relatively new concept in the study of the immune consequence of trauma and systemic infection. However, the studies outlined in this review support this notion and in many cases provide convincing evidence that regula-
tory lymphocytes, including NK, NKT, γδ, and Treg cells, indeed control both the adaptive and innate arms of immunity after injury and during sepsis. Although the precise mechanisms by which these cells regulate immunity remains to be determined in some instances, it is clear that a common mechanism by which regulatory lymphocyte subsets control immune responses is via the production of immunomodulatory cytokines, including IFN-γ, IL-4, IL-5, IL-10, and TGF-β. The advent of highly specific antibodies to block cell function (i.e., anti-CD1d mAb for NK T cells and anti-CD25 for Tregs) and the development of transgenic cell-deficient mice (i.e., CD1d and Jα281 ko for NK T cells, and δ TCR ko for γδ T cells) have allowed investigators to conduct complex studies to unequivocally identify roles for these cells in injury and sepsis-related immunopathology. Although considerably more research is needed to understand how rare lymphocyte subsets exert such profound regulation over immune function, the studies reviewed here allow us to develop new perspectives on the immunopathologic consequences of trauma and infection. As obvious sentinels of innate and adaptive immunity, regulatory lymphocytes represent attractive new targets for the development of therapeutics for injury-induced immune paralysis and sepsis.

REFERENCES

78. Venet F, Bohe J, Debard A, et al. Both the percentage of y8
T lymphocytes and CD3 expression are reduced during septic shock. Critical Care Medicine 2005;33:2836–40.