Injury-Induced Suppression of Effector T Cell Immunity Requires CD1d-Positive APCs and CD1d-Restricted NKT Cells

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Injury-Induced Suppression of Effector T Cell Immunity Requires CD1d-Positive APCs and CD1d-Restricted NKT Cells

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Overwhelming infection remains the leading cause of death from serious burn injury despite recent advances in the care of burn patients and a better understanding of immune and inflammatory consequences of injury. In this study, we report a critical requirement for CD1d-restricted NKT cells and CD1d expression by APCs in the immune dysfunction that occurs early after burn injury. Using a well-established murine scald injury model with BALB/c and BALB/c CD1d knockout mice, we investigated whether peripheral T cell immunity was affected by the presence or absence of CD1d-restricted NKT cells in the early stages after injury. Using Ag-specific delayed-type hypersensitivity, T cell proliferation, and cytokine production as indices of immune responsiveness, we observed that both CD1d expression by APCs and CD1d-restricted NKT cells are required for immune suppression after injury. Via adoptive transfer of splenocytes from injured mice to uninjured recipients, we found injury-induced suppression of immunity to be Ag specific, long lasting, and critically dependent on cell surface expression of CD1d by APCs. Together, our results suggest that the defects in T cell responsiveness that occur subsequent to severe burn injury are not merely the result of global or passive suppression, but instead represent an active form of CD1d/NKT cell-dependent immunologic tolerance. The Journal of Immunology, 2006, 177: 92–99.

Nearly 100,000 people each year in the United States suffer serious burn injury, 6% of which ultimately succumb to their wounds. Approximately 30% of burn patients that die do so as a result of serious infectious complications (1). For over five decades it has been recognized that burn injury results in marked suppression of immune function, resulting in life-threatening systemic infections, sepsis, multiple organ failure, and death. Despite profound advances in care for the critically injured and comprehensive knowledge of inflammatory and immunologic processes, the major cause of death among burn patients today continues to be infection (1) and not necessarily metabolic alterations or events directly related to the physical damage of the skin itself.

Immune function after major trauma has been well studied and is known to go awry due, in part, to the overproduction of numerous proinflammatory mediators (2–5), alterations in the production of granulocyte and monocyte progenitors (6, 7), and increased production of immunomodulatory cytokines, including IL-4, IL-10, and TGF-β (4, 8–10). Investigations over the past two decades have provided new insight into the roles of macrophages, dendritic cells, and T lymphocytes in regulating immunity during injury and sepsis (11–13). With the exception of a few isolated reports (8, 14), modulation of the immune response to injury by less numerous, nonconventional lymphocyte populations, such as γ-δ T cells, regulatory T cell subsets, and CD1d-restricted NKT cells in particular, has remained largely unexplored.

CD1d-restricted NKT cells are known to regulate T cell immunity during autoimmunity, immunologic tolerance, and cancer (15–19). We recently identified a role for CD1d stimulation of NKT cells in injury-induced suppression of T cell immunity (8). In this study, we expand upon those initial findings and identify a critical requirement for both CD1d expression and NKT cells themselves in the immune dysfunction that occurs early after burn injury. Moreover, our data show that injury-induced suppression of Ag-specific immunity can occur at the effector stage of immunity and can be adoptively transferred to both naive and Ag-presensitized hosts in a CD1d-dependent manner. Given the specificity and permanency of the adoptively transferred immune suppression, our results indicate that immune dysfunction that occurs secondary to severe injury may actually represent immunologic tolerance. The novel findings presented in this work provide new insight into the cellular mechanisms involved in immune suppression that occurs secondary to severe injury.

Materials and Methods

Animals

Eight- to 10-wk-old male BALB/c and BALB/c CD1d knockout (ko)3 mice used in these studies were obtained from The Jackson Laboratory. NKT cell-deficient BALB/c-Jtk281 ko mice were provided by M. Taniguchi (RIKEN, Yokohama, Japan). All mice used in this study were housed on a 12/12-h light/dark cycle and provided with food and water ad libidum. All mice were treated humanely and in accordance with guidelines set forth by the Loyola University Institutional Animal Care and Use Committee and the National Institutes of Health.

3 Abbreviations used in this paper: ko, knockout; DTH, delayed-type hypersensitivity; HSA, human serum albumin; WT, wild type.
**Burn injury model**

All mice were given dorsal scald (or sham) injury using a method previously described by Walker and Mason (20) and modified by Faunce et al. (21). Briefly, mice were anesthetized with a mixture of ketamine and xylazine (40 mg/kg, i.p.) and had their dorsal surfaces shaved with animal clippers. Mice were then placed into a plastic template that exposed 15–17% of their total body surface area, calculated by the method of Spector (22). The mouse and template were immersed into a 100°C water bath for 8 s. Sham control animals were anesthetized, placed into the template, and exposed to a room temperature water bath. After the water exposure, the mice were dried immediately to prevent further scalding, given 1.0 ml of i.p. fluid resuscitation (0.9% normal saline), and placed under warming lamps until recovery from anesthesia.

**Delayed-type hypersensitivity (DTH)**

DTH was induced, as previously described (18). Briefly, mice were inoculated s.c. at the nape of the neck with 100 μl of an emulsion containing 100 μg of OVA (Sigma-Aldrich) in CFA (Sigma-Aldrich). In some experiments, mice were comminuted with an emulsion containing 100 μg of OVA and 100 μg of human serum albumin (HSA) (Sigma-Aldrich) in CFA. Seven days later, mice had both ears measured with an engineer’s micrometer (Mitutoyo) and were given an intradermal inoculation of 10 μl of PBS containing 200 μg of OVA to the left ear pinna, and a similar inoculation of HSA to the right ear pinna as an Ag specificity control. Twenty-four hours later, ear measurements were made again, and the change in ear swelling was calculated as an index of DTH. All OVA and HSA solutions used for ear challenge as well as in vitro cultures (described below) were removed of potentially contaminating endotoxin by passage through DetoxiGel polysorb B chromatography columns (Pierce-Endogen).

**In vitro recall proliferative response to Ag**

To prepare splenocyte suspensions, spleens were minced and passed through fine wire mesh, and all debris was removed. Erythrocytes were removed by ammonium chloride lysis, and the remaining cells were resuspended in RPMI 1640 containing 5% FCS, penicillin-streptomycin, and glutamine. Cell viability was checked by trypan blue exclusion. Splenocytes were plated at 2.0 × 10^5 cells/well in 96-well plates in either RPMI 1640 alone or RPMI 1640 containing OVA (200 μg/ml) (Sigma-Aldrich) and cultured at 37°C, 5% CO2, for 48 h. After 48 h of culture, 1 μCi of [3H]thymidine ([3H]TdR) (Amersham Biosciences) was added to each well and the cells were cultured for an additional 16 h. [3H]TdR incorporation was assessed by scintillation counting and used as an index of OVA-specific splenocyte proliferation.

**Measurement of cytokine production**

Spleen cell suspensions were prepared as described above for in vitro proliferative assays and plated at 2.0 × 10^5 cells/well in 96-well plates with either RPMI 1640 alone or RPMI 1640 containing anti-CD3ε mAb (2.5 μg/ml, clone 145-2c11) and cultured for 24 h. At the end of the culture period, the plates were centrifuged at 1200 rpm for 10 min, and the supernatants were collected and stored at −80°C. The levels of IL-4 and IFN-γ in the supernatants were determined with commercially available ELISA kits (BD Pharmingen), conducted according to the manufacturer’s specifications. ELISA plates were read using an Amersham Biosciences SpectraMAX Plus 384 plate reader, and analyses of ELISA data were done with SoftMax Pro.

**Enrichment of F4/80- and CD11c-positive APCs**

Whole splenocytes were obtained from donor mice 24 h post-burn or post-sham injury. Erythrocytes were removed by ammonium chloride lysis, and remaining cells were washed and incubated for 10 min with anti-CD16/32 Ab (clone 93; eBioscience) to block Fc binding. Cells were then incubated with mouse anti-CD19 microbeads (Miltenyi Biotec), according to manufacturer’s instructions, and CD19-positive cells were depleted using magnetic AutoMACS columns. CD19-negative cells were then incubated with an allophycocyanin-conjugated anti-F4/80 (clone BM8; Caltag Laboratories) and anti-CD11c (clone N418; eBioscience) Ab mixture. Cells were washed and incubated with anti-allophycocyanin magnetic microbeads (Miltenyi Biotec), according to manufacturer’s instructions. APCs were positively selected using AutoMACS magnetic column separation. The F4/80- and CD11c-positive fractions were immunostained with PE-conjugated B220 (clone RA3-6B2; eBioscience). The enrichment of APCs from magnetic separation was determined to be 94–95% via flow cytometric analysis.

**Adoptive transfer of total splenocytes or enriched splenic T cells**

For adoptive transfer experiments, 1.0 × 10^7 total splenocyte cells obtained from either sham- vs burn-injured mice were resuspended in 200 μl of sterile HBSS and injected via the tail veins of syngeneic recipients that were either naive or immunized with OVA 7 days earlier. In some experiments, only IMMULAN-enriched splenic T cells (i.e., depleted of B cells and APCs) were injected. Splenic T cell enrichment was achieved by passage of total splenocyte cells over IMMULAN goat anti-mouse IgG-coated glass bead columns (Biotec Laboratories). Briefly, 24 h after either sham or burn injury, total splenocytes were prepared and removed of erythrocytes by ACK lysis. The splenocyte preparations were resuspended in serum-free medium and loaded onto 15 ml of IMMULAN columns, according to the manufacturer’s protocol. The enrichment procedure routinely resulted in removal of 94–96% of all B cells, macrophages, and dendritic cells, as determined by flow cytometry.

**Adoptive transfer of splenic APCs**

APCs were enriched by magnetic separation, as described above, from either burn- or sham-injured BALB/c or BALB/c CD1d ko donor mice 24 h postinjury. A total of 2.0 × 10^6 cells was resuspended in 200 μl of sterile HBSS and injected into the tail veins of age-matched BALB/c mice that were immunized with OVA 7 days before the transfer.

**Statistical analyses**

Statistical determinations were made by either Student’s t tests or ANOVA and Neuman-Keuls posthoc analyses, where appropriate. Statistical significance was determined when p < 0.05.

**Results**

Resistance to injury-induced immune dysfunction in the absence of CD1d and CD1d-reactive NKT cells

To identify a role for CD1d-restricted NKT cells in the immune dysfunction that occurs within the first 24 h after injury, we first examined the effect of injury on the ability of wild-type (WT) BALB/c vs NKT cell-deficient BALB/c CD1d ko mice to display Ag-specific DTH. Because CD1d is required for positive selection of NKT cell precursors in the thymus (in addition to stimulation of NKT cells in the periphery), CD1d ko mice lack the CD1d-restricted invariant NKT cell population (23, 24). Briefly, WT vs CD1d ko mice were immunized with OVA in CFA 7 days before being given a 15% total body dorsal scald injury, as previously described (25). Immediately following injury, all mice were given an intradermal ear challenge with OVA, and 24 h later, the DTH responses (i.e., differences in ear swelling) between groups were compared. At 24 h postinjury, burn-injured BALB/c WT mice demonstrated significantly lower OVA-specific DTH responses compared with sham-injured WT mice (Fig. 1A). The magnitude of the DTH response in burn-injured WT mice was only one-fifth of what was observed in sham-injured animals. In contrast, we found no statistically significant differences in the magnitude of DTH responses between sham- vs burn-injured CD1d ko mice (Fig. 1A). Moreover, the DTH responses of burn-injured CD1d ko mice were ~3-fold greater in magnitude compared with burn-injured WT mice. The observation that CD1d-deficient sham-treated mice had lower DTH responses than their WT counterparts is in line with studies by others identifying a role for NKT cells in amplifying T cell immunity and with our previous reports that showed lower OVA-specific DTH responses in WT mice treated with anti-CD1d mAb systemically (8).

As an additional measure of immune reactivity of WT vs CD1d ko mice subjected to injury, we also examined splenic T cell proliferation in response to rechallenge with OVA in vitro. Briefly, splenocytes were obtained from OVA-immunized WT vs CD1d ko mice 24 h after being given either sham or burn injury. Splenocytes were placed in culture in the presence or absence of OVA, and [3H]TdT incorporation was used as an index of cellular proliferation. Although total splenocytes were cultured with OVA, the

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spleenocytes were obtained from OVA-CFA-immunized mice; thus, the only cells that should proliferate in response to secondary challenge with OVA in vitro would presumably be T cells bearing an OVA-specific TCR. Similar to the DTH results described above, we observed that splenic T cells from burn-injured WT mice demonstrated a significantly lower proliferative response to a secondary Ag challenge in vitro compared with T cells from sham-injured mice (Fig. 1B). Likewise, we found that splenic T cells from burn-injured CD1d ko mice were not impaired in their ability to proliferate in response to secondary Ag challenge in vitro (Fig. 1B).

Absence of CD1d and CD1d-reactive NKT cells prevents injury-induced alterations in IL-4 and IFN-γ by splenic lymphocytes

We previously reported that during the first 24 h after burn injury, NKT cells fail to produce IFN-γ and acquire a predominantly IL-4-producing phenotype (8). In this study, we examined the production of IFN-γ and IL-4 in splenic lymphocyte cultures from sham- vs burn-injured WT vs CD1d ko mice collected at 24 h after injury and given polyclonal stimulus in vitro. Splenic lymphocytes from burn-injured WT mice produced nearly 3-fold greater the amount of IL-4 in response to immobilized anti-CD3e stimulation compared with lymphocytes from sham-injured animals (Fig. 2A). Interestingly, lymphocytes from burn-injured WT mice also had impaired production of IFN-γ relative to sham-injured animals (Fig. 2B). In contrast, IL-4 production was not elevated in response to injury in CD1d ko mice, nor was IFN-γ production impaired. Although we did not examine NKT cell-derived IL-4/IFN-γ per se in this experiment, we previously reported that at 24 h after burn injury, the only cells in the spleen that produce IL-4 are CD1d-restricted NKT cells (8). The effects of cytokines from other cell types within the cultures (i.e., T cells, NK cells, APCs, etc.) on NKT cell cytokine production or function cannot be ruled out, however. Together, these findings support the notion that injury-induced elevation in IL-4 and suppression of IFN-γ are CD1d dependent.

Adoptive transfer of injury-induced immune dysfunction to both naive and OVA-presensitized recipients

The ability of OVA-immunized CD1d ko mice to mount robust DTH responses after burn injury suggested that in WT mice, injury caused an active suppression of the efferent T cell response in a manner that required either CD1d expression on the APC population or the CD1d-restricted NKT cells themselves. To begin to address the cellular mechanisms and timing of active suppression after burn injury, we used a combination of adoptive transfer and OVA-specific DTH assays to test the ability of immune cell subsets to suppress the different phases of the immune reflex arc. First, total (i.e., unfraccionated) spleenocytes and enriched splenic T cells were prepared from OVA-immunized WT BALB/c mice that had sustained either sham or burn injury 24 h earlier. Enriched splenic T cells were obtained by passage of total spleenocytes over IMULAN columns to remove macrophages, dendritic cells, and B cells (Fig. 3A). The resultant enriched T cell preparations contained mainly T, NK, and NKT cells (88, 5.4, and 1.2%, respectively). The residual CD3-negative, Ly-49C-negative fractions contained 1% Gr-1+ cells and 3.3% residual B cells (data not shown). The enrichment procedure also removed 96% of all macrophages and dendritic cells (F4/80 and CD11c positive) (Fig. 3A). The different cell preparations were injected (1.0 × 107 cells/mouse, i.v.) into naive syngeneic WT BALB/c recipient mice. Twenty-four hours after cell transfer, all mice were immunized with OVA in CFA (s.c.), as described in Materials and Methods. A group of mice that received no cell transfer, but OVA immunization, served as an additional control. Seven days later, all mice received a secondary intradermal ear challenge with OVA and the DTH response was determined. Mice that received adoptive transfer with cells from sham-injured mice mounted DTH responses that were comparable with those seen in mice that received OVA immunization and secondary challenge, but no cell transfer (Fig. 3B).
peritoneal macrophages were collected from naive syngeneic mice. Briefly, thioglycolate-elicited APCs, as previously described (8). Seventy days post-injury, recipient mice were challenged intradermally with OVA in HBSS in the left ear pinnae.

In the experiments described above, cells from sham- or burn-injured animals were adoptively transferred to naive recipient mice before the recipient’s primary OVA immunization. Although adoptively transferred cells from burn-injured mice suppressed OVA-specific DTH in the recipients, the experiment did not allow us to distinguish whether the transferred suppression of T cell immunity occurred at the priming or effector stage. To determine whether cells from burn-injured mice regulated effector T cell immunity, we next adoptively transferred total spleen cells vs enriched splenic T cell preparations from OVA-immunized sham vs burn-injured mice to syngeneic recipients that had also been immunized with OVA in CFA 7 days earlier (i.e., presensitized). Mice that received no cells were included as an additional control. Twenty-four hours after transfer of cells, the recipients were given a secondary intradermal ear challenge with OVA, and the DTH response was determined 24 h later. Presensitized mice given either total spleen cells or enriched splenic T cell preparations from sham-treated donors mounted robust DTH responses after secondary challenge with OVA (Fig. 4). Presensitized mice given no cell transfer demonstrated robust DTH responses, as expected. In contrast, presensitized mice given total spleen cells or splenic lymphocytes from burn-injured donors had suppressed OVA-specific DTH responses 24 h after secondary challenge (Fig. 4). Thus, adoptively transferred cells (total splenocytes or just enriched splenic T cell preparations) suppressed OVA-specific DTH in non-injured recipients that were previously sensitized to the same Ag.

To identify whether the active suppression of T cell immunity via adoptive transfer of cells from burn-injured mice was permanent or transient, all transfer recipients were challenged intradermally in the ear pinnae at 14 and 28 days after the adoptive transfer. Because BALB/c mice, once immunized with OVA, are known to develop anaphylaxis in response to multiple cutaneous challenges with soluble Ag, we tested the persistence of the suppression of OVA-specific DTH in the adoptive transfer recipients 14 and 28 days later via intradermal injection of OVA-bearing APCs, as previously described (8). Briefly, thioglycolate-elicited peritoneal macrophages were collected from naive syngeneic mice and rested for 24 h in serum-free medium, followed by a 24-h culture with OVA (5 mg/ml). The OVA-pulsed cells were collected, extensively washed in HBSS, and injected into the ear pinnae in sterile HBSS (5.0 \times 10^4 cells/ear), and the DTH response was determined 24 h later. As expected, recipients of whole spleen cells from sham-injured mice mounted good DTH responses (Fig. 5), while recipients of total spleen cells from burn-injured animals continued to have suppressed OVA-specific immunity 28 days after the adoptive transfer of cells from injured donor mice. In contrast, we observed that recipients of splenic lymphocyte preparations from burn-injured mice no longer had suppressed DTH. Similar observations were made at 14 days after transfer (data not shown). Therefore, the adoptively transferred suppression of effector T cell immunity to OVA-primed, noninjured recipient mice was when enriched splenic T cell preparations were given, but long lasting when total splenocytes were used as the inoculum.

Transfer of injury-induced immune suppression is Ag specific

To determine whether the immune suppression generated in the adoptive transfer experiments was Ag specific vs global, we tested the ability of cells from burn-injured mice to suppress immunity to a third-party Ag in uninjured recipient mice. BALB/c donor mice were immunized with OVA in CFA and, 7 days later, given sham or scald burn injury. Twenty-four hours later, the OVA DTH response was measured in the donor animals to confirm burn-induced DTH suppression in animals that would serve as adoptive transfer donors (Fig. 6A). Ten million donor splenocytes were adoptively transferred to uninjured syngeneic recipients that were coimmunized simultaneously with OVA and HSA in CFA 7 days earlier. Recipient mice receiving no cells in the transfer, but immunized with OVA and HSA, served as an additional control. Twenty-four hours after transfer, recipient mice received a secondary challenge of OVA in the left ear pinnae and HSA in the right ear pinnae to test the Ag specificity of the transferred immune suppression. Recipients of both sham- and burn-injured cells generated a robust DTH response to HSA (Fig. 6B). However, the DTH response to OVA in recipients of cells from OVA-immunized, burn-injured donors was less than half that of the sham-injured recipients, demonstrating the specificity of the transferred immune suppression (Fig. 6B). Because the adoptive transfer of cells from OVA-challenged, injured donors suppressed the recipients’ DTH response to OVA, but not HSA, we concluded that the injury-induced suppression of T cell immunity was Ag specific and not the result of global or passive suppression.
CD1d is required for adoptive transfer of injury-induced immune suppression

In the experiments described above, we observed that splenocytes from burn-injured donors could induce long-term suppression of OVA-specific DTH when adoptively transferred to noninjured, OVA-immunized recipients. Because the adoptive transfer recipients were presensitized with OVA in CFA, our findings suggested that transfer of cells from the injured mice could suppress the recipient’s own effector T cell response in a manner that involved CD1d. Because we also observed that immune suppression in the injured mice themselves required either CD1d or NKT cells, we next tested whether CD1d was required for the adoptive transfer of injury-induced immune suppression. Briefly, mice that were presensitized with OVA in CFA received adoptive transfer of splenocytes from WT and/or CD1d ko mice that were given either sham or burn injury 24 h earlier. One day after adoptive transfer, all recipient mice were given an intradermal ear challenge with OVA, and changes in ear thickness were determined 24 h later. Recipients of CD1d ko cells from burn-injured mice did not exhibit the immune suppression that recipients of burn-injured WT cells did, showing that either the CD1d molecule itself or NKT cells are required to transfer Ag-specific immune suppression (Fig. 7).

APCs must have cell surface CD1d to induce Ag-specific immune suppression after injury

Because we observed that the CD1d molecule and CD1d-restricted NKT cells must be present in order for burn injury-induced immune suppression to occur or be transferred, and that total splenocytes (i.e., containing APCs), but not enriched splenic T cell preparations conferred long-term suppression, we next examined the requirement for CD1d cell surface expression on the APCs themselves. APCs were collected and enriched via magnetic bead isolation (see Materials and Methods) from either WT or CD1d ko OVA-immunized mice 24 h after burn or sham injury (Fig. 8A) and adoptively transferred to OVA-immunized WT mice. The enriched APCs used for transfer consisted of 94–95% macrophages and/or dendritic cells (F4/80+ CD11c+) or plasmacytoid dendritic cells (F4/80+ CD11c+ B220+) and were effectively depleted of B cells. All recipients were then given intradermal ear challenge with OVA, and DTH was measured 24 h later. Although recipients that received enriched APCs from burn-injured WT mice exhibited suppressed DTH, those that received enriched APCs from burn-injured CD1ko mice did not (Fig. 8B). Thus, injury-induced suppression was dependent upon APCs that expressed CD1d.

Injury-induced immune suppression by CD1d-positive APCs requires the presence of NKT cells

Using the adoptive transfer model, we observed that in order for APCs to confer long-term, Ag-specific, burn injury-induced immune suppression to uninjured recipients, the APCs must express CD1d on their surface (Fig. 8B). Because we also observed that suppression of T cell immunity in the burn-injured animals required NKT cells, we next tested whether adoptive transfer of injury-induced immune suppression by CD1d-positive APCs to uninjured recipients required NKT cells in those recipients. Briefly, WT-BALB/c donor mice were sensitized to OVA and 7 days later given sham or burn injury. Twenty-four hours after injury, APCs were purified from the injured mice and adoptively transferred to uninjured WT-BALB/c or NKT cell-deficient BALB/c-Ja281 ko mice that had been immunized with OVA-CFA 7 days earlier. One day later, the recipients were challenged intradermally with OVA on the ear pinnae and then examined for DTH reactivity 24 h later. WT recipients of burn APCs exhibited suppressed DTH responses, while NKT cell-deficient Ja281 ko mice that received burn APCs did not (Fig. 9A). As an additional measurement of immune responsiveness, we examined IL-2 and IFN-γ production by splenocytes from mice that received adoptive transfer of burn APCs. In agreement with the DTH findings, we observed suppressed production of IFN-γ by splenocytes from WT mice that received burn APCs. In contrast, splenocytes from Ja281 mice that received burn APCs produced IFN-γ levels that were comparable to mice that received sham control APCs (Fig. 9B). Interestingly, we observed that IL-2 production in both WT and Ja281 ko recipients was unaffected by transfer of burn APCs (data not shown). Together, these findings support the notion that adoptive transfer of injury-induced immune suppression by APCs requires NKT cells in the

**FIGURE 4.** Adoptive transfer of OVA-specific DTH with total splenocytes vs enriched splenic lymphocytes from sham- vs burn-injured mice to presensitized recipients. Seven days after OVA immunization, donor mice were given burn or sham injuries. Recipients that were immunized with OVA 7 days earlier received either 1 × 10⁷ total spleen cells or splenic lymphocytes (i.v.). Recipients were challenged intradermally 24 h later with OVA in HBSS in the left ear pinnae. Twenty-four hours later, ear thickness was measured in recipient mice as an index of DTH. Data are shown as mean change in ear thickness ± SEM. n = 5 mice per group. *, p < 0.05 vs recipients of cells from sham-treated mice.

**FIGURE 5.** Long-term effects of adoptive transfer of OVA-specific DTH with whole splenocytes vs enriched splenic lymphocytes from sham-vs burn-injured mice to presensitized recipients. Twenty-four hours after injury, cells were transferred to OVA-sensitized recipients, i.v. Transfer recipients were rechallenged in the ear pinnae with 5 × 10⁷ OVA-bearing APCs at 28 days postadoptive transfer. DTH was measured 24 h later. Data are shown as mean change in ear thickness ± SEM. n = 5 mice per group. *, p < 0.05 vs sham whole spleen cell recipients; **, p < 0.05 vs burn whole spleen cell recipients.
recipient; thus, CD1d-positive APCs and NKT cells must interact in some capacity to facilitate T cell suppression. Additionally, suppression of T cell immunity in uninjured recipients by burn APCs correlates with inhibition of IFN-γ production (but not IL-2) by recipient T cells.

Discussion

Previous studies by our laboratory showed that interruption of CD1d-NKT cell activation via systemic administration of anti-CD1d mAb prevented the suppression of T cell immunity that occurs subsequent to burn injury (25). Using CD1d ko mice and an adoptive transfer approach, we show in this study that suppression of T cell immunity after injury is not simply global or passive, but instead, it is an active form of suppression that is Ag specific, can be adoptively transferred, and is dependent upon APCs that express CD1d. Because adoptive transfer of purified CD1d-positive, but not CD1d-negative, APCs from injured mice could suppress a primed T cell response in uninjured recipient mice, we concluded that the APCs suppressed immunity in a manner that required CD1d-restricted NKT cells. Moreover, we observed that the CD1d-positive APCs from burn-injured mice induced long-lasting, if not permanent, suppression of peripheral effector T cell immunity, even after they were removed from the microenvironment of the injured host. Immune suppression was only transferred by CD1d-positive APCs if NKT cells were present in uninjured hosts on the injured host. Immune suppression was only transferred by CD1d-positive APCs from burn-injured mice induced long-lasting, CD1d-restricted NKT cells. Moreover, we observed that the CD1d-positive APCs from burn-injured mice induced long-lasting, if not permanent, suppression of peripheral effector T cell immunity, even after they were removed from the microenvironment of the injured host. Immune suppression was only transferred by CD1d-positive APCs if NKT cells were present in uninjured hosts. CD1d-negative APCs from uninjured mice could suppress a primed T cell response in uninjured recipient mice, whereas the APCs from burn-injured mice could suppress a primed T cell response in uninjured recipients. We conclude that the APCs suppressed immunity in a manner that required CD1d-restricted NKT cells. Moreover, we observed that the CD1d-positive APCs from burn-injured mice induced long-lasting, if not permanent, suppression of peripheral effector T cell immunity, even after they were removed from the microenvironment of the injured host. Immune suppression was only transferred by CD1d-positive APCs if NKT cells were present in uninjured hosts. CD1d-negative APCs from uninjured mice could suppress a primed T cell response in uninjured recipient mice, whereas the APCs from burn-injured mice could suppress a primed T cell response in uninjured recipients. We conclude that the APCs suppressed immunity in a manner that required CD1d-restricted NKT cells. 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Immune suppression was only transferred by CD1d-positive APCs if NKT cells were present in uninjured hosts. CD1d-negative APCs from uninjured mice could suppress a primed T cell response in uninjured recipient mice, whereas the APCs from burn-injured mice could suppress a primed T cell response in uninjured recipients. We conclude that the APCs suppressed immunity in a manner that required CD1d-restricted NKT cells. Moreover, we observed that the CD1d-positive APCs from burn-injured mice induced long-lasting, if not permanent, suppression of peripheral effector T cell immunity, even after they were removed from the microenvironment of the injured host. Immune suppression was only transferred by CD1d-positive APCs if NKT cells were present in uninjured hosts. CD1d-negative APCs from uninjured mice could suppress a primed T cell response in uninjured recipient mice, whereas the APCs from burn-injured mice could suppress a primed T cell response in uninjured recipients. 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Several regulatory lymphocyte subsets, including NKT cells, γδ T cells, and CD4+CD25+ cells, are now known to actively participate in host defense after injury or sepsis. In some cases, these regulatory lymphocytes appear to support host defense, while other subsets, including NKT cells, contribute to injury-induced defects in immunity. Studies by Schwacha et al. (14) showed that γδ T

![FIGURE 6](http://www.jimmunol.org/)  
Injury-induced immune suppression is Ag specific. Total spleen cells from sham- vs burn-injured donor mice (A) were adoptively transferred to recipients that were simultaneously presensitized with both OVA and HSA 7 days earlier. B. DTH responses to OVA and HSA were measured in recipients 48 h posttransfer. Data are shown as mean change in ear thickness ± SEM. n = 6 mice per group. *, p < 0.05 vs sham; **, p < 0.05 vs burn.

![FIGURE 7](http://www.jimmunol.org/)  
Adoptive transfer of spleen cells from burn-injured CD1d ko mice fails to suppress immunity in uninjured recipients. Uninjured, OVA-sensitized WT recipient mice received 1 × 10^7 total splenocytes from OVA-sensitized burn-injured WT or CD1d ko mice 24 h postinjury. Twenty-four hours posttransfer, recipients were challenged intradermally with OVA in the left ear pinnae. Twenty-four hours after the challenge, changes in ear thickness were calculated as an index of DTH. Data are shown as mean change in ear thickness ± SEM. n = 5 mice per group. *, p < 0.05 vs sham; **, p < 0.05 vs burn.

![FIGURE 8](http://www.jimmunol.org/)  
Injury-induced immune suppression is Ag specific. Total spleen cells from sham- vs burn-injured donor mice (A) were adoptively transferred to recipients that were simultaneously presensitized with both OVA and HSA 7 days earlier. B. DTH responses to OVA and HSA were measured in recipients 48 h posttransfer. Data are shown as mean change in ear thickness ± SEM. n = 6 mice per group. *, p < 0.05 vs sham; **, p < 0.05 vs burn.

![FIGURE 9](http://www.jimmunol.org/)  
Adoptive transfer of spleen cells from burn-injured CD1d ko mice fails to suppress immunity in uninjured recipients. Uninjured, OVA-sensitized WT recipient mice received 1 × 10^7 total splenocytes from OVA-sensitized burn-injured WT or CD1d ko mice 24 h postinjury. Twenty-four hours posttransfer, recipients were challenged intradermally with OVA in the left ear pinnae. Twenty-four hours after the challenge, changes in ear thickness were calculated as an index of DTH. Data are shown as mean change in ear thickness ± SEM. n = 5 mice per group. *, p < 0.05 vs sham; **, p < 0.05 vs burn.

![FIGURE 10](http://www.jimmunol.org/)  
Adoptive transfer of spleen cells from burn-injured CD1d ko mice fails to suppress immunity in uninjured recipients. Uninjured, OVA-sensitized WT recipient mice received 1 × 10^7 total splenocytes from OVA-sensitized burn-injured WT or CD1d ko mice 24 h postinjury. Twenty-four hours posttransfer, recipients were challenged intradermally with OVA in the left ear pinnae. Twenty-four hours after the challenge, changes in ear thickness were calculated as an index of DTH. Data are shown as mean change in ear thickness ± SEM. n = 5 mice per group. *, p < 0.05 vs sham; **, p < 0.05 vs burn.
cell-deficient mice were far more likely to succumb to experimental burn injury compared with WT controls. In those studies, Schwacha et al. (14) suggested that γδ T cells controlled macrophage proinflammatory cytokine production, namely IL-6 and TNF-α, after injury. Although NKT cells and γδ T cells are considered innate regulatory lymphocytes, CD4+CD25+ cells are considered part of the adaptive arm, and they were recently shown to regulate proinflammatory cytokine production after burns and sepsis (35).

In the studies shown in this work, we observed that efferent T cell immunity (as assessed by Ag-specific DTH, ex vivo lymphocyte proliferation, and IFN-γ production) was impaired in response to burn injury in a manner that required CD1d-positive APCs, CD1d-restricted NKT cells, or both. In this study, we relied upon Ag-specific DTH to show that CD1 expression by APCs was a requisite for suppression of T cell immunity after injury. DTH is a prototypical in vivo manifestation of peripheral T cell immunity in that it requires Ag presentation, costimulation, cytokine production, and effector T cell proliferation to proceed. In our hands, this assay has proven to be a reliable in vivo assessment of T cell immunity that closely replicates the observations seen with more commonly used in vitro experiments. Using an adoptive transfer approach, we observed that as few as 2 × 10⁶ purified APCs from CD1d-positive, but not CD1d-negative, mice could profoundly suppress OVA-specific DTH in uninjured, OVA-primed recipients. This finding strongly suggests that the transferred APCs stimulated the recipients’ own CD1d-restricted NKT cell population in a manner that led to suppression of peripheral T cell responsiveness in those recipients. Although our investigations in this study did not identify the precise molecular mechanism by which the CD1d-NKT cell-dependent suppression occurred, we previously showed that after injury, NKT cells lose their ability to produce IFN-γ and, instead, acquire a predominantly IL-4-producing phenotype (8). In those studies, we observed that early after injury, IL-4 was produced almost exclusively by the NKT cell population and not by conventional T cells. Indirectly, we provide additional evidence for this concept in this study, in that splenocytes from injured WT mice produced high levels of IL-4, while those from injured CD1d-deficient mice did not. Based on these findings together, we concluded that although NKT cells are critical for suppression of T cell immunity after injury, they require instruction from CD1d-positive APCs. Because APCs from injured CD1d-deficient mice could not suppress OVA-specific DTH, we conclude that APCs themselves are not sufficient to suppress T cell function, but that they must, instead, colocalize with and engage NKT cells via CD1d. This concept is supported by previous studies by Faunce et al. (18) that showed that APCs, NKT cells, and

FIGURE 8. Immune suppression by APCs from injured mice requires cell surface expression of CD1d. A. Splenocytes were obtained from sham- vs burn-injured donor mice, depleted of CD19-positive cells, and enriched for APCs using an aliphophycocyanin-conjugated F4/80 (clone BM8) and CD11c (clone N418) Ab mixture and magnetic beads. Cells were immunostained with PE-conjugated anti-CD1d and B220 (clone RA36B2) and checked on a flow cytometer for degree of APC enrichment. Data are expressed as mean percentage of F4/80- and CD11c-positive cells within the CD19-negative splenocyte fraction. B. Enriched APCs were transferred to OVA-sensitized uninjured recipients. Twenty-four hours posttransfer, recipient mice were challenged intradermally with OVA in HBSS in the left ear pinnae and DTH was measured another 24 h later. Data are shown as mean change in ear thickness ± SEM. n = 3 mice per group. *, p < 0.05 vs sham; **, p < 0.05 vs burn.

FIGURE 9. Burn-induced immune suppression by CD1d-positive APCs requires presence of NKT cells in the host. A. OVA-sensitized WT or Jα281 ko recipient mice received purifed APCs (1.0 × 10⁶) either burn-injured or sham OVA-sensitized WT mice via adoptive transfer. Twenty-four hours posttransfer, DTH was induced in recipient mice with OVA injections in the left ear pinnae. Twenty-four hours later, DTH was measured. Data are shown as mean change in ear thickness ± SEM. n = 6 mice per group (burn recipients). n = 4 mice per group (sham recipients). B, IFN-γ production in WT vs Jα281 ko recipient mice. Splenocytes from WT or Jα281 ko mice were cultured with or without plate-bound anti-CD3 for 24 h. IFN-γ content in the supernatants was measured by ELISA.
conventional T cells must form clusters in the lymphoid organs to suppress efferent T cell immunity during peripheral tolerance.

Work by our laboratory and others has previously identified a suppressive role for the APC (i.e., macrophages and/or dendritic cells) after burn injury in experimental mouse models (2, 21, 36, 37) and humans (10). In our studies, we did not distinguish whether the APCs responsible for injury-induced immune suppression were monocytes/macrophages vs dendritic cells, because we used a mixture of anti-F4/80 and anti-CD11c mAbs to isolate the cells. The novel findings that we present in this study though, as they pertain to the APC population, are that the APCs must express functional CD1d molecules on their surface to retain their suppressive capacity and that they can transfer long-lasting suppression, or immunologic tolerance, to uninjured hosts. The required involvement of CD1d on APCs in generating immunologic tolerance was also reported by Sonoda et al. (38), who showed that CD1d-positive APCs (but not CD1d-negative) could induce NKT cell-dependent efferent T cell tolerance toward Ags inoculated via the eye. Whether the suppression of T cell immunity observed in our experimental model represents an induced form of immunologic tolerance that results from secondary exposure to Ags in the early postburn period remains to be proven. However, the fact that the suppression was adoptively transferable to uninjured hosts, had considerable permanency, and was Ag specific, together support the idea that injury may lead to the induction of active immunologic tolerance, rather than global T cell suppression or anergy. In summary, our findings presented in this work advance our understanding of the cellular mechanism(s) that promotes immune suppression after injury, and identify CD1d or CD1d-restricted NKT cells as novel therapeutic targets for improving the clinical outcome of burn patients in the future.

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Disclosures

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References


