Mechanisms of allergy and clinical immunology

Innate responsiveness of CD8 memory T-cell populations nonspecifically inhibits allergic sensitization

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Background: Infection or stimulation of the innate immune system by non-specific microbial antigens is thought to educate the immune system to respond appropriately to allergens, preventing allergy.

Objective: To determine the immunologic pathways that might explain how infection/microbial exposure inhibits allergic sensitization.

Methods: Immunologic studies of non-antigen-specific functions of CD8 memory cells, their maturation in vivo, and their effects in a mouse asthma model, to test the hypothesis that CD8 memory is shaped by innate immunity in a way that can inhibit allergic disease.

Results: We found that CD8 memory T-cell (CD8 Tm) populations bridge innate and adaptive immunity by responding to either antigen or cytokines alone. CD8 Tm populations partially subvert the clonal selection process by activating their neighbors through induction of dendritic cell IL-12. Stimulation of innate or acquired immunity in the lung or gut causes expansion/maturation of CD8 Tm populations, which provide an early source of cytokines, enhance T(H)1 immunity, and inhibit allergic sensitization and airway inflammation/hyperresponsiveness in a non-antigen-specific fashion.

Conclusion: CD8 T-cell-mediated immune memory is long-lived and can retain its capacity for rapid cytokine release in a nonantigen-specific fashion. This novel type of memory enhances T(H)1 over T(H)2 immunity and prevents allergic sensitization after exposure to environmental antigens or infection. (J Allergy Clin Immunol 2008;122:1014-21.)

Key words: CD8 T cell, immunologic memory, innate immunity, allergy, T(H)1/T(H)2, dendritic cell, IL-12

Interaction between the innate and adaptive immune system is critical for establishing appropriate immune responses to newly encountered antigens and pathogens. In allergic disease, inappropriate immunity, skewed toward the T(H)2 phenotype, results in immunopathology triggered by allergen exposure. Cells of the innate system provide an early source of IFN-γ and other cytokines on first exposure to antigen, with IFN-γ central for the T(H)1 component of the response. Atopic patients are deficient in IL-12 after allergen challenge, and the immature phenotype of respiratory dendritic cells (DCs) contributes to poor IL-12 synthesis. Thus, early IFN-γ production is likely to be a limiting factor in ensuring a balanced response to inhaled allergens. Innate cells providing such DC-activating signals include NK cells, NKT cells, and γδ T cells. It is becoming apparent that CD8 T cells, normally considered a part of adaptive immunity, also participate in innate immune responses.

It has been proposed that exposure to various microbes nonspecifically suppresses allergy, increasing incidences of allergic diseases where infectious diseases are rare. Because early life events predict development of allergic disease, non-specific stimuli must have a long-lasting effect on adaptive responses to unrelated allergens. Memory lymphocytes with defined antigenic specificity emerge through the process of clonal selection. However, non-antigen-specific, innate immunity lacks immunologic memory because the receptors involved do not generate diverse specificities, and the cells are relatively short-lived. Therefore, epidemiologic evidence and data from rodent models suggest there must be non-antigen-specific or innate memory that develops in response to environmental antigens and pathogen-associated molecular patterns (PAMPs).

In the absence of Toll-like receptor (TLR) agonists, CD8 T cells may be essential for the T(H)1 component of immunity, and CD8 cells, triggered by cytokines alone, rapidly produce IFN-γ. Expression of nuclear factor of activated T cells c2 (NFATc2) in CD8 memory populations drives IFN-γ and suppresses T(H)2 and T(H)17 responses in the lung. The ability of respiratory syncytial virus infection to prevent airway inflammation in C57BL/6 mice is linked to CD8 IFN-γ secretion. Here we have investigated whether T(H)1-promoting nonspecific responsiveness is provided by resting CD8 memory T-cell (CD8 Tm) populations, providing an immunologic pathway linking infection with prevention of allergic disease.
Abbreviations used

BAL: Bronchoalveolar lavage
CD8 Tm: CD8 memory T cell
DC: Dendritic cell
NK: Natural killer
PAMP: Pathogen-associated molecular pattern
SEB: Staphylococcal enterotoxin B
TCR: T-cell receptor
TLR: Toll-like receptor

METHODS

Mice

C57BL/6 (B6) mice (6-8 weeks) were from Harlan UK (Oxford, United Kingdom [UK]). OT-1 and OT-2 T-cell receptor (TCR) transgenic mice (B6 background) were bred in our facility. CD8 Tm cells from lymph node/spleen, and lymphocytes from liver and lung (see this article’s supplementary Methods text in the Online Repository). Proliferation was measured by 3H-thymidine or carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling, and T-cell:DC cocultures were as detailed in this article’s supplementary Methods text in the Online Repository.

Cell isolations and T-cell:DC assays

For antibodies/reagents, see this article’s Online Repository at www.jacionline.org. Serum IgE was measured by ELISA10 and IL-12 p70 by ELISA kit (R&D Systems, Abingdon, UK). DCs were purified from spleens, CD8 naive/memory T cells from lymph node/spleen, and lymphocytes from liver and lung (see this article’s supplementary Methods text in the Online Repository). Proliferation was measured by 3H-thymidine or carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling, and T-cell:DC cocultures were as detailed in this article’s supplementary Methods text in the Online Repository.

Eimeria vermiformis infection experiments

B6 mice were infected 28 days after birth with 10 to 100 Eimeria vermiformis sporulated oocysts in sterile water by oral gavage. Three and 17 days later, some groups were allergen-sensitized with 1 dose of 100 μg ovalbumin in PBS intranasally. Spleocytes were analyzed for CD8 Tm activity on day 31. In some experiments, ovalbumin sensitization was not started until day 24 postinfection, and mice were killed on day 52. There was no change in spleen cellularity or proportions of CD8 cells in infected animals.

CD8 T cell depletion and adoptive transfer

Mice were depleted of CD8 T cells (>99%) as detailed in this article’s supplementary Methods text in the Online Repository. For transfer of CD8 memory, mice were injected intravenously with 5 × 10⁶ OT-1 CD8 cells, or PBS alone, and immunized on the same day with 50 μg SII peptide + 25 μg anti-CD40 subcutaneously. SII-specific CD8 cells were identified with H-2K¹-SII pentamer/CD8 staining.

T-cell–dependent peptide-induced airway inflammation model

B6 mice were adoptively transferred with 5 × 10⁶ OT-2 CD4 cells intravenously, sensitized with 10 μg ESQ2 mg alum intraperitoneally on day 0, and challenged daily with 5 μg ISQ in 50 μL PBS intranasally on days 10 to 14. On day 15, lung resistance and compliance (resistance-lung and compliance-dynamic) were measured as described.11 Data are expressed as percentage of baseline measurements. Bronchoalveolar lavage (BAL) inflammatory cells were counted by flow cytometry essentially as described,12 with addition of fluorescein isothiocyanate–Gr-1 antibody to identify neutrophils positively.

BAL cells were also stained for T1/ST2/CD3/CD4 to enumerate Tsl2 CD4 cells.

Statistics and data analysis

Nonparametric (Mann-Whitney) tests were used to compare results from groups of mice. Values of P < .05, P < .01, and P < .005 were considered significant and are indicated in Figs 1 through 5. Flow cytometry results are shown with mean percent staining ± SEM from 3 independent experiments.

RESULTS

Cytokine-stimulated CD8 Tms release early IFN-γ and develop into innate effectors

CD8 memory cells produced IFN-γ immediately after stimulation through the TCR or by stimulation with cytokines alone (see this article’s Fig E1 in the Online Repository at www.jacionline.org). Only CD44hi memory T cells produced early IFN-γ, and these were present in both lymphoid and nonlymphoid tissues such as lung and liver (Fig E1, A). In the absence of TCR ligation, a combination of IL-12 and IL-18, and to a lesser extent IL-12 + IL-15, induced rapid IFN-γ (Fig E1, B), and this cytokine-induced IFN-γ was similar in both central and effector memory CD8 cells (Fig E1, C). IFN-γ secretion kinetics were dramatically delayed in naive (CD44lo) compared with memory (CD44hi) fractions with either stimulus (Fig E1, D). Counterstaining for DX5, NKG2A/C/E, and αβ/γδ TCR suggested all CD8 IFN-γ+ cells were conventional αβ T cells and not NKT cells (not shown).

Proliferation assays of purified naive and memory CD8 cells (Fig 1, A) showed that IL-12 and IL-18 acted synergistically, mainly on memory cells, to induce proliferation. Progeny of IL-12/IL-18–induced cell division retained the capacity for early IFN-γ production (Fig 1, B), because restimulation of CFSE-labeled cells after 3 days with cytokines induced very high levels of IFN-γ in divided but not unresponsive cells. Proliferating cells also expressed CD25 after 3 to 4 cell divisions (Fig 1, C), indicating full activation and effector development. This was confirmed by kinetic analysis of IFN-γ production (Fig 1, D). Freshly isolated CD8 Tms, when stimulated with IL-12 + IL-18, produced IFN-γ at low frequency, with similar levels detected after 5 or 15 hours. After 3 days of priming in IL-12/IL-18, however, restimulated CD8 Tms were nearly all high-level IFN-γ+ by 5 hours, but staining had declined by 15 hours. Thus, IL-12/IL-18 induced blastlike cells that released IFN-γ with the kinetics and quantities characteristic of T effectors. TNF-α, but not IL-4 or IL-10, was detectable from CD8 Tms, but only after prolonged (24-hour) stimulation with both IL-12 and IL-18 (not shown). Priming/restimulation with IL-12 + IL-18 also induced expression of CD40 ligand, another critical IL-12–inducing signal, albeit at lower levels than in conventional TCR-induced effectors (Fig 1, E). Furthermore, perforin expression, indicative of cytotoxic function, was induced in CD8 Tms after IL-12/IL-18 culture (see this article’s Fig E2 in the Online Repository at www.jacionline.org).

CD8 Tms stimulate DC IL-12 and respond to TLR agonist–activated DCs

Because CD8 Tms were a major source of early IFN-γ, we hypothesized that collaboration between CD8 Tms, DCs, and inflammatory signals is critical for initiating DC IL-12 production. Purified CD8 Tms from OT-1 mice were stimulated with splenic
DCs and SIINFEKL (SII) peptide, an irrelevant peptide (RGY-VYQGL), or whole ovalbumin ± cytosine-phosphate-guanosine (CpG) DNA TLR-9 agonist (Fig 2, A; n = 3). Memory CD8 T lymphocytes stimulated secretion of IL-12 p70 from DCs, but very low levels were detected in naive CD8 cell cocultures. This was dependent on cognate interaction because only the antigenic peptide induced IL-12. Low-level IL-12 could also be elicited by cross-presented whole ovalbumin; this was enhanced by CpG. Neutralizing antibodies were then used to determine mechanisms of IL-12 induction (Fig 2, B; n = 4). Anti–IFN-γ consistently blocked IL-12 secretion induced by CD8 Tms; anti-CD40L had no significant effect. Mice were then depleted of CD8 T cells by using anti-CD8 antibody (see Fig E3 in the Online Repository at www.jacionline.org) and injected 24 hours later with staphylococcal enterotoxin B (SEB) superantigen, which induces circulating IL-12 secretion induced by CD8 Tms; anti-CD40L had no significant effect. Mice were then depleted of CD8 T cells by using anti-CD8 antibody (see Fig E3 in the Online Repository at www.jacionline.org) and injected 24 hours later with staphylococcal enterotoxin B (SEB) superantigen, which induces circulating IL-12 secretion induced by CD8 Tms; anti-CD40L had no significant effect. Mice were then depleted of CD8 T cells by using anti-CD8 antibody (see Fig E3 in the Online Repository at www.jacionline.org) and injected 24 hours later with staphylococcal enterotoxin B (SEB) superantigen, which induces circulating IL-12 secretion induced by CD8 T cells. Depletion of CD8 T cells significantly reduced the release of early IL-12 (Fig 2, C; n = 7), confirming a role for CD8 Tms in innate immunity.

We asked whether activation of DCs induced corresponding activation of CD8 Tms in a noncognate, antigen-independent fashion. CD8 T lymphocytes stimulated with TLR agonists, without antigen, failed to proliferate. However, if DCs were added, high levels of proliferation were induced by TLR-9 agonist CpG and TLR-1/2 agonist Pam3CysK4, but not TLR-3 agonist poly I:C (Fig 2, D; n = 3), with highest proliferation in the memory fraction. To test whether TLR agonists alone prime CD8 Tms in vivo, we challenged mice intranasally with CpG alone (Fig 2, E; n = 3). This primed CD8 Tms for heightened rapid IFN-γ production, most dramatically in lung T cells but also in draining lymph nodes. Bidirectional activation signals can therefore occur between CD8 Tms and DCs during innate/early immune responses.

**Infection enhances the responsiveness of CD8 Tms**

We predicted that microbial exposure, resulting in DC activation, would prime a non–antigen-specific response in CD8 cells, amplifying IL-12 secretion. To test this using a live infection, we used *Eimeria*, an intracellular protozoan parasite that infects intestinal epithelia, causes coccidiosis, and is commonly contracted in early life. Infection of mice with *E. vermiformis* significantly enhanced early IFN-γ in splenic CD8 Tms when assessed by using IL-12/IL-18 stimulation (Fig 3, A; n = 16). This effect was apparent at 31 and 52 days after infection, although parasites are cleared in these animals by day 16 (not shown). This suggested a lasting effect of infection on CD8 memory. Mean IFN-γ triggered by anti-CD3/CD28 appeared higher in infected mice, but this did not reach statistical significance in all groups. Although most mice were also immunized with ovalbumin, ovalbumin treatment had no significant effect on CD8 IFN-γ production. The degree of CD8 IFN-γ enhancement was dependent on the challenge dose of *Eimeria* oocysts (Fig 3, B; n = 8). Cells stimulated with *Eimeria* extract failed to stain positive for IFN-γ (not shown), suggesting the enhanced response was not a result of restimulation of *Eimeria* antigen-specific CD8 Tms.

**TCR triggering activates T-cell:T-cell activation via DCs**

Because CD8 Tms elicited cytokines from DCs and DC-cytokines stimulate CD8 Tms, we postulated that TCR-driven CD8 Tms would activate bystander, non–antigen-specific CD8 Tm. TCR transgenic cells were stimulated with peptide + DCs and labeled, nontransgenic bystander cells (Fig 4, A; n = 3). Nontransgenic cells produced no IFN-γ when stimulated with peptide + DC alone (not shown). However, antigen-triggered OT-1 CD8 Tm activated neighboring nonspecific CD8 Tm to produce IFN-γ. Addition of anti–IL-12/anti–IL-18 partially inhibited the bystander response, which was greater than that induced by IL-12/IL-18 alone, suggesting other DC-cytokines are involved. To demonstrate such bystander activation in vivo, we transferred nonspecific CFSE-labeled CD8 cells into B6 or OT-1 animals (Fig 4, B; n = 3) and challenged with SII. Cell division was observed in B6 CD8 cells when resident in an OT-1 mouse, but only if surrounding T cells were activated with SII. The same cells transferred to a nontransgenic mouse immunized with peptide failed to proliferate, demonstrating that B6 cell division was dependent on OT-1 T-cell activation and not the appearance of antigen-specific cells or presence of...
contaminating endotoxin in the antigen preparation. Furthermore, analysis of Vα2 and Vβ5 TCR elements, which are associated with ovalbumin-specific responses (Fig 4, B, lower panels), showed that bystander-activated cells were not enriched in either Vα2⁺ or Vβ5⁺ populations. Therefore, the response was not antigen-specific.

**Antigen-specific CD8 responses leave an imprint on non–antigen-specific CD8 Tm populations**

The predicted consequence of bystander activation was that non–antigen-specific memory would accompany the development of antigen-specific CD8 memory, providing a conduit for regulation of unrelated immune responses. To test this, we
induced strong in vivo CD8 responses entirely directed to SII peptide (Fig 4; C, n = 4). This allowed us to identify all antigen-specific CD8 T lymphocytes using MHC pentamer staining. OT-1 CD8 T lymphocytes, which all stain positive with MHC pentamer (not shown), were transferred to B6 recipients given SII in anti-CD40 adjuvant, resulting in strong expansion of SII-specific CD8 T lymphocytes. Control mice given peptide/anti-CD40 vaccine without OT-1 cells failed to respond. After the antigen-specific response had declined, we analyzed each group for early IFN-γ production in non–antigen-specific CD8 T lymphocytes (MHC pentamer–negative), using strict gating. Activation of nonspecific CD8 Tms was significantly enhanced in mice that had previously had a strong ovalbumin-specific response compared with controls. This effect was most dramatic in lymph nodes but was also apparent in spleen. Thus, antigen-specific CD8 responses result in a lasting imprint of enhanced maturity on nonspecific populations.

Non–antigen-specific CD8 Tms suppress allergic airway responses and IgE secretion induced by a neoantigen

Finally, we asked whether nonspecific CD8 Tms inhibit allergic airway disease by using 2 distinct approaches. First, we used anti-CD8β depletion (as in Fig 2, D). To confirm that CD8 memory was the critical factor, we also induced defined SII-specific CD8 memory pools in B6 recipients using OT-1 cells and peptide vaccine as in Fig 4, C. Because allergen-specific CD8 T lymphocytes are known to suppress allergic responses, we designed a model in which airway inflammation was entirely dependent on CD4 cells directed to a single class II–restricted peptide (ISQ). This excluded antigen-specific effects because there was no cross-reactivity between responses to the 2 peptides (not shown). OT-2 CD4 cells were transferred into B6 mice (predepleted of CD8 T lymphocytes or given control IgG 24 hours previously), sensitized with ISQ peptide/alum, and repeatedly challenged intranasally with ISQ. CD8β depletion greatly enhanced IgE levels (Fig 5, A; n = 6). Challenge of control mice recruited T cells into the airways, but few eosinophils or neutrophils (Fig 5, B). CD8β depletion increased eosinophil numbers with no significant change in neutrophils or T cells. Data from BAL. CD4+ T1/ST2 analysis using the T1/ST2 TH2 marker (Fig 5, E) were all significantly suppressed by transfer of the OT-1 population, implying that CD8 memory would regulate allergic asthma in a nonspecific fashion.
Our results are consistent with the theory that mature CD8 memory increases the availability of IL-12 from DCs early in responses to neoantigens, resulting in inhibition of Th2 development via immune deviation. How this might explain the role of microbial exposure in allergy is illustrated in Fig 6.

**DISCUSSION**

A central dogma of immunology has been that T-cell responses are controlled by 2 signals: signal 1 (TCR/antigen) and signal 2/3 (costimulatory molecules/cytokines) induced by danger signals and PAMPs. It has been assumed that only signal 1 results in immunologic memory, whereas signals 2 and 3 determine the nature and longevity of the response. In this study, we asked whether it is possible that signals 2/3 also result in a form of immunologic memory, not for specific antigen, but for the amount of environmental danger experienced or the intensity of previous immune responses. If true, this would explain a key proposal of the hygiene hypothesis: that dangerous environments strengthen the immune system in a non–antigen-specific fashion, leading to potent Th1 immunity and little allergy.

CD8 Tms possess unique characteristics that allow them to bridge innate and adaptive immunity. They are activated by cytokines alone in the absence of TCR triggering. They proliferate and secrete cytokines IFN-γ and TNF-α in response to IL-12, IL-18, IL-15. CD8 Tms are long-lived because of self-replication in response to IL-15 and migrate to peripheral tissues. Non–antigen-specific division of CD8 Tms is enhanced by IL-12. Our data indicate these cells participate in early activation of tissue DCs, enhancing IL-12 secretion. Although NK/NKT cells and γδ T cells can also perform this function, they are innate cells that do not mediate long-lived memory. Here we showed that CD8 T cells were partially required for early IL-12 release after SEB injection. It is likely that NKT cells may have provided another stimulus in this system because they can express Vβ7/8, which bind SEB. CD8 Tms are a major source of early IFN-γ after LPS challenge. By contrast, CD4 T-cell stimulation with TLR ligands may enhance survival but does
not induce proliferation. Like CD8 Tms, established Th1 effector cells respond to IL-12 + IL-18 by producing IFN-γ. However, Th1 effectors generate poor memory in experimental models, perhaps because CD4 Tms do not respond to IL-15. By contrast, CD8 Tms have a much lower threshold for IL-12 induction and can generate memory that is resistant to IL-15 and IL-18 suppression. Recently, data also indicate that Eimeria infection suppresses allergic airway inflammation after allergen challenge (D. Gibbons, S. Haque, A. Smith, and A. Hayday, unpublished data, September 2008). We therefore propose that increased maturity of CD8 Tms built up through extended antigenic experience reflects an innate memory of the levels of danger/infectious burden encountered. Development of this memory primes for rapid elimination of pathogens but also educates the immune system to respond appropriately to allergens to avoid allergic disease.

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Clinical implications: Lack of infection in early life could result in delayed maturation of CD8 Tms, resulting in increased incidence of allergy/asthma. Boosting immune memory could therefore prevent sensitization.

REFERENCES


METHODS

Antibodies and reagents

For in vivo experiments, azide-free anti-CD8β antibody (53-5.8; BD Biosciences, Oxford, United Kingdom [UK]), control rat IgG (Sigma, Poole, UK) or anti-CD40 (3/23; Serotec, Oxford, UK) were used. Anti-CCR3-phycocerythrin and anti–IL-12 were from R&D Systems (Abingdon, UK); anti–IL-18 was from MBL Ltd (Watertown, Mass). Biotin or phycocerythrin-labeled anti–CD44 (IM7.8.1), anti–CD25 (PC61), and Gr-1–fluorescein isothiocyanate were from Caltag-MedSystems (Towcester, UK) and anti-perforin-phycocerythrin (eBioOMAK-D) from Insight Biotec (Wembley, UK). Neutralizing azide/endotoxin-free anti–IFN-γ (R4-6A2), anti–CD40L (MR1), and control antibody were from BD Biosciences. mouse plasmacytoid dendritic cell antigen 1 (mPDCA1) antibody, specific for plasmacytoid DC, was from Miltenyi Biotec (Bisley, UK). The T1/ST2 (D93) T11–specific antibody was from BD Biosciences (Oxford, United Kingdom [UK]), control rat IgG (Sigma, Poole, UK) staining was used to identify MHC class I–restricted ovalbumin-specific CD8 cells. Cpg 1826 DNA (TLR-9 agonist) was from VHBio (Gateshead, UK), and palmitoyl-cysteinyl-seryl-lysyl-lysine lipo- peptide (Pam3CSK4, TLR1/2 agonist) was from EM Microcollections (Tubingen, Germany). SII, ISQ, and control vesicular stomatitis virus peptide GYVVQQGL (GRY) were synthesized by Perbio Mimotopes (Tattenhall, UK). Other reagents were purchased from Sigma.

Cell separations and flow cytometry

Dendritic cells were purified from B6 splenocytes by labeling with anti–CD11c biotin (0.5 μg/10^6 cells) followed by antibiotin microbeads (Miltenyi Biotec). CD11c− cells were selected by 2 rounds of magnetic selection (autoMACS; Miltenyi Biotec) and were >80% CD11c− MHC class II− CD14−. CD4/ CD8 T cells (>98% purity) were isolated from spleen/lymph node by using anti–CD4/CD8 microbeads. High purity, aliphophycocyanin (APC)-depleted CD8s and CD44hi/CD44hi CD8 separations were performed by negative selection after further magnetic separation and using 0.05 μg anti–CD44-biotin per 10^6 cells. Cell fractions and staining were performed in PBS + 1% FCS. Cell cultures were in Dulbecco modified Eagle medium (Invitrogen, Paisley, UK) + 10% FCS (Sera-Lab, Loughborough, UK) + 1-glutamine (2 mmol/L), nonessential amino acids (1 mmol/L each), gentamicin (50 μg/mL), and 2-mercaptoethanol (50 μmol/mL). Recombinant IL-12 (Peprotech, London, UK) and IL-18 (R&D Systems) were added at 10 ng/mL. Anti-CD3 (145-2C11) was used to coat 24-well plates (1 μg/mL in PBS, 4°C overnight), whereas anti–CD28 (37.51) was added to cultures at 1 μg/mL. Cytokine profiles were determined by restimulation of washed cells in the presence of 3 μmol/L monensin followed by intracellular cytokine staining as described. Flow cytometric analysis was using a FACScalibur (BD Biosciences), with gating on live cells determined by forward versus side scatter. IFN-γ secretion was measured in supernatants of cultured CD8 naïve or memory cells (2 × 10^6/mL) collected at sequential time points and measured by ELISA using antibody pairs (BD Biosciences according to the manufacturer’s protocol).

T-cell proliferation

In vitro proliferation was measured in triplicate cultures of 0.2 mL CD8 cells (2.5 × 10^6/mL) in 96-well plates. After 72 hours of culture, 0.5 μCi ^3H-thymidine (Amersham Ltd, Amersham, UK) was added to each well overnight, cells were harvested onto glass fiber filters, and scintillation counting was performed with the Topcount system (Perkin Elmer, Beaconsfield, UK). For tracking cell division by CFSE dilution, cells were washed twice in PBS, labeled with 2.5 μmol/L CFSE (Invitrogen) for 10 minutes at 37°C, washed, and cultured for 3 days before flow cytometry. For the in vivo proliferation experiment, B6 CD8 cells were labeled with CFSE as described and resuspended in PBS for intravenous transfer into tail veins of B6 or OT-1 recipient mice (5 × 10^6 cells in 100 μL). Mice then received 25 μg SII peptide in PBS or PBS alone intraperitoneally. Three days later, axillary, inguinal, and cervical lymph nodes were stained for CD44, Vα2, and Vβ5 and analyzed by flow cytometry.

T-cell:DC cocultures

For IL-12 induction, CD8 populations as described from OT-1 mice were cultured in U-shaped microtiter wells at 10^5/mL with addition of DCs at 5 × 10^3/mL, peptides at 2 μg/mL, ovalbumin at 500 μg/mL, Cpg at 1 μg/mL, and antibodies at 10 μg/mL. Supernatants were collected after 24 hours for IL-12 measurements. For proliferation assays, CD8 fractions from B6 mice were cultured at 2.5 × 10^5/mL ± DCs at 2.5 × 10^3/mL, with Cpg, Pam3CSK4, or polyl C at 1 μg/mL. For bystander activation experiments, CFSE-labeled B6 CD44hiCD8s, OT-1 CD44hiCD8s, and DCs were prepared and cocultured at 2 × 10^5/mL, 1 × 10^5/mL, and 1 × 10^5/mL, respectively. SII peptide (2 μg/mL) and antibodies to IL-12 and IL-18 (both 10 μg/mL) were added as indicated. After 15 hours, 3 μmol/L monensin was added, and intracellular staining was performed after a further 4 hours.

Analysis of lung and liver infiltrates

For intrahepatic lymphocyte isolation, livers were perfused with 5 mL cold PBS/FCS via the hepatic portal vein in situ, then dissociated by using a nylon mesh and washed before digestion in RPMI-1640 + 10% FCS + 0.7 mg/mL collagenase type IV for 1 hour at 37°C. Cells were released by pipetting and washed in PBS/FCS. Cells were then suspended in 24% metrizamide in situ and overlaid with culture medium before density gradient centrifugation at 1500g. Lymphocytes were harvested from the interface. For lung cell isolation, lungs were cut into fragments, digested as described, and used without density gradient purification.

CD8 T-cell depletion and adoptive transfer

Mice were depleted of CD8 T cells by injection of 75 μg anti-CD8β antibody intraperitoneally, or rat IgG control. Depletion was confirmed by flow-cytometric analysis of peripheral blood 24 hours later by using CD8α, CD3, CD11c, and mPDCA1 markers. This showed that there was >99% depletion of CD8 T cells with no effect on myeloid or plasmacytoid DC numbers (Fig E3). Effective CD8 T-cell depletion was also observed in lymph node, spleen, and lung cells and lasted for 3 weeks (not shown).
FIG E1. Early IFN-γ synthesis in central and effector memory CD8 Tms. A, CD8 Tms (CD44 hi) are the major source of TCR-triggered early IFN-γ. CD8 T cells purified from tissues of untreated mice were stimulated with anti-CD3/CD28 for 5 hours and stained for CD44 and intracellular IFN-γ. Gated CD8+ events are shown. Data refer to percent IFN-γ+ cells in total CD8 population. B, CD8 but not CD4 T cells from unprimed mice produce IFN-γ after primary stimulation with cytokines alone. Lymph node CD4 or CD8 T cells were stained for CD44 (memory marker) and CD62L (lymph node homing receptor) and then stimulated with indicated cytokines for 5 hours before intracellular IFN-γ analysis. C, Both central memory (CD44 hi CD62L hi) and effector memory (CD44 hi CD62L lo) CD8 cells produce early IFN-γ after IL-12/IL-18 stimulation. Splenic CD4 or CD8 cells were stained for CD62L and CD44 before IL-12/IL-18 stimulation. Spleen contained both central and effector memory cells. Percent IFN-γ+ cells within each subpopulation are shown. D, Time course of IFN-γ secretion from cultures of fractionated CD44 hi and CD44 lo CD8 cells stimulated with IL-12/IL-18 or anti-CD3/28. Data in A and B are representative of 3 experiments; data in C and D are means ± SEMs from 3 independent experiments. *P < .05; ***P < .005.
FIG E2. IL-12 + IL-18 induces perforin expression in CD8 Tms. Splenic CD8 T cells were labeled with anti–CD44-APC, washed, and cultured with IL-12 + IL-18 (10 ng/mL each) or platebound anti-CD3 + IL-2 or were unstimulated for 3 days. Cells were then fixed, permeabilized, with 0.1% Triton X100 (Sigma, Poole, UK), and stained with antiperforin-phycoerythrin or isotype control antibody before flow cytometry. Intracellular perforin staining in gated CD44^hi (memory CD8, upper graph) or naive CD8 (CD44^lo, lower graph) is shown. Data are representative of 3 experiments.
FIG E3. Proportions of CD8 T cells, total DC (CD11C<sup>+</sup>) and plasmacytoid DC (mPDCA-1<sup>+</sup>) in spleen 1 day after intraperitoneal injection of C57BL/6 mice with 75 μg anti-CD8β or control antibody.