

An Essential Role for IL-18 in CD8 T Cell-Mediated Suppression of IgE Responses¹

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The ability of CD8 T cells to suppress IgE responses is well established. Previously, we demonstrated that CD8 T cells inhibit IgE responses via the induction of IL-12, which promotes Th1 and suppresses Th2 responses. In this study, we show that IL-18 also plays an essential role in IgE suppression. In vitro, IL-18 synergized with IL-12 to promote Th1/T cytotoxic 1 and inhibit Th2/T cytotoxic 2 differentiation. OVA-specific TCR transgenic (OT-I) CD8 cells induced both IL-12 and IL-18 when cultured with OVA^{257–264} peptide-pulsed dendritic cells. In vivo, IL-18^{−/−} mice exhibited higher IgE and IgG1 levels compared with wild-type mice after immunization with OVA/alum. Furthermore, adoptive transfer of CD8 T cells from OVA-primed mice suppressed IgE responses in OVA/alum-immunized mice, but not in IL-18^{−/−} mice. IgE suppression in IL-18^{−/−} mice was restored if CD8 T cells were coadoptively transferred with IL-18-competent wild-type bone marrow dendritic cell progenitors, demonstrating an essential role of IL-18 in CD8 T cell-mediated suppression of IgE responses. The data suggest that CD8 T cells induce IL-18 production during a cognate interaction with APCs that synergizes with IL-12 to promote immune deviation away from the allergic phenotype. Our data identify IL-18 induction as a potentially useful target in immunotherapy of allergic disease. *The Journal of Immunology*, 2007, 178: 4771–4778.

Regulatory T cells were first described over three decades ago by Gershon et al. (1). Among the first described were CD8 T cells that inhibited IgE responses (2, 3). This function since has been confirmed by subsequent studies (4–9). Treatment with the toxin ricin depletes CD8 T cells in vivo, resulting in decreased IFN- γ and increased IL-4 production, leading to elevated IgE responses (8). Using the prototype allergen OVA, similar effects were observed when CD8 T cells were depleted using anti-CD8 (10). IgE-suppressive CD8 T cells were OVA specific, expressed the $\alpha\beta$ TCR, and were MHC class I restricted (11). CD8 T cells also down-regulated Th2 responses that were transiently induced through low dose *Leishmania major* infection (12), whereas CD8 T cell depletion abrogated protective Th1 responses (13). Similarly, in respiratory syncytial virus infection, depletion of CD8 T cells resulted in aberrant increases in IgA and the suppression of all other Ig isotypes (14). Furthermore, CD8 T cells have been shown to regulate airway hyperresponsiveness (15, 16).

The ability of CD8 T cells to down-regulate Th2 and IgE responses could be attributed to their ability to license dendritic cells (DC)³ for

the effective priming of Th1 responses (17). Consistent with this, CD8 T cells have been reported to produce IFN- γ during early interactions with DC, which can cooperate with CD40L-expressing CD4 T cells to induce IL-12 for the development of Th1 responses (18). However, in an IFN- γ -independent process, it has been demonstrated that CD8 T cell-mediated suppression of IgE responses required bone marrow DC precursor (BMDCp)-derived IL-12 (19). In these experiments, the eventual suppression of IgE responses required CD4 T cell IFN- γ , although the initial induction of IL-12 was independent of CD8 T cell-derived IFN- γ (19).

IL-18 is a proinflammatory, pleiotropic cytokine with complex functions in vivo (20). Originally named IFN- γ -inducing factor (21), it is now established that IL-18 synergizes with IL-12 to induce IFN- γ production and promote Th1 responses (22, 23). However, IL-18 alone can also promote Th2 responses (24, 25). Previously, we had established the importance of IL-12 in CD8 T cell-mediated IgE suppression (19). However, given that IL-18 synergizes with IL-12 to promote Th1 responses, we hypothesized that IL-18 is also an important factor in CD8 T cell-mediated IgE suppression. In this study, we determined the effects of IL-18 $\pm$ IL-12 on Th1/Th2 and T cytotoxic 1 (Tc1)/Tc2 development in vitro. We then examined the role of IL-18 in vivo during allergic sensitization in the presence or absence of transferred CD8 IgE-suppressor cells. The results indicate that IL-18 induced after interaction with CD8 T cells is crucial to the regulation of IgE responses in vivo.

Materials and Methods

Mice

C57BL/6 mice (6–8 wk) and Wistar rats were obtained from Harlan Olac. Unless stated otherwise, all C57BL/6 mice were wild type. OVA peptide-specific, class I-restricted V α 2V β 5 TCR transgenic mice (OT-I) were a gift from M. Merkenschlager (Royal Postgraduate Medical School, Imperial College, London, U.K.). IL-18^{−/−} knockout mice were a gift from K. Heeg (University of Heidelberg, Heidelberg, Germany). IL-12^{−/−} and IFN- γ ^{−/−} knockout mice were obtained from The Jackson Laboratory. All knockout mice and OT-I mice were on a C57BL/6 background.

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³ Abbreviations used in this paper: DC, dendritic cell; BMDCp, bone marrow DC precursor; IRF-1, IFN regulatory factor-1; PCA, passive cutaneous anaphylaxis; Tc1, T cytotoxic 1.

Reagents

RPMI 1640 and AIM V culture medium, PBS, and HBSS were purchased from Invitrogen Life Technologies. Complete medium comprised an equal mixture of AIM V serum-free medium and RPMI 1640 supplemented with L-glutamine (2 mM), nonessential amino acids (1%), streptomycin (100 ng/ml), penicillin (100 U/ml), sodium pyruvate (1 mM), and 2-ME (5 μ M) (all from Invitrogen Life Technologies). FCS was purchased from Globe-pharm. Purified OVA (grade V) was purchased from Sigma-Aldrich. Rodent lymphoprep 1.077 was purchased from Nycomed. Anti-mouse IgE H chain (LO-ME-3), anti-IgE κ -L chain biotin (OX-20), and mouse rIL-4 were purchased from Serotec. Anti-IgG1 alkaline phosphatase was purchased from The Binding Site. Anti-IL-12, anti-IFN- γ , and anti-IL-4 mAbs and rIL-12, rIFN- γ , and rIL-4 were purchased from BD Biosciences. Anti-IL-18 mAbs for ELISA and rIL-18 for cell cultures were purchased from R&D Systems. Anti-CD4 and anti-CD8 microbeads for MACS were purchased from Miltenyi Biotec. All other reagents were purchased from Sigma-Aldrich.

Immunization procedure

Groups of five age- and sex-matched C57BL/6 mice were immunized with alum-precipitated OVA prepared as follows. OVA was first dissolved in sterile saline at a concentration of 10 mg/ml. A total of 10 ml of the protein solution was then mixed with 4.5 ml of 1 M NaHCO₃ and 10 ml of 0.2 M KAlSO₄ and left at room temperature for 20 min. The mixture was then centrifuged at 3000 $\times$ g for 10 min. The precipitate was washed three times with sterile PBS, resuspended in 10 ml, and stored at 4°C. Recipient mice were immunized i.p. with 400 μ g of OVA-alum, diluted with 0.1 M Al(OH)₃ to the appropriate concentration. CD8 T cells from OVA-immunized C57BL/6 mice were obtained after 21 days.

Isolation of murine CD8 T cells from lymph node and spleen

To isolate CD8 T cells, OT-I, OVA/alum-immunized, or nonimmunized C57BL/6 mice were euthanized in CO₂ chambers, and lymph nodes and spleens were excised. Spleens and lymph nodes were combined, and single-cell suspensions were then obtained by pressing tissues through 70- μ m nylon filters (BD Biosciences) into chilled PBS. These were then layered on lymphoprep and subjected to density centrifugation at 600 $\times$ g, room temperature, for 20 min. Cells accumulating at the interface were then washed twice in PBS. CD8 T cells were then purified using MACS separation, according to manufacturer's instructions. CD8 T cell purity was determined by flow cytometry, using PE (FITC)-labeled anti-CD3 with either CyChrome-labeled anti-CD8 or anti-CD4. Purified CD8 T cells (>98% purity) were resuspended in PBS at a concentration of 5 $\times$ 10⁶ cells/ml, and 200 μ l of cells was adoptively transferred into recipient mice i.p.

Generation of murine DC and BMDCp

Femurs and tibia from nonimmunized C57BL/6 mice were harvested and placed in a petri dish containing PBS, and remaining muscle tissue was removed. Bone marrow cells were obtained by flushing the inside of each bone with 5 ml of PBS plus 1% FCS. Bone marrow cells were subsequently depleted of T and B cells using MACS beads conjugated to mAbs against CD4, CD8, CD19, and CD90. The remaining cells, which were <0.7% B cells and <1.2% T cells, were cultured at 2 $\times$ 10⁵ cells/ml in complete medium containing 20 ng/ml GM-CSF and 10 ng/ml IL-4. Cells were harvested on day 3 and used for adoptive transfer i.p. These cells were typically 20% MHC class II⁺/20% CD11c⁺ by FACS analysis and are termed BMDCp. Prolonged culture of these bone marrow cells for 7 days yielded DC >80% of cells expressing both MHC class II and CD11c. These DC were not used for adoptive transfer because they were able to inhibit Th2 responses in their own right, but were used in the CD8-DC coculture experiments.

Type 1 and type 2 polarizing conditions

Th1 cells were generated by culturing purified CD4 T cells (37°C, 5% CO₂) at 10⁶ cells/ml with 1 μ g/ml plate-bound anti-CD3, 1 μ g/ml soluble anti-CD28, and 10 μ g/ml anti-IL-4 in complete medium for 5 days. Th2 cells were generated by culturing purified CD4 T cells with plate-bound 1 μ g/ml anti-CD3, 1 μ g/ml soluble anti-CD28, and 10 ng/ml IL-4. Tc1 and Tc2 cells were generated from purified CD8 T cells under similar conditions as Th1 and Th2 cells, respectively, except that for Tc1, anti-IL-4 was excluded and for Tc2, 10 ng/ml PMA was included.

Intracellular cytokine staining

Cultured CD4 and CD8 T cells were washed thoroughly with PBS plus 1% FCS and then restimulated in complete medium containing 3 μ M monen-

sin, 10 ng/ml PMA, and 400 ng/ml ionomycin at 10⁶ cells/ml for 5 h at 37°C. Cells were then harvested and stained with anti-CD4⁺ or anti-CD8 CyChrome. Cells were washed, incubated for 20 min with 250 μ l of Perm/fix solution (BD Biosciences), and then washed twice with Perm/Wash buffer (PBS with 0.5% BSA and 0.1% saponin). Anti-IFN- γ FITC and anti-IL-4 PE were added at 1 μ l/tube, mixed, and then incubated for 30 min at room temperature. Cells then were washed with Perm/Wash buffer and resuspended in 500 μ l of 1% paraformaldehyde. Staining was analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

Cytokine ELISAs

Culture supernatants were harvested and frozen at -40°C until analysis. Throughout, 50- μ l volumes were used, and the assay was performed at 25°C. IL-12 (p40 chain) and IL-18 in supernatants were measured with capture and detector Ab pairs. Microtiter plates (Nunc Maxisorp; VWR) were coated with capture Ab at 1 μ g/ml in carbonate/bicarbonate (pH 9.6, 0.1 M) buffer overnight at 4°C. Plates were subsequently washed thrice with PBS with 0.05% Tween 20. Duplicate supernatant samples, diluted with assay diluent (PBS with 1% rat serum and 0.5% Tween 20), were added to coated plates for overnight incubation at 4°C. Plates were washed, and 1 μ g/ml biotinylated detector Ab was added. After 2 h, plates were washed and 1 μ g/ml streptavidin-conjugated alkaline phosphatase was added. After 45 min, the plates were washed and *p*-nitrophenyl phosphate substrate diluted to 1 mg/ml in diethanolamine buffer (0.1 M) was added. After 1 h, absorbance at 405 nm was detected with a plate reader (Molecular Devices), and the results were expressed as ng/ml or pg/ml with reference to a standard curve constructed using dilutions of recombinant cytokine.

Assessment of OVA-specific IgE by passive cutaneous anaphylaxis (PCA)

PCA was used to measure the levels of biologically active OVA-specific IgE Abs in mouse serum. Four-fold serial dilutions of mouse serum ranging from 1/8 to 1/2048 in PBS were made, and 50 μ l of up to 50 diluted samples was injected intradermally into the shaved back of an anesthetized Wistar rat. After 48 h, the rat was again anesthetized, and 500 μ l of 10 mg/ml OVA in 1% Evans blue dye was injected into the tail vein. After 30 min further anesthesia, extravasation of blue dye into the skin was recorded. OVA-specific IgE Ab titers from mouse serum were measured at days 0, 7, 14, and 21 and are represented as the mean $\pm$ SD of the highest dilution of test serum that produced a positive mast cell-dependent PCA reaction.

Statistics

Data were analyzed using unpaired Student's *t* test. A value of *p* < 0.05 was considered significant. Results are expressed as means $\pm$ SD.

Results

IL-18 synergizes with IL-12 to induce IFN- γ -producing CD4 and CD8 T cells

The effect of IL-18 on the generation of IFN- γ -producing CD4 and CD8 T cells was investigated in vitro. CD4 and CD8 T cells were isolated from C57BL/6 mice by positive selection using anti-CD4- and anti-CD8-coated magnetic beads and stimulated using plate-bound anti-CD3 and soluble anti-CD28 under type 1 (Fig. 1a) or type 2 (Fig. 1b) polarizing conditions (as described in *Materials and Methods*) together with IL-12, IL-18, or IL-12/IL-18. After 5 days, the cells were recovered, washed, and restimulated with PMA and ionomycin for 5 h in the presence of 3 μ M monensin and stained for intracellular IFN- γ and IL-4. Results in text are mean $\pm$ SD of four independent experiments, and a representative example of one experiment is shown in Fig. 1. Under type 1 polarizing conditions, half of the CD8 T cells made IFN- γ 48.1 $\pm$ 9.0%, whereas 4.1 $\pm$ 1.5% of CD4 T cells made IFN- γ , and very few made IL-4 (Fig. 1a). Addition of IL-12 and IL-18 separately increased the percentage of IFN- γ ⁺ CD8 T cells to 69.8 $\pm$ 5.8% and 66.8 $\pm$ 3.3%, respectively, whereas IL-12 and IL-18 together yielded 80.3 $\pm$ 1.5% IFN- γ ⁺ cells (Fig. 1a).

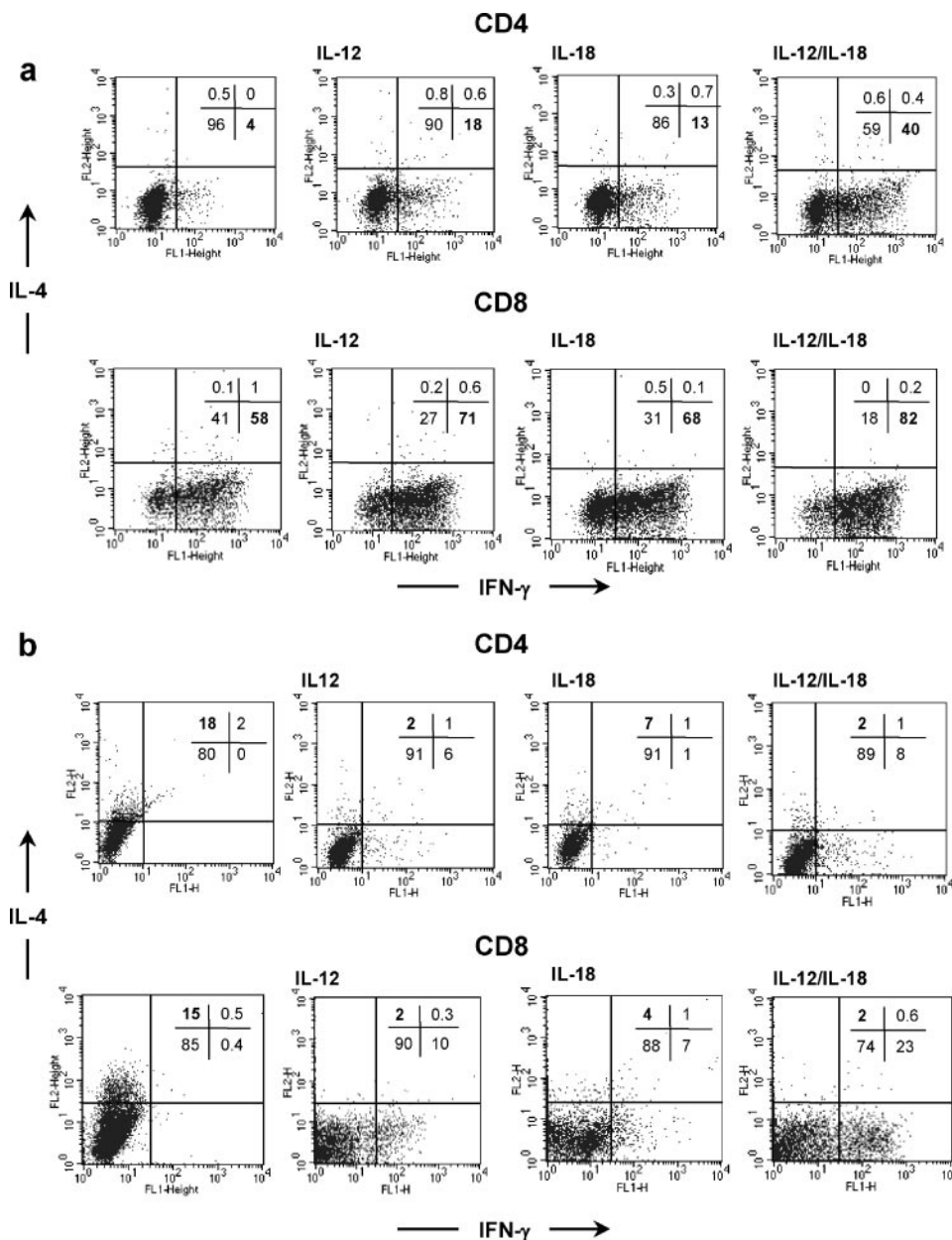


FIGURE 1. IL-18 and IL-12 induce IFN- γ -producing CD4 and CD8 T cells. CD4 or CD8 T cells from nonimmunized C57BL/6 mice were isolated by positive selection using magnetic bead-labeled anti-CD4 and anti-CD8 Abs, respectively, and cultured for 5 days under type 1 (a) or type 2 (b) polarizing conditions. In addition, IL-12 (10 ng/ml), IL-18 (10 ng/ml), or both IL-12 (10 ng/ml) and IL-18 (10 ng/ml) were included in the culture medium. After 5 days, the cells were recovered, washed, and restimulated for 5 h with PMA (10 ng/ml)/ionomycin (400 ng/ml) and monensin (3 μ M). Cells were subsequently stained with anti-IFN- γ FITC and anti-IL-4 PE. The numbers in the quadrants represent the percentage of cells making IL-4 (upper left), IFN- γ (lower right), or both (upper right). Data are representative of four independent experiments.

IL-12 and IL-18 had a more dramatic effect on the number of IFN- γ ⁺ CD4 T cells. Separately, IL-12 and IL-18 increased IFN- γ ⁺ CD4 T cells from $4.1 \pm 1.5\%$ to $18.4 \pm 3.2\%$ and $15.4 \pm 2.0\%$, respectively, whereas IL-12 and IL-18 together acted synergistically to increase the percentage of CD4 T cells that stained positive for IFN- γ by 10-fold to $37.1 \pm 3.5\%$ (Fig. 1a). The effect of IL-12 and IL-18 on cytokine production by CD4 and CD8 T cells stimulated under type 2 polarizing conditions was also investigated. In the absence of IL-12 or IL-18, $14.2 \pm 1.2\%$ of CD8 and $17.2 \pm 1.4\%$ of CD4 T cells were positive for IL-4, and very few T cells were positive for IFN- γ . Addition of IL-12 alone reduced the percentage of IL-4-positive CD4 and CD8 T cells to $1.2 \pm 0.4\%$ and $1.8 \pm 1.5\%$, respectively, and increased the percentage of IFN- γ -producing CD4 and CD8 T cells to $7.5 \pm 1.1\%$ and $11.2 \pm 2.8\%$, respectively (Fig. 1b). IL-18 alone was less effective, reducing IL-4⁺ CD4 T cells to $4.5 \pm 2.5\%$ and CD8 T cells to $8.1 \pm 2.5\%$, and increasing the percentage of IFN- γ -producing CD4 and CD8 T cells to $1.4 \pm 0.5\%$ and $8.1 \pm 2.5\%$, respectively. When added together,

IL-12 and IL-18 reduced the percentage of IL-4⁺ CD8 and CD4 T cells to $<2\%$, and increased the percentage of IFN- γ -producing CD4 and CD8 T cells to $8.2 \pm 1.2\%$ and $21.4 \pm 2.0\%$, respectively (Fig. 1b).

Effect of IL-18 and IL-12 on CD4 and CD8 T cell cytokine secretion in vitro

In addition to measuring the percentage of IFN- γ - and IL-4-producing T cells by intracellular staining, we also studied the effects of IL-12 and IL-18 on the levels of IFN- γ and IL-4 secreted by CD4 T cells (Fig. 2, a and b) and CD8 T cells (Fig. 2, c and d) in vitro by ELISA. Following culture of CD4 and CD8 T cells isolated from C57BL/6 mice for 5 days under type 1 (Fig. 2, left) or type 2 (Fig. 2, right) polarizing conditions with or without IL-12 and/or IL-18, the T cells were restimulated for 24 h with PMA and ionomycin, and supernatants were harvested for measurement of IFN- γ and IL-4 by ELISA. Moderate levels of IFN- γ were secreted by type 1 polarized CD4 T cells, and this could be augmented by IL-12, but not IL-18 alone

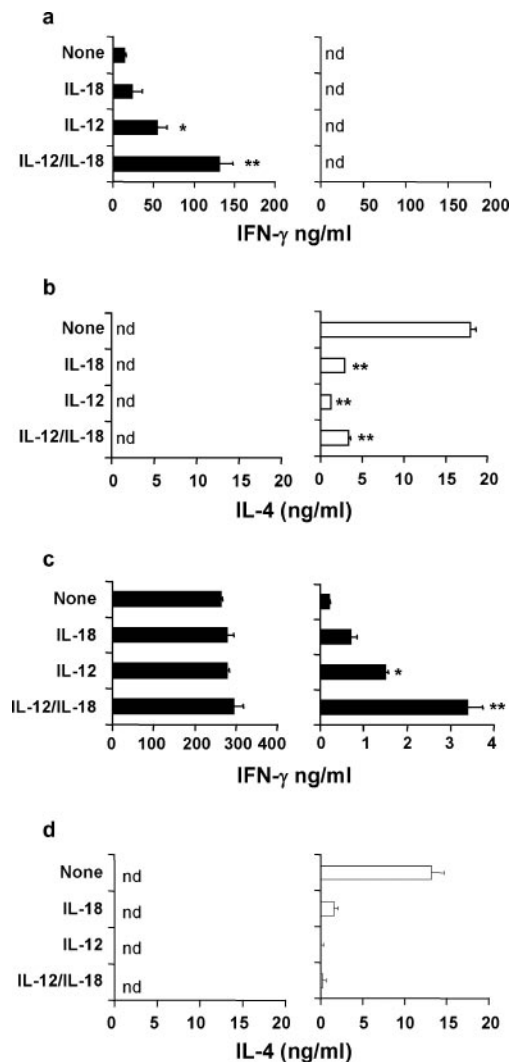


FIGURE 2. IL-18 and IL-12 induce IFN- γ and inhibit IL-4 production. CD4 (a and b) and CD8 (c and d) T cells from C57BL/6 mice were cultured at 1×10^6 cells/ml in 24-well plates with plate-bound anti-CD3 and soluble anti-CD28 under type 1 polarizing conditions (left diagrams) or type 2 polarizing conditions (right diagrams) together with IL-12 (10 ng/ml), IL-18 (10 ng/ml), or both IL-12 and IL-18, as indicated. After 5 days, cells were collected, washed, and stimulated for 24 h with PMA and ionomycin. Duplicate culture supernatants were analyzed for IFN- γ and IL-4 content by ELISA. Results are expressed as the mean $\pm$ SD of four experiments and compared with controls using Student's *t* test (*, $p < 0.05$; **, $p < 0.005$).

(Fig. 2a, left). However, IL-18 synergized with IL-12 to induce a >10 -fold increase in IFN- γ production. IFN- γ secretion by CD8 T cells polarized under type 1 conditions was not augmented by the addition of IL-12 and/or IL-18 (Fig. 2c, left). IL-4 was not detected in supernatants of both CD4 and CD8 T cells polarized under type 1 conditions (Fig. 2, b and d, left). Under type 2 conditions, IL-4 produced by CD4 and CD8 T cells could be inhibited by the addition of IL-12 and/or IL-18 (Fig. 2, b and d, right). Although IFN- γ secretion by type 2 polarized CD4 could not be detected even with the addition of IL-12 and/or IL-18 (Fig. 2a, right), IFN- γ secretion by type 2 polarized CD8 T cells could be augmented by the addition of IL-12 and/or IL-18 (Fig. 2c, right). IL-12 and IL-18 synergized to induce higher levels of IFN- γ from type 2 polarized CD8 T cells. Hence, our results suggest that IL-18, especially with IL-12, has a direct effect on promoting Th1/Tc1 over Th2/Tc2 responses.

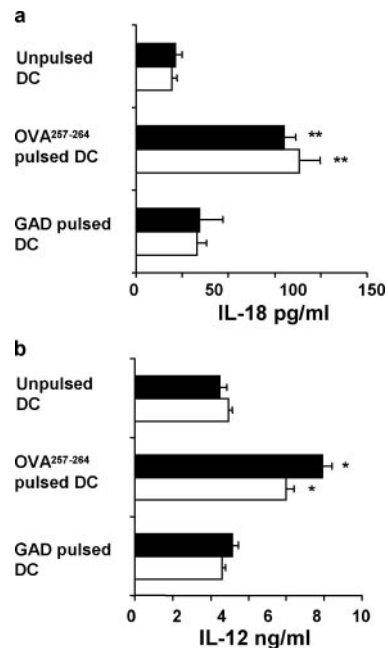


FIGURE 3. CD8 T cell induction of DC (a) IL-18 and (b) IL-12. CD8 T cells from OT-I mice were cocultured with DC from wild-type C57BL/6 mice for 72 h (■) or 144 h (□) at a ratio of 5:1 CD8 T cell-DC. DC were pulsed overnight with either medium alone, OVA²⁵⁷⁻²⁶⁴, or an irrelevant peptide GAD²⁷⁴⁻²⁸⁶ before coculture with CD8 T cells. At each time point, supernatants were harvested and IL-12 and IL-18 content was assessed by ELISA. The results shown are the means $\pm$ SD of two independent experiments.

CD8 T cells enhance the ability of DCs to produce IL-12 and IL-18

To determine whether CD8 T cells can induce DC to produce IL-18, DC were pulsed overnight with either OVA²⁵⁷⁻²⁶⁴ or irrelevant peptide GAD²⁷⁴⁻²⁸⁶, washed, and subsequently cultured with freshly isolated OT-I CD8 T cells at 5:1 T-DC ratio for 72 and 144 h. Supernatants were harvested and tested for IL-12 p40 (Fig. 3a) and IL-18 (Fig. 3b) by ELISA. CD8 T cells enhanced the secretion of both IL-12 and IL-18 from DCs pulsed with OVA²⁵⁷⁻²⁶⁴, whereas DC pulsed with irrelevant peptide GAD²⁷⁴⁻²⁸⁶ secreted levels of IL-12 and IL-18 that were similar to unpulsed DC. This suggests that CD8 T cells can enhance IL-12 and IL-18 production by DC during an Ag-specific CD8 T cell-DC interaction.

IgE and IgG1 Ab responses of IL-18^{-/-}, IL-12^{-/-}, IFN- γ ^{-/-}, and wild-type mice to OVA/alum immunization

Because IL-18, especially together with IL-12, can act directly on T cells to promote Th1/Tc1 and inhibit Th2/Tc2 differentiation, we ascertained the contribution of IL-18 to the suppression of Th2 responses in vivo by comparing the levels of IgG1 and IgE generated in IL-18 knockout mice that were immunized with OVA/alum i.p. For this, we compared the Ab responses of IL-18^{-/-}-immunized mice with IL-12^{-/-}- and IFN- γ ^{-/-}-immunized mice. Immunized C57BL/6 mice served as an indicator of the normal Ab response, and saline-injected C57BL/6 mice as the negative control. Peak IgE responses were obtained at day 14 for all OVA/alum-immunized mice (Fig. 4, a and b). As expected, the peak responses in IL-12^{-/-}- and IFN- γ ^{-/-}-immunized mice on day 14 were significantly higher than for wild-type immunized C57BL/6 mice. This was also true for IL-18^{-/-}-immunized mice. Interestingly, early phase IgE responses at day 7 were highest in

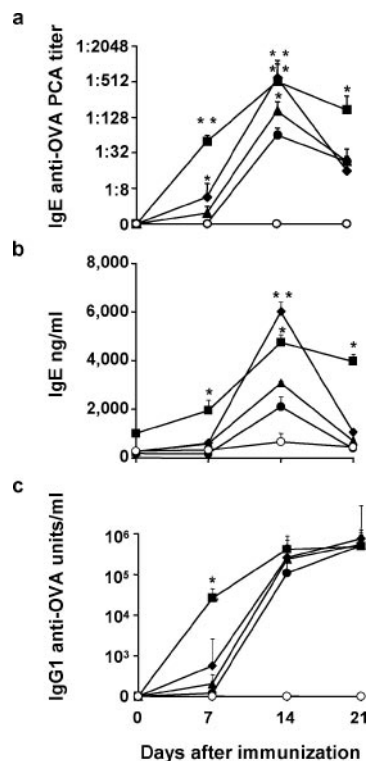


FIGURE 4. Ab responses of IL-18^{-/-}, IL-12^{-/-}, IFN- γ ^{-/-}, and wild-type mice to OVA/alum i.p. immunization. IL-18^{-/-} (■), IL-12^{-/-} (▲), IFN- γ ^{-/-} (◆), and wild-type mice (●) ($n = 6$) were immunized i.p. with either 400 μ g of OVA/alum or 100 μ l of saline (○). Blood was collected at weekly intervals, and serum OVA-specific IgE (a), total IgE (b), and OVA-specific IgG1 (c) were measured by PCA and ELISA, as described in *Materials and Methods*. Results are representative of two independent experiments and are shown as the mean $\pm$ SD. Differences were compared using Student's t test (*, $p < 0.05$; **, $p < 0.005$).

IL-18^{-/-}-immunized mice, even when compared with IgE levels of IL-12^{-/-}- and IFN- γ ^{-/-}-immunized mice at day 7. OVA-specific IgE levels were ~20-fold greater in IL-18^{-/-} mice than IL-12^{-/-} at this time point. This was also true for anti-OVA IgG1 responses (Fig. 4c). Furthermore, although IgE responses in IL-12^{-/-}- and IFN- γ ^{-/-}-immunized mice dropped to levels comparable to immunized wild-type C57BL/6 mice by day 21, the levels of IgE in IL-18^{-/-}-immunized mice were sustained at significantly higher (>10-fold at day 21) levels. This indicates that IL-18 plays an important nonredundant role in the regulation of both early and late phases of an IgE response.

CD8 T cells inhibit OVA-specific IgE responses in wild-type, but not in IL-18^{-/-}, mice

We previously showed that IgE responses induced by i.p. immunization of OVA/alum could be suppressed by the adoptive transfer of CD8 T cells from day 21 OVA/alum-immunized mice in an Ag-specific and IL-12-dependent manner (19). We used a similar approach to determine whether IL-18 is also involved in this mechanism of immune regulation. Consistent to our previous study, the adoptive transfer of CD8 T cells from nonimmunized C57BL/6 mice had no effect on the levels of IgE induced by OVA/alum immunization (Fig. 5a), whereas adoptive transfer of CD8 T cells from day 21 OVA/alum-immunized mice inhibited the IgE response in recipient wild-type mice (Fig. 5a) ($p < 0.005$). To determine the contribution of IL-18 to IgE suppression, CD8 T

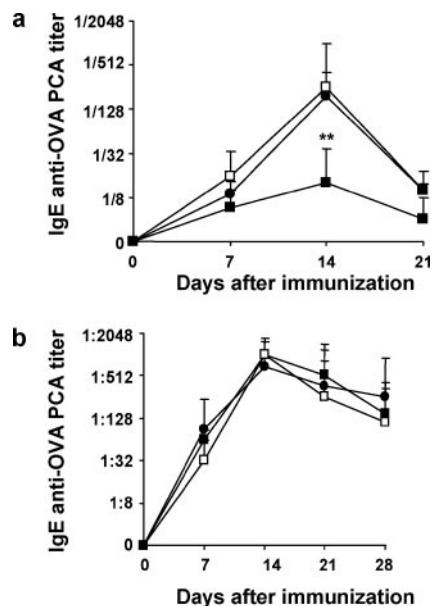


FIGURE 5. Effect of CD8⁺ T cells from OVA-primed mice on OVA-specific IgE response in wild-type and IL-18^{-/-} mice. Wild-type mice (a) and IL-18^{-/-} mice (b) ($n = 5$) were immunized with 400 μ g of OVA/alum i.p. This was immediately followed by the adoptive transfer of 1×10^6 day 21 OVA-primed CD8 T cells (■), CD8 T cells from nonimmunized mice (□), or PBS (●) i.p. Blood was collected at weekly intervals, and serum OVA-specific IgE was measured by PCA. Results are representative of three independent experiments and are shown as the mean $\pm$ SD. Differences were compared using Student's t test (*, $p < 0.05$; **, $p < 0.005$).

cells from nonimmunized C57BL/6 mice or CD8 T cells from day 21 OVA/alum-immunized C57BL/6 mice were also transferred to IL-18^{-/-} mice (Fig. 5b). No suppression of OVA-specific IgE was observed in IL-18^{-/-}-immunized hosts. This indicates that IgE suppression by CD8 T cells required the host to be competent in producing IL-18.

CD8 T cell-mediated IgE inhibition in vivo depends on IL-18

The inability of CD8 T cells from OVA-primed mice to suppress IgE responses in IL-18^{-/-} mice suggested that host animal-derived IL-18 as well as IL-12 was required for CD8 T cell-mediated IgE suppression. Because CD8 T cells could enhance IL-18 production from DC in vitro (Fig. 3a), we speculated that IgE suppression could be reinstated by transferring IL-18-competent cells into IL-18-deficient hosts. This was investigated by the reconstitution of IL-18^{-/-} mice with 10-fold incremental (10^2 – 10^5 per mouse) numbers of BMDCp from IL-18-competent, wild-type C57BL/6 mice (Fig. 6). CD8 T cells from OVA-immunized C57BL/6 mice were unable to inhibit IgE in IL-18^{-/-} mice when transferred without wild-type BMDCp (Fig. 6, group B), whereas suppression of IgE response was restored if CD8 T cells from day 21 OVA/alum-immunized C57BL/6 mice were cotransferred with IL-18-competent BMDCp. This effect was dependent on the number of BMDCp transferred, with the greatest IgE suppression achieved by cotransfer of 10^5 DC progenitors. The IgE anti-OVA Ab titer was significantly inhibited by as few as 10^3 cotransferred DC progenitors (Fig. 6, groups D–F). The inability of IL-18-competent BMDCp from wild-type C57BL/6 mice to suppress IgE responses in the absence of CD8 T cells from OVA-immunized mice (Fig. 6, group A) suggests that the inhibition of IgE responses induced by transfer of both cells is due to CD8 T cell induction of IL-18. These results show that IL-18 produced by BMDCp was an

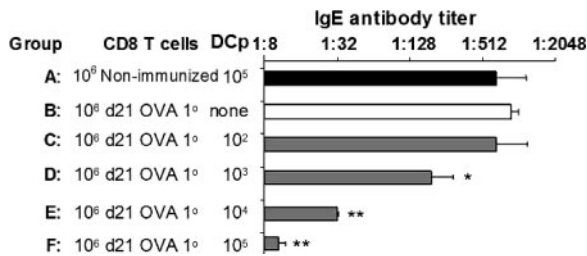


FIGURE 6. Restoration of CD8 T cell-mediated IgE inhibition by adoptive transfer of wild-type BMDCp into IL-18^{-/-} mice. Groups of IL-18^{-/-} mice ($n = 5$) were immunized with OVA/alum i.p. In addition, the following cells were also adoptively transferred: 1×10^6 CD8 T cells from nonimmunized mice and 10^5 BMDCp from wild-type mice (group A), 1×10^6 CD8 T cells from OVA-primed mice only (group B), and 1×10^6 CD8 T cells from OVA-primed mice together with increasing numbers of BMDCp from wild-type mice (1×10^2 – 1×10^5) (groups C–F). Blood was collected at weekly intervals, and serum OVA-specific IgE was measured by PCA, as described in *Materials and Methods*. Results are representative of two independent experiments and are shown as the mean $\pm$ SD. Differences were compared using Student's t test (*, $p < 0.05$; **, $p < 0.005$).

important element in the suppression of IgE responses by CD8 T cells.

Discussion

In the present study, we established for the first time the role of IL-18 in CD8 T cell-mediated IgE suppression. In vitro, IL-18 synergized with IL-12 to promote Th1/Tc1 development and IFN- γ secretion. CD8 T cells also induced the production of IL-18 and IL-12 by DCs in an Ag-specific manner. In vivo, IgE responses to OVA/alum immunization were dramatically elevated in the absence of IL-18.

Adoptive transfer of CD8 T cells into wild-type mice results in the suppression of IgE responses. To determine whether IL-18 played a role in this process, we used IL-18^{-/-} mice as recipients of CD8 T cells (Fig. 5). OVA-specific IgE responses in IL-18^{-/-} mice that had received CD8 T cells from OVA-immunized mice were significantly higher than those of comparably treated wild-type mice. This suggested that IL-18 was crucial for CD8 T cell-mediated IgE suppression. To confirm this finding, we restored the IL-18-producing ability of IL-18^{-/-} mice by the adoptive transfer of BMDCp from wild-type mice. The transfer of wild-type cells capable of producing IL-18 reinstated the ability of CD8 T cells to suppress IgE responses. This was dependent on the number of coadoptively transferred BMDCp. The possibility of an alternative factor that bypassed the need for IL-18 was introduced by BMDCp in these experiments while possible seems unlikely because these cells were unable to inhibit IgE in the absence of OVA-specific CD8 T cells (Fig. 6, group A). Furthermore, if an alternative factor was involved, it is hard to explain the lack of IgE suppression by CD8 T cells in IL-18^{-/-} mice, whereas it was clearly demonstrated that IL-12 and IL-18 synergized to induce Th1 cells in vitro (Figs. 1 and 2).

Our results differ from a previous study by Pollock et al. (26), who reported no observable difference between the levels of OVA-specific IgE in response to OVA/alum challenge between wild-type and IL-18^{-/-} mice. This difference raises interesting questions. Pollock et al. (26) used IL-18^{-/-} BALBc mice in which CD8 T cell IgE suppression has not been described. In addition, the route of immunization, timing, and dose of OVA were different in the two studies. Induction of OVA-specific CD8 T cells requires cross-presentation of OVA. At this stage, it is not clear whether

IgE suppression is mediated by a specific subpopulation of CD8 T cells. IgE inhibitory CD8 T cells may need to be of a particular activation stage or differentiation status. However, Thomas et al. (19) showed that both Tc1 and Tc2 CD8 T cells could trigger this pathway. It is clear that recognition of Ag by CD8 T cells is important because IgE suppression was not observed in mice that received CD8 T cells from nonimmunized mice or in mice that were immunized with a different Ag (27). Based on pentamer staining of mouse blood, we find that after immunization, percentage of OVA-specific CD8 T cells in blood was increased from 0.2 to 1.0% of total CD8 T cells (data not shown). The proportion in the spleen and lymph nodes is likely to be higher.

Consistent with our in vitro experiments, IL-18 and IL-12 production were enhanced during an Ag-specific interaction between CD8 T cells and DCs (Fig. 3). The factor(s) involved in IL-18 induction is not known, and it would be of particular interest to determine whether IFN- γ secretion by CD8 cells was required, although for regulation of IgE we have clearly shown that CD8 T cell IFN- γ is not required (19). The roles of other cytokines and molecules such as IFN regulatory factor-1 (IRF-1) also remain undefined. IRF-1 is a transcriptional factor that induces Th1 responses (28, 29); by regulating caspase-1 and IL-18-binding protein expression, IRF-1 regulates IL-18 assembly and release (30).

Although IL-18 was first described as an IFN- γ -inducing cytokine (21), it is clear from subsequent studies that IL-18 can influence both Th1 and Th2 immunity (20). In general, IL-18 enhances IL-12-driven Th1-immune responses, whereas IL-18 alone stimulates Th2 responses (20). The present study suggests that IL-18 deficiency results in enhanced Th2 responses. We also show that IL-18 antagonizes Th2 and Tc2 cell development in vitro. IgE responses in IL-18^{-/-} mice were significantly higher compared with wild-type mice in response to OVA/alum immunization (Fig. 4). Furthermore, although peak IgE responses were not significantly different between IL-18^{-/-}, IL-12^{-/-}, and IFN- γ ^{-/-} mice, the IgE response of IL-18^{-/-} mice occurred earlier and was sustained.

Consistent with our observation that the lack of IL-18 augments Th2 responses, other investigators have shown that IL-18^{-/-} mice lack protective Th1 responses during *Candida albicans*, *Propionibacterium acnes*, and *Mycobacterium bovis* infection (31, 32). Immunization using *Helicobacter pylori* with cholera toxin adjuvant also failed to elicit protective Th1 responses in IL-18^{-/-} mice (33). In a model of allergic asthma, IL-18^{-/-} mice had increased levels of eosinophilia and lung damage, which could be reverted with administration of IL-18 (34). Collectively, these and other studies support our observation that IL-18 deficiency leads to increased Th2 responses. In contrast, administration or overexpression of IL-18 can also result in enhanced Th2 responses. For instance, stimulation of naive CD4 T cells via TCR and IL-18 is reported to induce the development of IL-4-producing cells in vitro (24). In vivo, the administration or overexpression of IL-18 resulted in increased levels of Th2 cytokines and IgE Abs, and can potentially induce allergic sensitization (25, 35–38). Furthermore, histamine was also reported to stimulate IL-18 production without induction of IL-12 (39).

Further studies show that IL-18 can act in ways that defy the Th1/Th2 paradigm. Transgenic mice overexpressing IL-18 produced higher levels of IFN- γ , IL-4, IL-5, and IL-13; thus, aberrant expression of IL-18 in vivo results in increased production of both Th1 and Th2 cytokines (40). Memory Th1 cells, which do not normally induce airway hyperresponsiveness, acquired the ability to induce airway hyperresponsiveness after stimulation with IL-18 (41). In another report, the systemic neutralization of IL-18 resulted in the accumulation of allergen-associated eosinophils,

whereas airway instillation of IL-18 increased peribronchial eosinophil accumulation (42). These studies and others demonstrate that depending on conditions, IL-18 can promote either a Th1 or Th2 response. It is evident that the cellular source and locality of IL-18 secretion are crucial to its role in allergic sensitization. For example, the presence of IL-18 in the lung, where IL-12 production may be lacking due to DC immaturity, enhances IgE responses and eosinophilic inflammation (36). However, IL-18 produced in conjunction with IL-12 from mature DC after migration to draining lymph nodes would promote Th1 responses and suppression of allergy. Likewise, the down-regulation of IgE production from B cells in lymphoid organs would require IL-18 as shown in this study, whereas prolonged IL-18 secretion from inflammatory cells in the lung could exacerbate allergic asthma, for example, via eotaxin-mediated eosinophil recruitment (40). Our data indicate that the potent role of CD8 T cells in regulating developing immunity needs to be considered to explain the complex effects of IL-18. Specifically, the ability of CD8 cells to simultaneously induce IL-12 and IL-18 from DC may explain the dominance of IL-18-dependent IgE suppression seen in our model. By contrast, the ability of IL-18 to induce IgE responses is dependent on CD4 Th2 responses triggered by early NKT cell secretion of IL-4 (43).

The current study defines a novel role for IL-18 in CD8 T cell-mediated suppression of IgE responses. The effect described in this study may be important for defining the set point at which a Th1 or Th2 dominant immune response ensues. This might allow the prevention of allergic sensitization by inhibition of Th2 responses and consequent IgE production. In fact, a number of novel therapeutic approaches for the treatment of allergy, including the use of oligodeoxynucleotides containing CpG motifs (44), chitin particles composed of *N*-acetyl-D-glucosamine polymer (45), and heat-killed *Listeria monocytogenes* (46), appeared to exert their effect by the regulation of IL-12 and IL-18. DNA vaccination with OVA cDNA fused to IL-18 cDNA inhibited allergic sensitization in an IFN- γ - and CD8-dependent manner (47). Similarly, an anti-CD3/IL-18 fusion protein inhibited Th2 responses to OVA (48, 49). Hence, therapeutics that target IL-18 should simultaneously target the CD8 T cell, MHC class I-restricted pathway via DC activation to ensure effective suppression of allergy.

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Disclosures

The authors have no financial conflict of interest.

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CORRECTIONS

Pier, G. B., D. Boyer, M. Preston, F. T. Coleman, N. Llosa, S. Mueschenborn-Koglin, C. Theilacker, H. Goldenberg, J. Uchin, G. P. Priebe, M. Grout, M. Posner, and L. Cavacini. 2004. Human mAbs to *Pseudomonas aeruginosa* alginate that protect against infection by both mucoid and nonmucoid strains. *J. Immunol.* 173: 5671–5678.

In **Results**, in the third sentence of the first paragraph under the heading *Characteristics of the initial hybridomas and their variable region genes*, the first, third, and fourth GenBank accession numbers are incorrect. The corrected sentence is shown below.

Analysis of the nucleotide and amino acid sequences indicated that clones F428 and F429 contained the same H chain V region but different L chain V region, whereas clone F431 had a distinct H chain V region but shared the L chain V region of clone F428 (GenBank accession numbers: AY626661, IGLV-J of mAbs F428 and F431; AY626662, IGLV-J of mAb F429; AY626664, IGHV-D-J of mAbs F428 and F429; and AY626663, IGHV-D-J of mAb F431).

Brown, H. J., H. R. Lock, S. H. Sacks, and M. G. Robson. 2006. TLR2 stimulation of intrinsic renal cells in the induction of immune-mediated glomerulonephritis. *J. Immunol.* 177: 1925–1931.

Sentence seven of the **Abstract** is incorrect. The corrected sentence is shown below.

Nephrotoxic Ab and TLR2 ligation caused a neutrophil influx in both types of chimera above that seen in the sham chimera totally TLR2 deficient.

In Fig. 3, the statistics presented represent p values from t tests performed on data that should have been transformed, as stated in **Materials and Methods**. In addition, p values were not given for several comparisons that are relevant. The figure below shows new p values on transformed data. The revised p values do not change the message of the paper, since the same comparisons are still significant when performed on the transformed data. The corrected figure and legend are shown below.

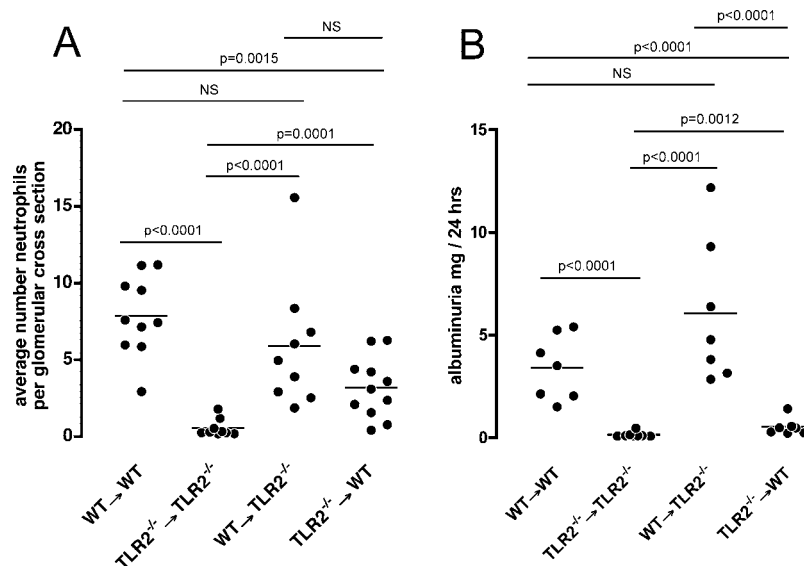


FIGURE 3. A, Glomerular neutrophil influx at 2 h. B, Albuminuria in 24 h following i.v. Pam₃CysSK₄ and NTS in chimeric and sham chimeric animals. The graphs show results from all statistical analyses.

Collins, J. T., J. Shi, B. E. Burrell, D. K. Bishop, and W. A. Dunnick. 2006. Induced expression of murine γ 2a by CD40 ligation independently of IFN- γ . *J. Immunol.* 177: 5414–5419.

As a part of other studies, the authors found that the I γ 2a primer used to amplify I γ 2aC μ products in one panel of Fig. 4 was contaminated with a C γ 2a primer. Therefore, the products designated as “I γ 2aC μ ” in the second from the top panel of Fig. 4 are actually I γ 2aC γ 2a germline transcripts. The authors have prepared new I γ 2a and new C μ primers and redone the RT-PCR for I γ 2aC μ transcripts. As this required new cDNA, the authors also repeated the RT-PCR for hypoxanthine phosphoribosyltransferase (HPRT) of the same samples.

The original version of the second from the top panel of Fig. 4 is incorrect in that only one authentic RT-PCR product is detected for I γ 2aC μ RNA (see revised Fig. 4); the original Fig. 4 showed the two products that the authors now know derive from the two splice variants of γ 2a germline transcripts (Ref. 43 in original article). As expected, revised Fig. 4 demonstrates that the expression of authentic I γ 2aC μ RNA (a postswitch RNA) lags behind that of germline transcripts; the expression of CD40L-induced I γ 2aC μ transcripts cannot be detected at day 2 of induction and does not peak until day 3 (or even later, not tested). Nevertheless, the major point of this figure remains correct. CD40 ligation of B cells results in switch recombination to γ 2a. Postswitch I γ 2aC μ transcripts do not pre-exist in the B cell population (they cannot be detected at 5 h or 1 day of culture), but γ 2a postswitch transcripts do appear at day 3, after germline transcription. This correction does not alter any major conclusion of the publication. The corrected figure and legend are shown below.

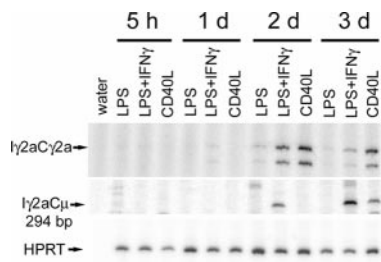


FIGURE 4. T-depleted splenic lymphocytes from IFN- $\gamma^{-/-}$ mice were cultured in LPS, CD40L, or LPS plus IFN- γ for 5 h, 1 d, 2 d, or 3 d. Transcripts, as indicated on the *left side* of the figure, were detected in RNA from these cells by RT-PCR with incorporation of ^{32} P-dATP. The primers for amplification (35 cycles) of I γ 2aC μ were GCTGATGTACCTACCTGAGAG (I γ 2a) and CCAGGTGAAGGAAATGGAGC (C μ), and annealing was at 67°C. Other aspects of the RT-PCR reaction, including HPRT amplification, were as described (28). Other RT-PCR products, none of which was the correct size, were inconsistently amplified in the I γ 2aC μ reaction. To verify that the designated 294-bp fragment is indeed an authentic I γ 2aC μ product, we cloned and sequenced it from RNA of both C57BL/6 and BALB/c cells cultured in CD40L plus IFN- γ . DNA sequence verified that the 294-bp product is the authentic I γ 2aC μ product with 169 bp of I γ 2a sequence joined to 125 bp of C μ product, using the expected splice site (43).

Kim, I.-J., C. E. Burkum, T. Cookenham, P. L. Schwartzberg, D. L. Woodland, and M. A. Blackman. 2007. Perturbation of B cell activation in SLAM-associated protein-deficient mice is associated with changes in gammaherpesvirus latency reservoirs. *J. Immunol.* 178: 1692–1701.

In Table II, the SAP^{-/-} rows were shifted to the left. The corrected Table is shown below.

Table II. Number of cells in the spleen of γ HV68-infected wild-type and SAP-deficient mice^a

Days Postinfection	Group	No. of Spleen Cells ($\times 10^{-6}$ /spleen)	No. of B Cells ^b ($\times 10^{-6}$ /spleen)	No. of Memory B Cells ($\times 10^{-3}$ /spleen)
90	SAP ^{+/+}	80.0	10.5	49.4
	SAP ^{-/-}	44.4	6.2	4.6
150	SAP ^{+/+}	104.0	23.2	371.0
	SAP ^{-/-}	29.6	3.2	13.3
210	SAP ^{+/+}	69.7	8.2	41.8
	SAP ^{-/-}	6.21	1.2	1.2
420 ^d	SAP ^{+/+}	89.6 $\pm$ 34.1 ^e	37.7 $\pm$ 8.9	ND ^f
	SAP ^{-/-}	52.8 $\pm$ 23.1 ^e	26.1 $\pm$ 9.9	ND ^f
540 ^d	SAP ^{+/+}	58.8	20.1	147.0
	SAP ^{-/-}	3.1	9.9	18.2

^a Three to eight spleens were pooled and counted after RBC lysis. Total pooled spleen cells were divided by the number of mice pooled to calculate number of cells per spleen. CD19⁺ B cells and memory B cells were analyzed and calculated based on percent positive cells by FACS analysis. A representative experiment at each timepoint is shown.

^b CD19⁺ cells were gated and number of CD19⁺ B cells was calculated based on the frequency of CD19⁺ cells by FACS analysis within the total spleen cell number.

^c Memory B cells are isotype switched sIgG⁺ CD38^{high} cells.

^d Spleen cells were dissociated with collagenase D to improve cell yield.

^e Data shown are the mean + SD of five individual mice per group.

^f ND, Not done.

Sa, S. M., P. A. Valdez, J. Wu, K. Jung, F. Zhong, L. Hall, I. Kasman, J. Winer, Z. Modrusan, D. M. Danilenko, and W. Ouyang. 2007. The effects of IL-20 subfamily cytokines on reconstituted human epidermis suggest potential roles in cutaneous innate defense and pathogenic adaptive immunity in psoriasis. *J. Immunol.* 178: 2229–2240.

In **Materials and Methods**, under the heading *Microarray analysis and real-time RT-PCR*, the accession number should have been included as the last sentence in the second paragraph. The omitted sentence is shown below.

The microarray data were deposited in the public Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo/) under accession number GSE7216.

Xu, Q., J. Lee, E. Jankowska-Gan, J. Schultz, D. A. Roennburg, L. D. Haynes, S. Kusaka, H. W. Sollinger, S. J. Knechtle, A. M. VanBuskirk, J. R. Torrealba, and W. J. Burlingham. 2007. Human CD4⁺CD25^{low} adaptive T regulatory cells suppress delayed-type hypersensitivity during transplant tolerance. *J. Immunol.* 178: 3983–3995.

The last name of the fifth author was misspelled. The correct name is Drew A. Roenneburg.

In Table I, “off insulin” in footnote *b* and “ceased insulin” in footnote *f* should be “off immunosuppression.” The corrected footnotes are shown below.

^b Kidney transplant from a deceased donor in 1993 for reflux nephropathy. Off immunosuppression 1995; clinical course described as patient DS elsewhere (25) and in Fig. 3.

^f Kidney transplant from a deceased donor in 1986 for chronic glomerulonephritis; off immunosuppression in 2003 due to renal carcinoma in native kidney that was removed in 2004. Current serum creatinine is 1.1 mg/dl.

In Table II, under “Peptide” in the middle column, the labels to the peptides “allo” and “self” are transposed. The corrected table is shown below.

Table II. HLA-B peptide sequences

HLA-B Ag	Peptide	Amino Acid Sequences
B*1501	p106 (p106–123) allo	NH ₂ -DGRLLRGHD Q SAYDGKDY-COOH
B*1501	p149 (p149–166) allo	NH ₂ -AAREAE Q WRAYLEGLCVE-COOH
B*1501; B*5701	p37-MA (p37–54) ^a allo	NH ₂ -DSDAASPR MA PRAPWIEQ-COOH
	p37-TE (p37–54) self	NH ₂ -DSDAASPR TE PRAPWIEQ-COOH
B*0801	p61-F (p61–77) ^b allo	NH ₂ -DRNTQ I FKTNTQTDRES-COOH
	p61-C (p61–77) self	NH ₂ -DRNTQ I CKTNTQTDRES-COOH

^a For the peptide defined by the region from aa 37 to aa 54 we designated the donor allopeptide p37-MA where “MA” refers to the polymorphic residues methionine and alanine at positions 44 and 45 (in boldface type), while the corresponding “self” peptide p37-TE has threonine and glutamic acid. The MA polymorphism is found predominantly in only two common Caucasian HLA-B antigens, HLA-B*1501 and HLA-B*57, a mismatched HLA-B antigen for patient K2.

^b The polymorphic phenylalanine (F) amino acid is present at position 67 (in boldface type) in all HLA-B8 family members, while the cysteine (C) amino acid (boldface type) is present in the HLA-B14 of patient K2.

In **Results**, there are several errors as follows. In the last sentence of the paragraph under the heading *Nonregulated and regulated PBMC samples contain different proportions of IFN- γ vs TGF- β 1-inducible T cells*, “TGF- β 1” is missing from “anti-TGF- β 1 Ab.” The corrected sentence is shown below.

DTH responses to the control peptide, p37-TE, were negative with or without anti-TGF- β 1 Ab (Fig. 5C).

In the first sentence of the first paragraph under the heading *CD4⁺CD25^{low} T cells show variable CD25 expression but retain allopeptide-specific TGF- β 1 responsiveness after flow sorting and short-term culture*, “CD25^{low} T cells” should be “CD25^{low} T_R cells” and “TGF- β 1” is missing. In the last sentence of the same paragraph, the word “retained” should be “remained.” The corrected sentences are shown below.

The adoptive transfer data, coupled with the finding that mainly CD25^{low} T_R cells were induced to express surface TGF- β 1 by allopeptide stimulation in vitro (Fig. 4C), raised the possibility that adaptive T_R cells are among the CD4⁺TGF- β 1⁺ T cells in the graft that showed variable CD25 expression by immunostaining.

However, in cultures with medium alone or peptides, >85% remained negative for CD25 and there was no induction of surface TGF- β 1 expression by p37-MA relative to p37-TE.

Salagianni, M., W. K. Loon, M. J. Thomas, A. Noble, and D. M. Kemeny. 2007. An essential role for IL-18 in CD8 T cell-mediated suppression of IgE responses. *J. Immunol.* 178: 4771–4778.

The second author’s name is incorrect. The correct name is Kok Loon Wong.