Interferon-γ and interleukin-4 reciprocally regulate CD8 expression in CD8+ T cells

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The CD8 co-receptor can modulate CD8+ T cell function through its contributions to T cell receptor (TCR) binding and signaling. Here we show that IFN-γ and IL-4 exert opposing effects on the expression of CD8α mRNA and surface CD8 protein during CD8+ T cell activation. IL-4 caused down-regulation of surface CD8 on ovalbumin (OVA257-264) specific TCR-transgenic OT-I CD8+ T cells activated with OVA257-264 coated antigen presenting cells or polyclonal stimuli, and on wild type CD8+ T cells activated with polyclonal stimuli. This effect was enhanced in each case when the cells lacked a functional IFN-γ or IFN-γR gene. When WT or IFN-γ-deficient OT-I CD8+ T cells were analyzed 9 days after co-injection with control or IL-4-expressing OVA+ tumor cells into Rag-2-/-γ-/- mice, CD8 levels were highest on WT donor cells from mice that received the control tumor and lowest on IFN-γ-deficient donor cells from mice that received the IL-4-expressing tumor. The latter CD8low cells displayed markedly impaired binding of OVA257-264/MHC tetramers and peptide/MHC-dependent degranulation. The data reveal an unexpected role for IFN-γ in tuning the CD8 co-receptor during primary CD8+ T cell activation both in vitro and in vivo.

Results

CD8 Expression Is Reduced in CD8+ T Cells Activated in the Absence of IFN-γ and the Presence of IL-4. Approximately 90% of peripheral CD8+ T cells from OT-I mice carry the transgenic TCR specific for the OVA257-264 epitope SIINFEKL and bind SIINFEKL/H-2Kd tetramers in a CD8-dependent manner (17). Fig. 1A shows that CD8+ cells freshly isolated from pooled lymph nodes (LN) and spleen of OT-I and OT-I x IFN-γ-/- mice bound anti-CD8 Ab at similar levels when co-stained with SIINFEKL tetramers. Most cells of both genotypes displayed a naïve (CD44low CD62Lhigh) phenotype (Fig. 1B). No consistent differences were detected in surface CD8 levels (median fluorescence intensity, MFI) or in the frequencies or absolute numbers of CD4+ CD8+, CD4+ CD8+ or CD4+ CD8- TCRβ cells in thymus, LN or spleen between C57BL/6 and C57BL/6 x IFN-γ-/- mice (data not shown). The data indicate that disruption of the IFN-γ pathway does not alter CD8 expression in naïve CD8+ T cells, consistent with earlier reports (18).

We have previously shown that activation of naïve CD8+ T cells in the presence of IL-4 led to progressive loss of surface CD8 expression over 7–10 days (13, 14). As IL-4 and IFN-γ exert reciprocal effects on some T cell functions, the effect of IL-4 was compared in purified intact and IFN-γ-deficient OT-I CD8+ T cells activated with immobilized Ab to CD3, CD8 and CD11a essentially all conventional CD8+ T cells could eventually give rise to committed CD8low cells that maintained this phenotype in the absence of continued IL-4 exposure (13, 14). By contrast, Park et al. have recently reported that IL-4 and other γc cytokines up-regulated surface CD8 levels when CD8+ T cells were cultured in vitro without a TCR stimulus (7).

IL-4 and IFN-γ reciprocally regulate a number of lymphocyte functions, including the expression of type 1 and type 2 cytokines by CD8+ and CD8+ effector cells (15, 16). The present study was therefore undertaken to determine whether this was also true for CD8 expression in newly activated CD8+ T cells. We showed here that IFN-γ not only counteracts the down-regulatory effect of IL-4 on CD8 mRNA and protein levels but also contributes to the maintenance of CD8 levels in the absence of exogenous IL-4. The reciprocal effects of IL-4 and IFN-γ were observed both in vitro and in the response to an IL-4-expressing tumor in vivo and point to an unexpected role for these key immunoregulatory cytokines in tuning TCR signaling thresholds during the primary CD8+ T cell response.


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observations were made when intact and IFN-γ−/− mice were cultured with (A) anti-CD8α Ab and SIINFEKL tetramer or (B) Ab to CD44 and CD62L. The CD8 MFI and the percentage of cells with a naïve (CD44low CD62Lhigh) phenotype (C) the two CD8+ populations were cultured with anti-receptor Ab and IL-2 with or without IL-4 for 6 days then stained with anti-CD8α Ab (filled histograms) or isotype control Ab (open histograms). (D) The same CD8+ populations were cultured with SIINFEKL-coated APC or PMA and ionomycin, and IL-2 with or without IL-4. CD8 expression is shown as relative fluorescence intensity, obtained by normalizing the MFI to the highest sample in the experiment (100%). (E) CD8 expression by CD8+ cells from C57BL/6 WT (IFN-γ+/+) IFN-γ−/− or IFN-γ−/− mice was measured after culture with anti-receptor Ab, IL-2 and with or without IL-4 for 10 days. Groups were compared by unpaired t test (see Materials and Methods).

CD8 Expression Is Determined by the Balance Between IFN-γ and IL-4 During Primary Activation. We have previously found that IL-4 and IFN-γ deficiency and replacement exerted only minor effects on CD8β mRNA levels (Fig. 2B). Fig. 2C shows that mixing of exogenous IFN-γ and IL-4 in varying concentrations modulated surface CD8 expression, particularly in the absence of endogenous IFN-γ. The data demonstrate the striking sensitivity of CD8+ T cells to cytokine-dependent regulation of surface CD8 levels during primary activation and show that these effects are mediated mainly by controlling expression of the α-chain of the CD8 heterodimer at the mRNA level. As CD8α is required for surface expression of CD8β (19), regulation of the α-chain determines levels of surface expression of both components of the heterodimer. The down-regulation of CD8 expression was maintained for at least 21 days in vitro [supporting information (SI) Fig. S1], indicating that the effect was not a transient response to activation.

CD8 Expression Is Reciprocally Regulated by IL-4 and IFN-γ in Vivo. To test the ability of IL-4 and IFN-γ to regulate CD8 expression in vivo, Va2+ CD8+ cells from OT-I or OT-I x IFN-γ−/− mice were adoptively transferred into RAG-2−/−γc−/− hosts and activated with OVA-expressing tumor cells transplanted with an IL-4-expressing or a control vector. Donor (Va2+) cells exhibited a similar hierarchy of CD8 expression levels to that observed in vitro, with maximal down-regulation in cells from IFN-γ−/− mice that received the IL-4-secreting tumor (Fig. 3A). Median CD8 levels differed significantly between all groups (Fig. 3B). The down-regulation of CD8 expression observed in this protocol apparently depended on T cell activation as CFSE-labeled CD8+ T cells from OT-I and OT-I x IFN-γ−/− mice proliferated in vivo but maintained their surface CD8 levels when recovered 5 days after s.c. injection into RAG-2−/−γc−/− hosts in the absence of the tumor (Fig. S2). Fig. 3C shows that the hierarchy of CD8 expression observed ex vivo was maintained at the level of CD8α mRNA when the cells from the four groups were restimulated in vitro with anti-receptor Ab. The data demonstrate that the IL-4-dependent down-regulation of CD8 expression we and
MFI is shown for each population following staining with the indicated tetramer dilutions in the absence of anti-CD8 receptor Ab for 6 days.

IL-4 but is normally dampened by endogenous IFN-γ. Apte et al.

Fig. 4. Modulation of CD8 levels by IL-4 and IFN-γ. (A) The restimulated cell populations in Fig. 3 were co-stained with anti-CD8α Ab and (A) SIINFEKL tetramers at a dilution of 16.7 × 10^{-4} or (B) anti-Vα2 Ab. Values within the frames indicate the percentage of cells in that region. (C) The tetramer MFI is shown for each population following staining with the indicated tetramer dilutions in the absence of anti-CD8α Ab. (D) The restimulated cell populations were rested for 6 days then cultured with SIINFEKL or SIIGFKL peptide-coated APC or with PMA, ionomycin, and IL-2, in the presence of anti-CD107 Ab and monensin. Upper frames show CD107 profiles of the indicated populations from (D); the open histogram in the left frame shows CD107 Ab binding to cells cultured with APC without peptide. Lower frames show corresponding CD8 profiles and CD8 MFI following gating on CD107^− (dark) and CD107^+ (light) cells.

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against an association of CD8 down-regulation with sub-
WT CD8 expression during effector CD8 T cell development both in vitro and in vivo. We report that IFN-γ not only counteracts the down-regulatory effect of IL-4 on CD8α mRNA and CD8αβ surface protein levels but also contributes to their maintenance in the absence of exogenous IL-4.

Several recent reports have shown that modulation of CD8 expression, or tuning, can alter the signaling threshold through the TCR, with even moderate changes in CD8 levels markedly affecting TCR-dependent effector function (7, 9, 10). Here we show that IFN-γ- and IL-4-dependent modulation of CD8 levels correlated with changes in peptide/MHC tetramer binding and in degranulation in response to specific TCR stimulation. These data are consistent with the work of others showing that reduced CD8 expression correlated with a reduction in tetramer binding (9, 10) and our earlier observations that CD8+ T cells that down-regulated CD8 during activation in the presence of IL-4 exhibited lower perforin-dependent cytolytic activity in 51Cr-release assays than their CD8high counterparts (13). In preliminary experiments, we have also found that IFN-γ−/− donor T cells recovered from host mice injected with IL-4-producing tumor cells (in the protocol used in Fig. 3) not only expressed lower levels of CD8 but also showed reduced ability to control EG7 tumor cells when co-injected into RAG-1−/− recipients, compared with IFN-γ−/− cells recovered from mice injected with control tumor cells (unpublished data).

A number of earlier studies have shown that IL-4 induces both the de novo production of IL-4- and down-regulation of CD8 expression in activated CD8+ T cells (12–14, 22). Important clinical correlates have been reported in HIV infection and chronic B cell lymphocytic leukemia, in which IL-4-expressing CD8+ T cells with reduced CD8 expression have been described and may be associated with disease progression (23–25). The data in HIV have recently been supported experimentally by the observation that therapy of simian HIV-infected macaques with antisenese IL-4 DNA was associated with an increase in CD8 expression by CD8 T cells (26). In our earlier studies, we found that IL-4 induced the down-regulation of CD8 expression in essentially every conventional CD8+ T cell during activation in vitro. However, the extent of CD8 down-regulation was variable in vitro (14) and exposure of WT CD8+ T cells to IL-4-expressing tumor cells in vivo affected their IL-4 and granzyme mRNA levels but did not lead to a significant reduction in CD8 expression (27). The findings reported here offer an explanation for these results, namely that endogenous or host-derived IFN-γ might counteract the effects of IL-4 on CD8 levels, inhibiting the emergence of CD8low effector cells. An alternative explanation might be that CD8 is down-modulated in the absence of IFN-γ because lower MHC Class I expression leads to sub-threshold T cell priming. We think this is unlikely as we recovered similar numbers of IFN-γ−/− and IFN-γ−/− cells following adoptive transfer with tumor cells in the experiment shown in Fig. 3 and all displayed a CD44high phenotype (unpublished observations), suggesting comparable activation efficiency. Our data in vitro also argue against an association of CD8 down-regulation with sub-threshold priming as cells progressively lost CD8 over time and many cell divisions in the presence of optimal concentrations of immobilized anti-receptor Ab (14).

The finding that IFN-γ contributes to the maintenance of CD8 expression following TCR-dependent activation in the absence of IL-4 was unexpected. One implication is that “normal” CD8 levels on effector cells are determined, at least in part, by ambient IFN-γ concentrations and are therefore subject to tuning in response to changes in the levels both of this cytokine and of its receptor. It is notable that IFN-γR1 levels on CD8+ T cells varied over the course of one viral infection (28) and that IFN-γ exposure altered the expansion, immunodominance and death of antigen-specific CD8+ T cells during the course of infection in other models (29, 30), although a direct link between altered CD8 levels, TCR avidity and immunodominance remains to be established.

In our hands, cytokine-induced alterations in CD8 expression are maintained over several weeks in cultures of proliferating CD8+ T cells, suggesting heritable epigenetic changes in the expression machinery. The molecular mechanisms by which IFN-γ and IL-4 modulate CD8 levels are not yet known. Several transcription factors have been implicated in the control of CD8α gene expression, including STAT5 and STAT6 for which a putative binding site has been identified within the CD8 enhancer element E8α (7), MAZR which binds enhancer E8α (8), negatively regulates this enhancer (31), cKrox which binds E8α and negatively regulates both CD8 and IFN-γ expression while up-regulating GATA-3 (32), and GATA-3 for which binding sites have been identified in both mouse and human CD8 enhancer regions (33, 34). Two observations suggest that GATA-3 may have a role as a negative regulator: first, in the thymus, GATA-3 promotes CD4 lineage development while inhibiting CD8 lineage development (35); second, GATA-3 expression is induced in CD8+ T cells exposed to IL-4 (36) or transduced with cKrox which, as noted above, down-regulates CD8 expression (32).

Selective regulation of CD8α was recently reported by Park et al. (7) who found that the γc cytokines IL-2, IL-4, IL-7, and IL-15 increased CD8α (not β) mRNA and protein levels in LN CD8+ T cells in the absence of TCR signaling; IL-4 and IL-7 acted by increasing the CD8α transcription rate, at least in part via the E8α enhancer. Their data are consistent with our observation that IFN-γ and IL-4 act preferentially on CD8α mRNA levels and suggest that the effects we observed might be mediated via one or more of the CD8 enhancer elements, most of which are located within the CD8 locus and lie downstream of the CD8α genes (37). On the other hand, their observation that IL-4 enhanced CD8α expression on T cells in the absence of TCR signaling contrasts with our finding that IL-4 inhibits CD8 expression following TCR-dependent activation. It will now be interesting to investigate the immunological significance of this shift in CD8 responsiveness to IL-4 following primary activation.

In summary, IFN-γ and IL-4 reciprocally modulate CD8 levels on CD8+ T cells activated with various stimuli in vitro and in response to tumor cells in vivo, acting at the level of CD8α mRNA to alter surface CD8αβ protein levels. These changes in CD8 expression are correlated with functional alterations in tetramer binding and antigen-specific degranulation, reflecting their potential to exert profound effects on the sensitivity of CD8+ T cells to peptide/MHC levels and the resultant response to pathogens.

**Materials and Methods**

**Mice**. Specific pathogen-free C57BL/6 (CD43.2) and B6.SJL-Ptprc<sup>−/−</sup> (CD45.1) mice (Animal Resources Centre) were used at 6–9 weeks of age. RAG-2−/− γc−/− [provided by Dr. Alberto Pinzon-Charrly, Queensland Institute of Medical Research (QIMR), OT-I (243.2) (Dr. William Heath, The Walter and Eliza Hall Institute of Medical Research)] mice were used at 6–9 weeks of age. RAG-2γc−/− mice (Animal Resources Centre) were used at 6–9 weeks of age. RAG-2−/− γc−/− mice were used at 6–9 weeks of age.
were enriched from spleen (9 days after injection) by fluorescence-activated cultured at 24°C (Melbourne). Cells were washed and resuspended with 1 ml growth medium supplemented as above. (3) Peptide activation. Naïve CD8+ T cells were cultured at 24 × 10^5/well with 10 μg/ml PMA and 250 ng/ml ionomycin (Sigma) in 12-well plates with 4 ml growth medium supplemented as above. (3) Peptide activation. Naïve CD8+ T cells were cultured at 24 × 10^5/well with 5 × 10^5/μl irradiated congenic CD45.1+ splenocytes previously incubated with 10 μg/ml SIINFEKL peptide (Minotopes) for 1 h and γ-irradiated with 3000 cGy, in 12-well plates with 4 ml growth medium supplemented as above.

**Activation of CD8+ T Cells in Vivo**. (1) Anti-receptor Ab. Naïve CD8+ T cells were cultured at 125 × 10^5/well in 6-well plates coated with purified Ab to CD3ε (145–2C11, 10 μg/ml), CD80 (53.6; 10 μg/ml) and CD11a (2I7/7.7; 5 μg/ml) in 8 ml growth medium (modified DMEM, 50 μM 2-mercaptoethanol and 216 mg/ml t-glutamine), 10% heat-inactivated FCS and 20 IU/ml human rIL-2 (National Institutes of Health AIDS Research & Reference Reagent Program) (39) with or without 25 ng/ml mouse rIL-4 and the indicated concentrations of mouse rIFN-γ (both from ProSpec-Tyan Technogeno, Revohot, Israel) (2). PMA/ionomycin. Naïve CD8+ T cells were cultured at 24 × 10^5/well with 10 μg/ml PMA and 250 ng/ml ionomycin (Sigma) in 12-well plates with 4 ml growth medium supplemented as above. (3) Peptide activation. Naïve CD8+ T cells were cultured at 24 × 10^5/well with 5 × 10^5/μl irradiated congenic CD45.1+ splenocytes previously incubated with 10 μg/ml SIINFEKL peptide (Minotopes) for 1 h and γ-irradiated with 3000 cGy, in 12-well plates with 4 ml growth medium supplemented as above.

**Fluorescence-Activated Cell Sorting and Analysis**. Cells were incubated on ice with combinations of fluorochrome-conjugated reagents: Ab to CD8ε (S3–6.7), CD8β (CD8βj), CD26L (ME-14) and CD45.2 (104) and isotype controls (BioLegend); Ab to CD44 (IM7), CD107a (1D4B) and CD107b (H4B4) and isotype controls (BD Biosciences); Ab to Vα2 (B2.10) and isotype control (ebioScience); SIINFEKL/K-2K6 tetramers (provided by Dr. Stephen Turner, University of Melbourne). Cells were washed and resuspended with 1 μg/ml propidium iodide (PI; Calbiochem). CD8+ T cells were purified by using a MoFlo cytometer (DakoCytomation) with exclusion of dead cells based on forward scatter and PI uptake. For analysis without sorting, a FACSCalibur was used with CellQuest version 3.1f software (BD Biosciences). Post-acquisition data analyses were performed by using Summit Software v4.3 (Dako).

**In Vitro Restimulation of in Vivo-Activated T Cells**. Donor Vα2+CD8+ T cells were enriched from spleen (9 days after injection) by fluorescence-activated cell sorting and cultured at 2 × 10^6/well with anti-receptor Ab under the same conditions as naïve T cells except that plates were coated with a reduced concentration of anti-CD3 Ab (1 μg/ml).

**RNA Preparation and Real-Time PCR Analysis**. RNA was extracted by Nonidet P-40 hypotonic lysis of 3 × 10^6 cells and cdDNA was prepared as described (13). Triplicate cdDNA samples were prepared from each culture and each cdDNA sample was assayed in duplicate. cdDNA was quantified by real-time PCR using the following primers and probes for β2-microglobulin (β2M) (5′: 5′-TTCCTGGGCTCTGGTCAAC-3′; 3′-GTCCTGCTCCACTTCACTCT-5′; probe: 5′-JOECGAGGTATGCTATCCAGAACAAC-BHQ-1′-3′), cdDNA (5′: 5′-GAGATGGCATCTCAGAAATGAG-3′; 3′: 5′-CCGCAATCTTGCTCTTCT-5′; probe: 5′-FAM-CTACACGAGGCTGTCGCAACT-BHQ-1′-3′) and CD8j (kit ID number Mm00438116_m1, Applied Biosystems). Samples were amplified in a Corbett Rotor-Gene 3000 (Corbett Research) for 40 cycles at 95°C for 2 min, 95°C for 5 s, and 60°C for 30 s.

All results except those comparing CD8β and CD8βj are reported in 2′ units (target gene copy number/r2 gene copy number) and represent the mean of triplicate cdDNA samples. Relative CD8β and CD8βj mRNA expression was determined by comparison to a standard curve obtained by titrating CD8+ T cell cdDNA containing known CD8α and unknown CD8β cdDNA copy numbers; the derived values of the test samples were then divided by the j2 M copy number and expressed as a percentage of the highest mean value of the test samples.

**CD107 Mobilization Assay.** After restimulation in culture, CD8+ T cells were rested in 20 μl growth medium human rIL-2 for 6 days. Live cells were then isolated on Ficoll-Paque and cultured at 5 × 10^5 cells/well with either 10^5 APC (CD45.1+ splenocytes incubated with or without 10 μg/ml SIINFEKL or SIIGFEKL peptide at 37°C for 1 h) or 40 ng/ml PMA and 0.67 μl/ml monensin (all from BD Biosciences) for 5 h at 37°C (20). Cells were then washed and analyzed for CD45.2 and CD8 expression by using a FACSCalibur with exclusion of dead cells by PI. The percentage of CD107ab+ cells was determined by subtraction of the unstimulated control from the test sample using Summit Software v4.3 (Dako).

**Statistical Analyses.** Data were evaluated by unpaired t test (Prism 4.02 software package, GraphPad Software1). P values are indicated in the figures by the symbols: >0.05, ns; 0.01–0.05, *; 0.001–0.01, **; <0.001, ***.

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Supporting Information
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Fig. S1. CD8 down-regulation persists in long-term culture. CD8⁺ cells from C57BL/6 WT (IFN-γ⁺⁺⁺), IFN-γR⁻⁻⁻ and IFN-γ⁻⁻⁻ mice were cultured with anti-receptor Ab and IL-2 with or without IL-4 for 21 days then stained with anti-CD8α Ab (filled histograms) or isotype control Ab (open histograms). The mean fluorescence intensity (MFI) of CD8 binding is shown.
Fig. S2. CD8 expression is maintained during homeostatic proliferation. Vα2⁺ CD8⁺ cells from OT-I (IFN-γ⁺⁺) or OT-I x IFN-γ⁺⁻ (IFN-γ⁻⁻) mice were labeled with CFSE and injected into RAG-2⁻⁻⁻ mice. After 5 days, splenic Vα2⁺ cells were identified (upper panels, R3 gate) and analyzed for surface CD8 expression and CFSE dilution by flow cytometry (lower panels). The mean fluorescence intensity (MFI) of CD8 binding is shown.
**Fig. S3.** TCR expression is maintained on most in vivo activated cells. Vα2⁺ CD8⁺ cells from OT-I (IFN-γ⁻/⁻) or OT-I x IFN-γ⁻/⁻ (IFN-γ⁻/⁻) mice were co-injected with EG7-IL-4⁺ (IL-4⁺) or EG7-IL-4⁻ (Ctrl) tumor cells into RAG-2⁻/⁻γ⁻/⁻ mice. After 9 days, splenic Vα2⁺ CD8⁺ cells were isolated and restimulated in vitro with anti-receptor Ab for 6 days and then co-stained with anti-CD8α Ab and anti-Vα2 Ab. Values within the panels indicate the percentage of cells in that region.