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Gene Regulation and Chromatin Remodeling by IL-12 and Type I IFN in Programming for CD8 T Cell Effector Function and Memory

Pujya Agarwal,*† Arvind Raghavan,2*‡ Sarada L. Nandiwada,*‡ Julie M. Curtsinger,*† Paul R. Bohjanen,*‡§ Daniel L. Mueller,*‡ and Matthew F. Mescher3*†

A third signal that can be provided by IL-12 or type I IFN is required for differentiation of naive CD8 T cells responding to Ag and costimulation. The cytokines program development of function and memory within 3 days of initial stimulation, and we show here that programming involves regulation of a common set of ~355 genes including T-bet and eomesodermin. Much of the gene regulation program is initiated in response to Ag and costimulation within 24 h but is then extinguished unless a cytokine signal is available. Histone deacetylase inhibitors mimic the effects of IL-12 or type I IFN signaling, indicating that the cytokines relieve repression and allow continued gene expression by promoting increased histone acetylation. In support of this, increased association of acetylated histones with the promoter loci of granzyme B and eomesodermin is shown to occur in response to IL-12, IFN-α, or histone deacetylase inhibitors. Thus, IL-12 and IFN-α/β enforce in common a complex gene regulation program that involves, at least in part, chromatin remodeling to allow sustained expression of a large number of genes critical for CD8 T cell function and memory. The Journal of Immunology, 2009, 183: 1695–1704.

 naive CD8 T cells that encounter Ags on mature dendritic cells (DC)4 proliferate, clonally expand, and differentiate to acquire effector functions and altered adhesion-migration properties that facilitate trafficking to peripheral tissues (1, 2). Many of the effector cells then die, but about 10% persist long term as memory cells that respond rapidly upon re-encountering Ags. In contrast, Ag presentation by immature DCs results in normal CD8 T cell proliferation, but clonal expansion is limited by poor survival, compromised effector functions, and tolerance results. Although Ag and costimulatory ligands, usually B7-1 and B7-2, are essential for the activation and proliferation of naive cells, there is now considerable evidence that these two signals are not sufficient to promote survival, differentiation, and establishment of memory. Instead, a third signal is required that can be provided by either IL-12 or IFN-α/β (3–9).

The requirement for a third signal was initially demonstrated in vitro using highly purified naive CD8 T cells and artificial APCs (aAPC); microspheres with class I/peptide Ag complexes and B7-1 ligands on the surface (5, 6, 10). Consistent with these in vitro results, coadministration of peptide Ag with IL-12 (11) or IFN-α (7) bypassed the requirement for adjuvant to generate strong in vivo responses and avoided the tolerance induced when peptide alone was administered. The relative contributions of IL-12 and IFN-α/β in vivo depend upon the physiological response being examined. In an ectopic heart transplant model it was shown that CD4 T cells conditioned DCs to produce IL-12 and that this IL-12 was necessary to support development of CD8 effector functions and graft rejection (12). In contrast, the response to lymphocytic choriomeningitis virus (LCMV) infection was reduced by >99% when the virus-specific CD8 T cells lacked the receptor for type I IFNs (7), whereas the response to vaccinia virus (VV; Ref. 2) infection was only partially reduced (3). Both IL-12 and type I IFNs support CD8 T cell responses to Listeria monocytogenes and VV infections (13). For both L. monocytogenes and VV expressing the OVA257–264 peptide Ag, OT-I CD8 T cells that lacked both IL-12 and type I IFN receptors underwent strong primary clonal expansion, but function is compromised at the peak of the response, and a memory population does not form. Similarly, the weak CD8 T cell response that occurs to E.G7 thymoma tumor depends on IL-12 and/or type I IFN (14). Thus, IL-12 and type I IFNs appear to be the major sources of the third signal for CD8 T cell activation in normal mice.

Interaction of naive CD8 T cells with Ag and B7-1 for 2–6 h is sufficient to program the cells to subsequently undergo multiple rounds of division over the next 3 days, but survival is poor and effector functions do not develop in the absence of IL-12 or IFN-α/β signals (15, 16). Optimal clonal expansion and development of function require stimulation with Ag, B7-1, and IL-12 for a prolonged period (16), consistent with the prolonged interaction that occurs between CD8 T cells and DCs in lymph nodes under conditions that lead to effective activation (17, 18). The programming required for development of a memory population also occurs as a result of signals received while the cells are initially responding to Ag. In vitro stimulation of splenocytes for 3 days is sufficient to yield a memory population when the cells are then transferred into normal mice (19). When naive cells are stimulated
for 3 days with Ag and B7-1 on aAPC and transferred into normal mice, very few of the cells survive to day 30 and those that do are tolerant, whereas cells stimulated with aAPC and IL-12 for 3 days form a protective memory population on transfer (13). Thus, during the first 3 days of a response to Ag and costimulation, IL-12 not only signals for development of effector functions, but also programs the cells to survive as functional memory cells.

To determine the extent and molecular nature of the differentiation program induced by IL-12 and IFN-α/β in concert with TCR and CD28 signals, we have conducted oligonucleotide microarray analysis of naive cells responding to Ag and B7-1 alone, and in the presence of IL-12 or IFN-α. The results demonstrate that the differentiation program induced by the cytokines includes changes in expression of many genes that have important roles in CD8 T cell survival, function, signaling, and migration, as well as of genes for transcription factors that are likely to have important roles in controlling these expression patterns, including T-bet and eomesoderm (Eomes). The temporal pattern of IL-12/IFN-α/β-regulated gene expression suggests that histone-dependent chromatin remodeling mechanisms play a central role in determining the differentiation program, and evidence in support of this idea is presented.

Materials and Methods

Mice and in vitro cell stimulation

Naive (CD44low) CD8+ T cells from lymph nodes of OT-1 mice, having a transgenic TCR specific for H-2Kb/OVA257-264 (20), were negatively enriched (MACS; Miltenyi Biotec) to >97% purity. aAPC were made using 5 μM latex microspheres coated with Dimer-X-H-2Kb/Ig fusion protein (BD Pharmingen; 2.5 μg/107 beads) and B7-1/Fc chimeric protein (R&D Systems; 0.15 μg/107 beads) and Ag complexes formed using 200 nM OVA257-264 peptide. Cells were cultured in vitro at a 1:4 ratio with the aAPCs in the absence or presence of murine IL-12 (Genetics Institute; 2 U/ml) or Universal type I IFN (PBL Biomedical Laboratories; 1000 U/ml). All cultures were supplemented with human rIL-2 at 2.5 U/ml (TECNIC: National Cancer Institute Biological Resources Branch (NCBIBethesda, MD). Trichostatin A (TSA; Upstate Biotechnology; 7.5 ng/ml), sodium butyrate (Sigma-Aldrich; 1 mM), and curcumin (Sigma-Aldrich; 2–5 μg/ml) were added from the beginning of the cell culture when used. In the presence of TSA, cells exhibited good viability but proliferation at 72 h was reduced. Cells were harvested at the indicated times for staining, and total RNA was isolated (RNeasy Mini Kit; Qiagen) for cRNA preparation. Mice were housed under specific pathogen-free conditions at the University of Minnesota (Minneapolis, MN) and were used in compliance with relevant laws and institutional guidelines and with the approval of the Institutional Care and Use Committee of the University of Minnesota.

Intracellular staining and in vitro cytolytic assay

Cells were harvested at the indicated times, with addition of 0.6 μM GolgiStop (BD Pharmingen) for the last 3 h of culture, and intracellular staining was performed as previously described (4) using PE-conjugated anti-human granzyme B (grzB) and mouse IgG1 (Caltag Laboratories). APC-conjugated anti-IFN-γ and rat IgG1 (eBioscience) Abs and analyzed by flow cytometry using FlowJo software. For T-bet intracellular detection, fixed cells were permeabilized with 0.12% Triton X and 2% FCS in PBS and stained for 2 h with fluorescein isothiocyanate-conjugated mouse anti-T-bet mAb (Santa Cruz Biotechnology). Cytolytic activity was determined in a standard 4-h 51Cr release assay using E/G7 cells (EL-4 thymoma transfected with OVA) as targets, with EL-4 cells included as a control for specificity. Triplicate measurements were done in all assays with SD <0.05%.

cRNA preparation and microarray data analysis

Biotin-labeled transcripts were prepared from 10 μg of RNA according to the manufacturer’s protocol for hybridization onto Affymetrix MG U74Av2. The quality of cRNA was evaluated using test chips. GeneChips were probed, hybridized, and scanned at the University of Minnesota BioMedical Genomics Center Facility. Triplicate arrays were done for naive (0 h) and three-signal-stimulated cells (48 h), and four arrays were done for two-signal stimulated (48 h) RNA samples obtained from independent experiments and single arrays were done for 24- and 72-h samples. For triplicate samples, transcripts were included in the analysis if present in two out of three experiments and for Ag-B7 (48 h) if present in at least two experiments. Signal log ratios were generated using GCRMA and fold change calculated as 2^Δlog ratio. Significant differentially expressed genes were sorted that expressed an average fold change of ±1.70 and a change p value of ≤0.05 (Wilcoxon’s signed rank test). Gene treeview clustering, hierarchical linkages, and mean-centered clustering GeneSpring (version 7.2 (Agilent Technologies). The microarray data have been deposited in the NCBI GEO database and can be accessed at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE15930.

Semiquantitative RT-PCR

Total RNA was reverse-transcribed into cDNA using Superscript RNASense H-III (Invitrogen). cDNA (75 ng) was amplified using a PCR setting (94°C for 5 min; 94°C for 30 s, 60–65°C for 60 s, 92°C for 60 s, 72°C for 10 min; 27 cycles of T-bet/oesomes; 21 cycles of β-actin) with primers sequences designed using Primer3 software as follows: T-bet (575 bp), 5’-caggatgtagtttgatggg-3’ and 5’-catgaaagggctgtagaa-3’; eomes (642 bp), 5’-ggtgtgctttagcagagga-3’ and 5’-gcagctctttcatctgggc-3’. β-actin (224 bp), 5’-caggctcatactggcaagca-3’ and 5’-gaggatgtaaccttctgac-3’. Amplification was done for 20, 22, 25, 27, and 30 cycles to determine the linear range of amplification for T-bet and eomes mRNA. β-Actin expression was the same in all samples at 15, 17, and 21 cycles.

Chromatin immunoprecipitation assay and quantitative real-time PCR

Purified naive CD8 T cells stimulated in vitro as described previously were harvested at 48 h to obtain ~15 × 10^6 cells that were processed for chromatin immunoprecipitation assay and real-time PCR using a described procedure (21). Anti-acetyl H3 and anti-acetyl H4 (Upstate Biotechnology) Abs were used with normal rabbit IgG (Santa Cruz) as isotype controls. The primer pairs used for analysis of DNA were: greb promoter (292 bp), forward 5’-aactagctgtagctgggctg-3’ and reverse 5’-ttgctgacgctctgagcatag-3’; eomes promoter (149 bp), forward 5’-gctcaagactcagcagcbe-3’ and reverse 5’-actgacagctatgcacctc-3’. Quantitative real-time PCR was performed on the Cepheid SmartCycler II system with a cycle of 95°C for 5 min, 95°C for 15 s; 62°C (eomes)/65°C (grzB) for 30 s, 72°C for 30 s and 40 cycles. Template copy numbers for PCR cycle thresholds were extracted using standard graphs. For each sample, template copy numbers were internally normalized with their respective input control. Relative expression was calculated as the ratio of template copy numbers of a sample relative to the naive control after normalizing with their respective isotype control IgG and is shown as the mean ± SEM. Statistical significance was determined by a one-tailed pair Student’s t test.

Online supplemental material

Supplemental Table I provides a list of genes regulated by two signals (two signals) or with IL-12 or IFN-α/β-mediated changes in gene expression with respect to Ag-B7; supplemental Table III shows IFN-α-mediated changes in gene expression with respect to Ag-B7; Supplemental Table IV lists genes regulated in common by IL-12 and IFN-α (from supplemental Tables II and III); supplemental Figs. 1 and 2 depict various kinetic patterns of gene expression in response to IL-12 and IFN-α, respectively, as determined using the k-means clustering algorithm. Supplemental Tables V and VI provide lists of genes as illustrated in supplemental Figs. 1 and 2, respectively; supplemental Table VII shows genes regulated by TSA in common with IL-12 and IFN-α.

Results

Gene expression regulation in response to Ag and B7-1 alone (two signals) or with IL-12 or IFN-α (three signals)

When naive (CD44low) OT-I CD8 T cells expressing a transgenic TCR specific for H-2Kb and OVA257-264 peptide (20) are stimulated with microspheres coated with H-2Kb/OVA257-264 complex and B7-1 ligand (Ag-B7), the cells proliferate and clonally expand. However, they fail to become cytolytic effectors (Fig. 1A), express low granzyme B (grzB) levels (Fig. 1C), and produce little IFN-γ

5 The on-line version of this article contains supplemental material.
upon restimulation (Fig. 1B) unless IL-12 or type I IFN is also present in the cultures. Use of aAPC and purified naive TCR-transgenic CD8 T cells provides an ideal system for determining the contributions of cytokines to gene regulation and differentiation. The signals available to the cells are precisely defined because unlike APCs, the aAPCs do not display additional ligands or produce other cytokines, and they do not contribute to the pool of RNA isolated for analysis. In these experiments, IL-2 was added to all of the cultures to achieve maximum clonal expansion and avoid expression of genes involved in cell cycle control, DNA synthesis and repair, and protein translation were coordinately regulated by this cytokine by the activated T cells.

Gene expression was analyzed using Affymetrix murine MG U74Av2 gene chips displaying 12,488 genes/expressed sequence tags (ESTs). Naive OT-1 CD8 T cells expressed 4273 transcripts over 3 days, and Ag-B7 and IL-12 must be present for optimal clonal expansion and development of effector function (from Ref. 16). E, Expression patterns for 408 probe set identifiers (ids) representing the 355 genes regulated by both IL-12 and IFN-α. The stimuli used and time in culture for each sample are shown at the top. Red represents high expression (signal value); blue represents low expression on a log2 scale of 6.0 to 0.0 as shown at the bottom. Data not meeting the selection criterion was excluded and appears gray. The signal value was normalized to −1. F, Hierarchical clustering linkage for cells treated with the various stimuli at 0, 24, 48, and 72 h.

When compared with expression in cells stimulated only with Ag-B7, addition of IL-12 or IFN-α was found to further regulate expression of a modest number of genes/ESTs (Table I). In contrast to Ag-B7 stimulation, where the greatest number of genes was regulated at 24 h, the numbers of genes regulated in response to IL-12 and IFN-α/β progressively increased at longer times. Because the functional outcome of signaling via IL-12 or IFN-α is similar, the genes regulated in common by both cytokines were of greatest interest. This set included 355 genes commonly regulated

<table>
<thead>
<tr>
<th>Table I. Numbers of genes regulated by IL-12 and IFN-α</th>
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<tr>
<td>---------</td>
</tr>
<tr>
<td>24</td>
</tr>
<tr>
<td>48</td>
</tr>
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<td>72</td>
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</tbody>
</table>

<sup>a</sup> The total number of genes with increased or decreased expression at 24, 48, or 72 h by ≥1.7-fold in response to Ag-B7 and IL-12 or IFN-α relative to expression in naive cells.

<sup>b</sup> The number of genes that increased or decreased in expression at 24, 48, or 72 h by ≥1.7-fold in response to Ag-B7 and IL-12 or IFN-α relative to cells stimulated with only Ag-B7.
at least one time point. Previous work showed that prolonged signaling by Ag-B7 and IL-12 are necessary for full activation, and defined temporal windows required for IL-12 to optimally support clonal expansion (15–35 h) and development of effector functions (30–60 h) (16). This is depicted in Fig. 1D, along with the numbers of genes regulated in common by IL-12 and IFN-α at these times. Distinct patterns of regulation are seen, with the majority of genes being regulated late, during the period when IL-12 signaling is critical for development of effector functions.

Expression levels of the set of genes regulated in common by IL-12 and IFN-α are shown in Fig. 1E. In comparison to naive cells, expression of the majority of these genes changes within 24 h in response to Ag-B7, and most exhibit similar expression levels whether or not IL-12 or IFN-αβ are present. By 72 h, however, expression levels of most of these genes in cells stimulated with Ag-B7 revert to a pattern quite similar to that of naive cells, and very distinct from the patterns observed in cells stimulated with IL-12 or IFN-α. Hierarchical clustering (Fig. 1F) confirmed that at 72 h the expression levels of genes in cells stimulated with Ag-B7 were most closely linked to those of naive cells, whereas the 72-h expression profiles of cells stimulated in the presence of IL-12 or IFN-α were closely linked to each other. The expression profiles for cells stimulated with IL-12 and IFN-α for 72 h reveal striking similarities, but there are also differences, including genes with expression that is changed in opposite directions by the two cytokines. For many of the genes regulated by both IL-12 and IFN-α, it appears that signals provided by Ag-B7 initiate changes in expression levels within 24 h, but the changes are transient and expression reverts to naive levels by 72 h. In the presence of either IL-12 or IFN-α/β, the changes in expression increase and persist at 72 h, and additional changes occur, consistent with these cytokines inducing a differentiation program in the cells.

**Genes regulated by IL-12/IFN-αβ and patterns of temporal expression**

Complete data sets at 24, 48, and 72 h for genes that change in expression level in cells stimulated with Ag-B7 in comparison with naive cells, and for genes that change in response to IL-12 and IFN-α in comparison to Ag-B7 alone, are included as supplemental Tables I–III. Supplemental Table IV lists the subset of those genes that are regulated in common by IL-12 and IFN-α. Gene regulation in response to only Ag-B7 is briefly described in Discussion. A partial listing of the genes regulated by IL-12 and IFN-α is shown in Fig. 2 and Table II. Analysis based on biological function (National Center for Biotechnology Information database, GenBank annotation, and literature search) demonstrates that IL-12 and IFN-αβ regulate expression of numerous genes that encode proteins for CTL effector functions, cell surface receptors, signal transduction, defense and homeostasis, transcription regulation, cell adhesion and migration, cytoskeleton, secretion, metabolism, transport, and other functions (Table II and supplemental Tables II–IV). Some of these are of obvious relevance for acquisition of effector functions, including grzB, Fas ligand, and IFN-γ. Others are known or likely to be important in conferring the ability to survive (and thus undergo clonal expansion) and regulate migration to peripheral tissues. Time courses for expression of a number of genes in response to two or three signals are shown in Fig. 2 (these are not included in Table II to avoid duplication), and illustrate several ways in which IL-12 and IFN-α regulate expression. For several, including grzB, IFN-γ, CXCL-10, OX40, 4-1BB, and serine protease inhibitor 6, Ag-B7 up-regulates expression by 24 h but levels then decline, whereas IL-12 and IFN-α act to enhance and sustain the expression. Numerous other genes are regulated in a similar manner (Fig. 1E). Other genes, including CD25 and Spie2, are up-regulated in response to Ag-B7 and further increased in response to either IL-12 or IFN-α. Still others do not change expression in response to Ag-B7 but are up-regulated in response to IL-12 (grzC and grzF, CCR5, CCR2), IFN-α (CXCL-10, ISG-15), or both (FasL). The expression levels of a few genes (including IFN-regulated genes, calcyclin and Schlafen genes) are inversely regulated in the presence of IL-12 or IFN-α. A more complete description of the different temporal patterns of regulation for groups of genes is included in supplemental Figs. 1 and 2 and supplemental Tables V and VI.

The data obtained using oligonucleotide microarray analysis is quite robust in that it agrees well with results obtained on examination of both mRNA levels by RT-PCR and protein levels for a number of genes including grzB, IFN-γ, CD25 (6, 22, 23), FasL, OX40, 4-1BB, and others (our unpublished results). Some of the genes with expression that is up-regulated in a signal 3-dependent manner have previously been reported to be regulated by IL-12, for example CCR5 (24), or by IFN-α, for example CXCL-10 (25).

IL-12 and IFN-αβ regulate expression levels of a number of transcription factors (Table II and supplemental Tables II and III). Of particular interest was comesdermin (Eomes), a T-box family transcription factor that has a role in regulating genes important for...
CD8 T cell functions (26). T-bet (27, 28), a highly homologous member of the T-box family, also has an important role in CTL differentiation but is not represented on the Affymetrix MG U74Av2 gene chip. We therefore examined the regulation of eomes and T-bet mRNA in response to different signals using RT-PCR. T-bet mRNA expression was low in naive cells, increased in response to Ag-B7, and was further increased and sustained when either IL-12 or IFN-α were present (Fig. 3A). In contrast, and consistent with the array analysis, eomes mRNA was expressed at a high level in naive cells and decreased upon stimulation with Ag-B7 but was maintained at a high level when either IL-12 or IFN-α were present (Fig. 3A). Expression of eomes mRNA was somewhat higher in response to IFN-α than to IL-12 (Fig. 3A), and two independent experiments using real-time PCR showed that expression was 1.7 ± 0.1-fold higher in response to IFN-α than to IL-12 (data not shown). Thus, both IL-12 and IFN-α/β cause the sustained high expression of these important transcription factors to allow them to contribute to CD8 T cell differentiation. IL-12-dependent up-regulation of T-bet

Table II. Changes in gene expression in response to Ag-B7 alone and with IL-12 or IFN-α*

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<th>48 h</th>
<th>72 h</th>
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*a For cells stimulated with Ag and B7, the fold change was calculated by comparing signal intensities with naive (0 h) cell signal intensities. For cells stimulated with Ag-B7 in the presence of IL-12, IFN-α, or TSA, the fold change was determined by comparison with cells stimulated with only Ag-B7 at each of the respective time points. IL-2 was present in all cultures. Fold changes are shown only if they were ≥1.70 and had a change p value of ≤0.05. * No change or no significant change based on the selection criteria. Only selected genes are shown; a complete listing of regulated genes is provided in the supplemental tables. To avoid duplication, the genes shown in Fig. 2 are not included in Table II.
expression at the protein level was confirmed by intracellular staining with an anti-T-bet mAb (Fig. 3B).

Our results would appear to be in partial contradiction to a recent study concluding that IL-12 represses eomes expression during a response to *L. monocytogenes* (29). They found that eomes was expressed in naive cells and increased somewhat at the peak of the effector response, as we do, but that cells in IL-12-deficient mice expressed eomes at a higher level. Largely from this, they concluded that IL-12 represses eomes expression. Given our results, the more likely interpretation is that because IFN-γ levels are increased in IL-12-deficient mice, IFN-γ becomes the predominant signal 3 cytokine, whereas IL-12 is the predominant signal 3 cytokine in the wild-type (WT) mice as our studies of responses to *L. monocytogenes* have shown (13). Thus, it is likely that eomes expression in WT mice is lower not because it is being repressed by IL-12, but because IL-12 does not induce expression to as high a level in the WT mice as does IFN-α/β in the deficient mice.

Chromatin remodeling in CD8 T cell differentiation

Remodeling of chromatin to establish open or condensed structures that promote gene activation or repression, respectively, plays a major role in differentiation of many cell types, including differentiation of CD4 T cells to become Th1 or Th2 effector cells (30). Chromatin remodeling by DNA demethylation and/or histone acetylation has been demonstrated for a few genes expressed during CD8 T cell differentiation (31, 32), and there is emerging evidence that these mechanisms may contribute to the more rapid responsiveness of memory CD8 T cells (33–36). The temporal, coordinated gene expression patterns observed in response to IL-12 and IFN-γ (Figs. 1E and 2) suggest that these global events may be mediated, in part, by chromatin remodeling. The expression of genes for grzB, IFN-γ and many other proteins are up-regulated in response to Ag-B7 within 24 h but then decline at later times, whereas IL-12 or IFN-γ maintain and increase the expression at later times (Fig. 1, B, C, and E), raising the possibility that the cytokines may promote an open chromatin structure to allow continued and increased transcription.

Gene accessibility and transcriptional activity can be regulated by histone acetylation levels, with increased histone acetylation generally leading to greater accessibility, and de-acetylation mediated by histone deacetylases (HDAC) leading to decreased transcriptional activity. We therefore examined the effects of pharmacological agents that alter histone acetylation levels by inhibiting HDAC activity. When naive CD8 T cells were stimulated with Ag-B7 for 3 days, the addition of TSA, an HDAC inhibitor, resulted in a large increase in grzB expression (Fig. 4A), development of the capacity to produce IFN-γ (Fig. 4B), and induction of

**FIGURE 4.** Inhibition of histone deacetylation mimics signal 3 cytokine effects on CD8 T cell differentiation. Purified CD44low OT-1 CD8 T cells were stimulated for 3 days with Ag-B7-coated aAPC alone or with IL-12, IFN-α, or TSA (7.5 ng/ml). TSA was dissolved in DMSO, and controls having Ag-B7 and DMSO were included. A and B, Expression of grzB and IFN-γ by intracellular staining. Isotype controls were run for all samples (not shown), and gates set so that >98% of events fell into the lower quadrant for the controls. C, Cytolytic activity measured by ⁵¹Cr release assay using E.G7 target cells.
potent cytolytic activity (Fig. 4C). Essentially the same results were obtained with sodium butyrate (1 mM), another HDAC inhibitor (data not shown). For both TSA and sodium butyrate, the responses required the presence of an Ag stimulus. Thus, inhibition of HDACs mimics the effects of IL-12 and IFN-α/β on the development of functional activities by naive CD8 T cells responding to Ag-B7.

The effects of HDAC inhibitors suggested that IL-12 and IFN-α/β might act, in part, by promoting histone acetylation of genes expressed during differentiation, and we examined this directly for the grzB locus. The grzB proximal promoter region ~250 bp upstream from the transcriptional start site becomes DNase I hypersensitive upon TCR ligation in the presence of a mitogenic signal (37), suggestive of chromatin remodeling, and we therefore examined the histone acetylation status of this region (Fig. 5). Quantitative chromatin immunoprecipitation was conducted using Abs specific for acetylated H3 and H4 histones. Naïve CD8 T cells exhibited low basal H3 and H4 acetylation at the grzB proximal promoter region (Fig. 5, A and B). After in vitro stimulation with Ag-B7 for 48 h, the promoter exhibited little or no increase in H3 acetylation and an increase in H4 acetylation in comparison with results in naïve cells. In contrast, when cells were stimulated with Ag-B7 and IL-12, IFN-α, or TSA there was a substantial increase in H3 acetylation over the levels seen in cells stimulated with two signals, whereas relatively little further change was seen in H4 acetylation.

Histone hyperacetylation is often found to extend to distal regions of genes, and we therefore also examined the region of the grzB locus spanning the end of exon 3 and extending into intron C. H3 and H4 acetylation was low for this region in naïve cells, and H3 acetylation increased little upon Ag-B7 stimulation, but was strongly increased when IL-12, IFN-α, or TSA were present (Fig. 5C). H4 acetylation increased upon stimulation with Ag-B7 and exhibited significant further increases in the presence of IL-12, IFN-α, or TSA. These results therefore directly demonstrate that for at least one of the genes with expression that depends on a signal 3 cytokine, the cytokines induce histone hyperacetylation in both the promoter and distal regions that could contribute to the increased and sustained expression of the critical differentiation gene.

We also examined the associations of acetylated histones with the promoter region of eomes. Unlike grzB, eomes mRNA is expressed at high levels in naïve cells and declines upon stimulation with Ag-B7 but is maintained at a high level if IL-12 or IFN-α is present (Fig. 3A). The promoter region of eomes ~250 bp upstream of the transcription start site was examined, because this region does not exhibit similarity with any other T-box family member (NCBI/Basic Local Alignment Search Tool). There was a significant decrease in association of acetylated H3 histone with this region upon stimulation of naïve cells with Ag-B7 alone, and a substantial increase if IL-12, IFN-α, or TSA was present (Fig. 5D). Levels of associated acetylated H4 histone were low in naïve cells and did not change upon stimulation with Ag-B7, but also showed a substantial increase in response to IL-12, IFN-α, and TSA. Thus, IL-12 and IFN-α/β stimulate increased associations of acetylated histones with the eomes promoter region that parallel the mRNA expression levels of this critical transcription factor.

To further compare the effects of TSA with those of IL-12 and IFN-α/β, we conducted oligonucleotide array analysis of cells stimulated with Ag-B7 in the presence or absence of 7.5 ng/ml TSA for 72 h. In comparison with Ag-B7 alone, TSA significantly altered (fold change ≥1.7, p ≤ 0.05) the expression levels of 1473 genes. This included 185 of the 355 genes regulated in common by IL-12 and IFN-α, and

![FIGURE 5.](http://www.jimmunol.org/)

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**FIGURE 5.** IL-12 and IFN-α promote histone hyperacetylation at the grzB and eomes gene loci during CD8 T cell differentiation. Purified CD44<sup>+</sup> OT-I CD8 T cells were used as naïve control or were stimulated with Ag-B7 aAPC alone or with IL-12, IFN-α, or TSA (7.5 ng/ml). Cells were harvested at 48 h, processed, and examined by cross-linked chromatin immunoprecipitation and quantitative RT-PCR for acetylated histones H3 and H4. A, Association of H3 and H4 histones with the grzB promoter, expressed as template copy numbers as a percent of whole genome copy number. Bars, ranges for duplicate samples. B–D, Association of H3 and H4 histones with the grzB promoter (B), grzB distal region (C), and the eomes promoter (D) regions. Template copy numbers for each condition were internally normalized with their respective input control. Relative expression is calculated as the ratio of template copy numbers of each sample relative to the naïve control in each experiment, and is shown as the mean ± SEM of two or three independent experiments with duplicate samples for each. In comparison with naïve cells, activation with Ag-B7 resulted in significant increases of acetylated H3 and H4 histones with the promoter and exon regions of GrzB (p < 0.05), a significant decrease in acetylated H3 histone association with the eomes promoter (p < 0.001), and no change in association of acetylated H4 with the eomes promoter (p = 0.5). In comparison with cells stimulated with only Ag-B7, association of acetylated H3 and H4 histones was significantly greater when IL-12, IFN-α, or TSA was added (p < 0.03), with one exception. The exception was acetylated H4 association with the GrzB promoter, where the additions did not cause a significant increase (p > 0.05).
140 of these 185 were changed in the same direction as with the cytokines, including eomes, grzB, IFN-γ, RGGTase B, CD25, CCR3, CCR2, XCI-1, and others (Table II and supplemental Table VII). Thus, almost one-half of the genes the expression of which is sustained by IL-12 or IFN-α are regulated in a similar manner by inhibiting histone deacetylation.

Discussion

TCR and CD28 signals stimulate multiple rounds of CD8 T cell division (15, 16) but in the absence of a signal from IL-12 or IFN-α/β, survival is compromised, effector functions and memory do not develop, and the small number of cells that do survive are tolerant (4, 11, 38). Use of aAPC provides a powerful approach for studying gene regulation by IL-12 and IFN-α; the signals are well defined and all of the cells become activated in a narrow time frame, thus allowing analysis of the time course of changes in gene expression levels. In contrast, this level of definition would not be feasible for cells responding in vivo due to the redundancy of the effects of IL-12 and type I IFNs, potential modifications of the genetic program due to other signals present in vivo, and the fact that all of the T cells will not be stimulated simultaneously, instead being recruited over time as the infection progresses. Thus, the in vitro studies described here provide a baseline definition of the signal 3-dependent differentiation program and will form the basis for studying in vivo responses in more detail by examining expression of gene products known to be regulated as part of this program. The program includes ~355 genes regulated in common by IL-12 and IFN-α (Fig. 1, D and E, and supplemental Table IV). This regulation is superimposed on the regulation of >3000 genes by TCR and CD28 signals, many of which are involved in cell cycle regulation, DNA synthesis and repair, protein translation, and metabolism (supplemental Table I). Much of the signal 3-dependent program involves regulation of genes that encode proteins of obvious relevance to the differentiation process that leads to effector and memory cells.

Once CD8 T cells have undergone an initial burst of proliferation over about 3 days, expansion and survival can be further increased by additional signals. IL-2 produced by CD4 helper T cells can sustain and expand effector CD8 T cell populations (39, 40). IL-12 increases the expression of CD25 (Fig. 2), thus increasing IL-2Rα protein expression and making the effector cells more responsive to this signal (23). Receptors belonging to the TNF receptor superfamily can also increase the survival of effector cells by increasing expression of anti-apoptotic genes (41). Expression levels of genes for several of these receptors, including OX-40, 4-1BB, and GITR, are up-regulated IL-12 and IFN-α (Fig. 2 and Table II), and this has been confirmed at the level of cell surface protein expression for OX-40 and 4-1BB (42). In contrast CD27, a member of the TNF receptor superfamily that acts earlier in the response (43), was up-regulated with just two signals, and expression decreased in the presence of IL-12 or IFN-α (Table II). IL-12 and IFN-α/β also up-regulate a number of cell-intrinsic defense genes including cysteine protease inhibitors (CTLA-2α and 2B) and serine protease inhibitors (spil6, spil2e, serpin5, serpin1; Fig. 2 and Table II). spil6 is important for protecting effector CD8 T cells from death induced by their own lytic machinery (44). In addition, IL-12 (45) and IFN-α (our unpublished results) also up-regulate expression of Bcl-3, a member of the IkB family of proteins that can enhance T cell survival (46). Thus, a critical part of the signal 3-dependent differentiation program involves increased expression of numerous genes that encode for proteins important for clonal expansion and survival of the effector and memory cells that develop.

Major functions of effector CD8 T cells include production of cytokines and killing of target cells by the perforin/granzyme-dependent degranulation mechanism or the Fas ligand pathway, and IL-12 and IFN-α regulate expression of genes for several of the critical proteins of these pathways. GrzB is expressed weakly and transiently in response to two signals and is increased to high, sustained levels by IL-12 or IFN-α (Figs. 1C and 2). Although IL-12 and IFN-α both support development of cytolytic activity (Fig. 1A), there may be differences in functional capacities depending on which signal a cell receives, given that grzC and grzF mRNAs were up-regulated by IL-12 but not by IFN-α (Fig. 2 and Table II). Unlike granzymes, perforin mRNA and protein are strongly up-regulated by two signals alone, and only marginally increased in the presence of IL-12 or IFN-α (22). The Fas-dependent killing pathway also appears to require signal 3, given that Fas ligand mRNA expression is not up-regulated by two signals but is strongly up-regulated by IL-12 or IFN-α (Fig. 2). IFN-γ mRNA and protein are also strongly up-regulated by both IL-12 and IFN-α (Figs. 1B and 2). In contrast, TNF-α mRNA is highly expressed in naive cells and is initially down-regulated (24 h) but later increased (72 h) to naive levels in response to two signals (Table II), and expression does not significantly change in response to IL-12 or IFN-α, and we have confirmed this at the protein level by intracellular staining (our unpublished results). This is consistent with the fact that naive CD8 T cells rapidly produce TNF-α upon Ag stimulation, and this capacity declines as the cells become effectors (47). Thus, unlike cytolytic activity and IFN-γ production, the capacity to produce TNF-α does not require differentiation of the naive cells nor does it depend upon a third signal.

To carry out its effector functions, a CD8 activated in a draining lymph node must migrate to the site of the foreign Ag. Two signals (Ag-B7) are sufficient to increase expression of a number of genes for cell adhesion and homing receptors, including CD44, MAC-2, and ICAM-1, CCR5, and CXCR3 (Fig. 2), and to down-regulate genes for secondary lymphoid homing receptors, including CD62L and CCR7. Regulation of these receptors by just two signals is consistent with the observation that CD8 T cells activated in the absence of a third signal can nevertheless migrate to peripheral sites of Ag but fail to mediate autoimmunity (38), graft rejection (12), or elimination of infected cells (48). IL-12 and IFN-α/β do regulate expression of genes for several of the other receptors and chemokines involved in controlling migration, but differing patterns of regulation were observed for IL-12 vs IFN-α (Fig. 2 and Table II). IL-12 up-regulated CCR2 and CCR5, chemokine receptors that promote migration to inflammatory and allergic response sites, whereas IFN-α did not increase CCR2 expression and only weakly increased CCR5 expression. IL-12 also induced chemokines that may aid recruitment of CD4 Th cells, DCs, and monocytes, including CCL9, MIP-1α, and XCI-1, whereas IFN-α increased expression of only XCI-1. CXCL10, a chemokine that helps attract NK cells, B cells, neutrophils, and type I T cells, was induced by IFN-α but not by IL-12. Galectin-3, an adhesion molecule, was strongly up-regulated by IL-12, whereas galectin-9, an eosinophil attractant, was up-regulated by IFN-α. Finally, integrin β7, which affects migration to mucosal regions, was down-regulated by both IL-12 and IFN-α. Thus, the signal 3-dependent differentiation program includes alterations in expression of genes for a number of receptors and chemokines important for migration into sites of foreign Ag and recruitment of additional effector cells to the sites, but the migration phenotypes of the effector cells may differ depending on whether the third signal was provided by IL-12 or type I IFN.
Numerous genes encoding proteins involved in signal transduction pathways are regulated by IL-12 and/or IFN-α, including both cell surface receptors (e.g., IL-18R1, IL-18Rα, IL-12Rβ1 and β2, IL-2Rα) and intracellular signaling intermediates (e.g., MyD88, Traf4, Traf6, Gadd45β, and Gadd45γ). This may contribute to enhanced signaling for effector responses. IL-12 and IFN-α also up- or down-regulate expression of a number of genes for transcriptional factors, including ATF-3, -4, and -5, Jun-B, CREM, STAT5β, Map2k1, Sox2, regulators of G-protein signaling, and others. Two particularly interesting transcription factors regulated by IL-12 and IFN-α/β are T-bet and eomes, both of which are involved in CD8 T cell activation of effector functions upon stimulation with Ag (26, 28), in part through a role in up-regulating grzB expression. T-bet expression is weakly induced in response to just two signals, and increases in response to IL-12 or IFN-α. In contrast, eomes is expressed in naive cells and decreases in response to Ag and B7-1 but is sustained at a high level when either IL-12 or IFN-α are present (Fig. 3). Thus, it appears likely that these signal 3 cytokines are promoting differentiation in part by maintaining these critical transcription factors at high levels. Many additional transcription factors and regulators are also positively or negatively regulated by IL-12 and IFN-α/β (Table II) and are likely to have important roles in the differentiation program.

Histone-dependent chromatin remodeling appears to be a major mechanism by which IL-12 and IFN-α/β regulate the differentiation program. Expression of many of the IL-12/IFN-α-regulated genes increases early in response to Ag and B7-1 but then declines by 72 h (Fig. 2 and supplemental Figs. 1 and 2). Expression is increased and sustained by IL-12 or IFN-α, suggesting that these loci might initially be accessible to transcription factors induced by Ag and B7-1, but that alterations in chromatin structure then render them inaccessible unless a signal 3 cytokine is present. The ability of TSA, a class I and II HDAC inhibitor, to substitute for IL-12 or IFN-α/β (Fig. 4) indicated that the cytokines might act by increasing the level of histone acetylation at critical gene loci to allow their continued transcription. We directly demonstrated this for the grzB and eomes genes, where IL-12 or IFN-α caused increased acetylation of histones H3 and H4 at the proximal promoter regions, and at the distal exon region of grzB (Fig. 5). In a similar approach using naive CD8 T cells stimulated with anti-TCR and anti-CD28 mAb, IL-12 was shown to cause long-range hyperacetylation in the promoter and exon regions of the IFN-γ gene (31). Thus, it appears that IL-12 and IFN-α/β enforce the gene regulation program, at least in part, by promoting chromatin remodeling to allow sustained expression of critical genes. Both cytokines up-regulate expression of mRNA for cyclin-dependent kinase inhibitor p21 (Cdkn1A, p21), a G1-S arrest gene, by 24 h, and it continued to be expressed at 72 h (Fig. 1D). This may contribute to chromatin remodeling by prolonging the G1-S phase, because CD4 Th cells in this phase of the cell cycle have been shown to be the most susceptible to epigenetic modifications of chromatin (49, 50).

There is increasing evidence that chromatin remodeling involving histone acetylation may play an important role in differentiation of CD8 T cells to develop memory. Memory CD8 T cells exhibit increased histone acetylation levels for many of the genes that are expressed more rapidly than in naive cells following activation, including eomes, perforin, and grzB (33–36). The results described here, together with the evidence that a signal from IL-12 or type I IFN is required to program for memory development (13), suggest that much of the chromatin remodeling may be initiated by these cytokines. Epigenetic memory of the remodeling is likely to account for the more rapid responses of memory cells upon re-encountering Ag, and would be consistent with the fact that memory CD8 T cells do not require a third signal to efficiently respond to Ag and costimulation (10, 51).

CD4 T cells can provide help to CD8 T cells through CD40-dependent stimulation of DCs to produce IL-12 (12), thus making this critical third signal available to the responding CD8 T cells. In experimental models that require CD4 T cell help for CD8 memory formation, increased acetylation in the memory cells has been shown to be CD4 T cell dependent (35, 36). CD8 T cells activated with anti-TCR and anti-CD28 mAb in splenocyte cultures in the absence of CD4 T cells remain hypoaetylated and do not develop into functional memory cells upon transfer into mice, but addition of TSA to these cultures to inhibit histone deacetylation results in generation of cells that survive and become functional memory cells upon transfer (36). It would seem reasonable to speculate that CD4 T cells are providing help, at least in part, by stimulating production of IL-12 and/or IFN-α/β, and that TSA can bypass this requirement for memory formation as it does for induction of effector functions (Fig. 4).

Kaeche et al. (52), compared gene expression levels in naive, effector, and memory CD8 T cells responding to LCMV infection, a response that depends almost completely on type I IFNR (7). Comparison of our results to those shows that of the 355 genes regulated by both IL-12 and IFN-α, ~25% were similarly up- or down-regulated in memory vs naive cells. This suggests that once these genes are up- or down-regulated by a signal 3 cytokine during the primary response, they continue to be expressed at similar levels in the memory cells. For transcription factors up-regulated by IL-12 and IFN-α (Table II and supplemental Tables II–IV), several were also seen to be increased in the effector cells (day 8) responding to LCMV, including Blimp-1, BhlhB2, eomes (Tbr2), several were also seen to be increased in the effector cells (day 8) responding to LCMV, including Blimp-1, BhlhB2, eomes (Tbr2), and nfll3/E4BP4, whereas Lef-1, Tcf-7, Pou2af1, Id3, and others were also down-regulated. Nfll3/E4BP4 and BhlhB2 genes continued to be expressed at increased levels in memory cells (day 40), whereas Id3 continued to be repressed. Thus, the results of our analysis of in vitro stimulated cells agree well with those for cells responding in vivo, and the comparison suggests that many of the changes in expression level that occur in response to IL-12 and IFN-α/β during primary stimulation persist in resting memory cells. When naive cells respond to Ag and costimulation in the absence of a signal 3 cytokine, survival is compromised, effector functions do not develop, and the cells that do survive long term are tolerant. This raises the possibility that the response to Ag and B7 alone may lead to permanent silencing of critical genes to result in tolerance.

Disclosures

The authors have no financial conflict of interest.

References


Supplementary Table and Figures Legends

Table SI: Two-signal (Ag-B7.1) – dependent gene expression changes  Gene expression intensities of Ag and B7-1 stimulated CD8 T cells at 24-h, 48-h and 72-h each were compared with naive CD8 T cells using the Affymetrix murine MG U74Av2 gene chip displaying 12,488 genes/ESTs. Fold differences are shown for only those genes that were altered by at least 1.70 fold at any one time point. The total numbers of genes regulated at each time point are summarized in Table 1 of the published text.

Table SII: IL-12 – dependent gene expression changes (IL-12 + Ag-B7 versus Ag-B7 only)  The complete set of genes that changed in response to IL-12 was determined by comparing IL-12+Ag-B7 stimulated gene expression to Ag-B7 at 24, 48 and 72-hrs. The genes ‘present’ in at least one of the comparing samples, and altered by FC of at least 1.70 (p value of at least 5%) at any one time point, were included. A partial list from this table is included in the Table 2 of published text. The gene annotations were extracted from www.affymetrix.com.

Table SIII: IFN-α/β - dependent gene expression changes (IFNα + Ag-B7 versus Ag-B7 only)
The complete set of genes that changed in response to IFNα was determined by comparing IFNα +Ag-B7 stimulated gene expression to Ag-B7 at 24, 48 and 72-hrs. The genes ‘present’ in at least one of the comparing samples, and altered by FC of at least 1.70 (p value of at least 5%) at any one time point, were included. A partial list from this table is included in the Table 2 of published text. The gene annotations were extracted from www.affymetrix.com.

**Table SIV: Genes regulated in common by IL-12 and IFNα**

This table shows the genes from Tables S2 and S3 that were regulated by both IL-12 and IFNα at any time point. The fold changes are shown for Ag-B7 (72-h) compared to naïve (0h), and for IL-12 or IFNα treated compared to Ag-B7 only, at each time.

**Table SV: Identification of IL-12 – dependent genes in clusters determined by K-mean clustering (as shown in Figure S1)**

**Table SVI: Identification of IFNα– dependent genes in clusters determined by K-mean clustering (as shown in Figure S2)**

**Table SVII: Genes regulated in common by IL-12, IFNα and trichostatin A**

Gene expression analysis was determined for naïve CD8 T cells stimulated with Ag-B7 alone or in the presence of either trichostatin A or IL-12 or IFNα for 72-h.
Genes whose expression changed by ≥ 1.7 or ≤ 1.7 in comparison to Ag-B7 alone are shown.

**Figure S1: Patterns of gene expression in response to IL-12**

The 920 probe ids altered in presence of IL-12 compared with Ag-B7 alone were clustered using K-mean analysis (GeneSpring version 7.2). Each cluster was individually visualized and grouped into sets of closely related patterns as shown. Genes in each cluster are listed in Table SV.

**Figure S2: Patterns of gene expression in response to IFNα**

The 714 probe ids altered in presence of IFNα compared with Ag-B7 alone were clustered using K-mean analysis (GeneSpring version 7.2). Each cluster was individually visualized and grouped into sets of closely related patterns as shown. Genes in each cluster are listed in Table SVI.
Set I: Genes with expression induced, enhanced and/or sustained by IL-12 at 72 hr

Cluster (I, 1) 170 genes
Cluster (I, 2) 153 genes
Cluster (I, 3) 40 genes (induced at single time point)

Set II: Genes with expression downregulated by Ag-B7 but sustained by IL-12

Cluster (II, 1) 61 genes
Cluster (II, 2) 64 genes

Set III: Genes with expression decreased by IL-12 at 72 hr

Cluster (III, 1) 120 genes
Cluster (III, 2) 117 genes
Cluster (III, 3) 80 genes
Cluster (III, 4) 31 genes (induced at single time point)

Set IV: Other

84 genes
Set I: Genes with expression induced, enhanced and/or sustained by IFN-α at 72hr

Cluster (I, 1) 191 genes
Cluster (I, 2) 68 genes
Cluster (I, 3) 19 genes (induced at single time point)

Set II: Genes with expression downregulated by Ag-B7 but sustained by IFN-α

Cluster (II, 1) 60 genes
Cluster (II, 2) 53 genes

Set III: Genes with expression by IFN-α at 72hr

Cluster (III, 1) 107 genes
Cluster (III, 2) 112 genes
Cluster (III, 3) 44 genes
Cluster (III, 4) 24 genes (induced at single time point)

Set IV: Other

36 genes

(induced at single time point)