IL-6, in Synergy with IL-7 or IL-15, Stimulates TCR-Independent Proliferation and Functional Differentiation of CD8+ T Lymphocytes

Julien Gagnon, Sheela Ramanathan, Chantal Leblanc, Alexandre Cloutier, Patrick P. McDonald and Subburaj Ilangumaran


http://www.jimmunol.org/content/180/12/7958

**References**

This article cites 90 articles, 48 of which you can access for free at:
http://www.jimmunol.org/content/180/12/7958.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
IL-6, in Synergy with IL-7 or IL-15, Stimulates TCR-Independent Proliferation and Functional Differentiation of CD8$^+$ T Lymphocytes

Julien Gagnon, Sheela Ramanathan, Chantal Leblanc, Alexandre Cloutier, Patrick P. McDonald, and Subburaj Ilangumaran

Recent reports have shown that IL-21, in synergy with IL-15, stimulates proliferation of CD8$^+$ T lymphocytes in the absence of signaling via the TCR. In this study, we show that IL-6, which induces phosphorylation of STAT3 similarly to IL-21, also can stimulate proliferation of CD8$^+$ T cells in synergy with IL-7 or IL-15. IL-6 displays a stronger synergy with IL-7 than with IL-15 to stimulate naive CD8$^+$ T cells. Concomitant stimulation by IL-6 or IL-21 augments phosphorylation and DNA-binding activity of STAT5 induced by IL-7 or IL-15. Like IL-21, IL-6 reduces the TCR signaling threshold required to stimulate CD8$^+$ T cells. Prior culture of P14 TCR transgenic CD8$^+$ T cells with IL-6 or IL-21 in the presence of IL-7 or IL-15 augments their proliferation and cytolytic activity upon subsequent stimulation by Ag. Furthermore, cytokine stimulation induces quantitatively and qualitatively distinct phenotypic changes on CD8$^+$ T cells compared with those induced by TCR signaling. We propose that the ability of IL-6 to induce TCR-independent activation of CD8$^+$ T cells in synergy with IL-7 or IL-15 may play an important role in the transition from innate to adaptive immunity. The Journal of Immunology, 2008, 180: 7958–7968.

Received for publication September 19, 2007. Accepted for publication April 6, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was supported by a Canadian Institutes of Health Research grant to S.I. (MOP-62800) and by the Faculté de Médecine et des Sciences de la Santé, Université de Sherbrooke, and Centre de Recherche Clinique Étienne-Le Bel, Centre Hospitalier Universitaire de Sherbrooke. S.I. was a scholar of the Fonds de la Recherche en Santé du Québec and is a Canadian Institutes of Health Research new investigator. P.P.M. is a Fonds de la Recherche en Santé du Québec scholar. J.G. is supported by a Fonds de la Recherche en Santé du Québec doctoral studentship. A.C. is a recipient of Canadian Institutes of Health Research doctoral studentship.

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/$2.00
with IL-15 or IL-7 to stimulate TCR-independent proliferation and effector functions of CD8+ T lymphocytes.

Materials and Methods

Mice

C57BL/6 and Rag1-/- mice in C57BL/6 background were purchased from The Jackson Laboratory. H-2Y TCR transgenic (H-2Y TCRtg) mice (43) were obtained from P. Poussier (Sunnybrook and Women’s College Hospital, Toronto, Canada). P14 TCRtg mice (44) were provided by P. Ohashi (University of Toronto, Toronto, Canada). The H-2Y TCR recognizes the male-specific H-Y Ag, whereas the P14 TCR recognizes the gp33–41 (KAVYN FATM) epitope from lymphocytic choriomeningitis virus glycoprotein, both in the context of MHC class I molecule H-2Dd. Rag1-/-/H-2Y TCRtg mice were generated in our animal facility. Six- to 8-wk-old mice were used in all experiments. All experiments on mice were conducted with strict adherence to institutional guidelines.

Abs and reagents

Abs against mouse CD4, CD8, CD16/CD32, CD44, CD62L, CD69, CD122, CD127, TRα/B (H57), and Ly6C conjugated to FITC, PE, or biotin were purchased from BD Biosciences. Abs to mouse IL-6Rα and IL-21Rα were purchased from Biologend and eBioscience, respectively. T37 mAb was purified from hybridoma supernatant and conjugated to FITC or biotin. Streptavidin-spectral red was from Southern Biotechnology Associates. Recombinant cytokines IFN-γ, IL-2, IL-6, IL-7, IL-15, and IL-21 were purchased from R&D Systems. RPMI 1640 cell culture medium and PBS were from Sigma-Aldrich. CFSE was purchased from Molecular Probes. Phospho-specific rabbit polyclonal Abs to STAT5, STAT3, and STAT1 were obtained from Cell Signaling Technology. Rabbit polyclonal Abs to STAT5, STAT3, and STAT1 were from Santa Cruz Biotechnology. Pharmacological inhibitors of Src family protein tyrosine kinases (PP2), PI3K (LY 294002), p38 MAPK (SB 202190), MEK (PD 98059), JNK inhibitor II, and the JAK-STAT3 pathway (JSI-124) were purchased from Calbiochem.

Flow cytometry and magnetic cell sorting

Single-cell suspensions in PBS containing 5% FBS and 0.05% sodium azide were preincubated with anti-CD16/CD32 Ab for 10 min to block FcRs. Expression of cell surface markers was estimated by standard two- or three-color staining using FITC-, PE-, and biotin-conjugated primary Abs, followed by streptavidin-spectral red. Data acquisition and analysis were done on a FACSCalibur using CellQuest software (BD Biosciences). To analyze the expression of granzyme B, stimulated cells were stained with anti-CD8 Ab, fixed in 1% paraformaldehyde, permeabilized with 0.5% saponin in PBS containing 1% BSA, stained with anti-human granzyme B (Invitrogen-Caltag Laboratories), and analyzed by flow cytometry. CD8+ T cells were purified using magnetic beads (Miltenyi Biotec), following the manufacturer’s instructions, to >99% purity. To purify CD8+ CD44low and CD8+ CD44high subsets, CD8+ T cells from lymph node was first stimulated with indicated cytokines using anti-CD4 and anti-CD19 magnetic beads, followed by positive selection of the CD44low subset using anti-CD44 Ab, and using two MACS columns in succession to attain desired purity.

Cell proliferation assays

Proliferation of total splenocytes or purified T cell subsets was measured by [3H]thymidine incorporation assay. Total splenocytes or lymph node cells (2 × 10^5 cells/well), or purified T cell subsets (1 × 10^5 cells/well) were stimulated with indicated concentrations of cytokines in 200 μl of RPMI 1640 medium in 96-well culture plates for 72 h. Alternately, cells were stimulated with anti-mouse CD3/CD28 Dynabeads (Invitrogen) or gp33–41 peptide in presence of irradiated splenocytes from C57BL/6 mice as APC. A total of 1 μCi of methyl-[3H]thymidine (NEN Life Sciences) was added per well during the last 8 h of culture. The cells were harvested onto glass fiber filter mats, and the incorporated radioactivity was measured in a Top Count microplate scintillation counter (PerkinElmer).

The CFSE dye dilution assay was used to estimate the number of divisions of the cell cycle within each T cell subset following cytokine stimulation. Total splenocytes or lymph node cells were incubated at 1 × 10^7 cells/ml in PBS containing 5 μM CFSE for 5 min at room temperature. The reaction was quenched with an equal volume of PBS. The cells were washed twice with PBS, resuspended in 40 μg/ml poly(1–4C), and then labeled for surface markers. Sequential reduction in dye content, which reflects successive divisions of the cell cycle, was followed within gated CD4 and CD8 T cell subsets.

Generation of memory H-Y TCRtg CD8+ T cells

Memory H-Y TCRtg CD8+ T cells were generated following a published protocol (45). Briefly, 0.5 × 10^6 MACS-purified naive H-Y TCRtg CD8+ T cells from Rag1-/-/H-2Y TCRtg female mice were injected into Rag1-/- male mice with 1 × 10^7 irradiated total bone marrow cells from Rag1-/- males as immunogen and 0.5 × 10^6 purified CD4+ T cells from female C57BL/6 mice as Th cells. Memory H-Y TCRtg CD8+ T cells were recovered from the recipient mice 8 wk after immunization.

Preparation of nuclear extract and EMSA

MACS-purified 5 × 10^6 P14 TCRtg CD8+ T cells were starved in serum-free RPMI medium for 2 h before stimulation with cytokines. At indicated time points after stimulation, the cells were washed in ice-cold PBS and nuclear extracts were prepared (46). Briefly, the cells were lysed in cold hypotonic lysis buffer (10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, and protease inhibitors), followed by lysis of the sedimented nuclei in high salt buffer (20 mM HEPES (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF). A small aliquot of the clarified nuclear extract was saved for protein quantification, and the rest was stored frozen at −70°C until EMSA was conducted. To analyze the binding of STAT proteins to DNA, 3 μg of nuclear protein was incubated in DNA-binding buffer (20 mM Tris (pH 7.5), 50 mM KCl, 1 mM EDTA, 1 mM DTT, 0.1% Nonidet P40, 5% glycerol, 40 μg/ml poly(dI–dC), and 300 μg/ml acetylated BSA) for 15 min at room temperature with a 32P-Radio-labeled 39-bp oligonucleotide probe corresponding to the IFN-γ response region (GRR) located within the Fc-γRI/CD64 gene promoter (5’-ACGAT GTTTCAGAATTGATGTTATTTCCAGAAGG-3’) (47, 48). In supershift analyses to determine the specificity of STAT binding, 1 μg of anti-STAT3 or anti-STAT5 Ab (Santa Cruz Biotechnology) was added to the nuclear extract 30 min before incubation with the labeled GRR oligonucleotide probe. DNA-binding reactions were electrophoresed on 5.5% acrylamide gels in 0.75× Tis-borate buffer at 4°C. The gels were dried and exposed to Hyperfilm MP (GE Healthcare) with intensifying screens at −80°C.

CTL assay

EL-4 cells (H-2b) were prepared by incubation with 400 μg/ml Citrate (NEN Life Sciences) and the stimulator gp33 (KAVYNFATM) or nonstimulator AV (SGSNTPPPEI) peptide for 2 h at 37°C (49). These target cells were washed three times and plated in 96-well round-bottom microtiter plates with indicated effector P14 TCRtg CD8+ T cells at different E:T cell ratios. After incubation for 7 h at 37°C, 100 μl of supernatant was counted for 60 s using a gamma counter (PerkinElmer). Maximal release was achieved by adding Triton X-100 to a final concentration of 1%. Percentages of specific lysis was calculated as (cpm sample release – cpm spontaneous release)/cpm maximal release – cpm spontaneous release) × 100.

Results

IL-6 stimulates Ag-independent proliferation of CD8+ T lymphocytes in synergy with IL-7 or IL-15

IL-21 can stimulate proliferation of CD8+ T lymphocytes in synergy with IL-7 or IL-15 independently of signaling via the TCR (Fig. 1A) (19, 20). Whereas IL-7 and IL-15 induce phosphorylation of STAT5 in CD8+ T lymphocytes, IL-21 induces phosphorylation of STAT3 and STAT1 (Fig. 1B) (20, 27). Because IL-21 alone does not induce proliferation of CD8+ T lymphocytes, its ability to induce Ag-independent proliferation of CD8+ T cells in the presence of IL-7 or IL-15 (Fig. 1A) could result from synergy between STAT3 and STAT5 signaling pathways. To test this hypothesis,

Downloaded from http://www.jimmunol.org/ by guest on July 26, 2017

The Journal of Immunology 7959
we investigated whether other cytokines that induce STAT3 phosphorylation also showed a capacity to stimulate proliferation of CD8\(^+\) T cells in synergy with IL-7 or IL-15. IL-6 induces Ag-independent proliferation of CD8\(^+\) T cells from C57BL/6 mice were stimulated with IL-7 (5 ng/ml) or IL-15 (10 ng/ml) either alone or in the presence of IL-21 (10 ng/ml). Cell proliferation was measured after 3 days by \([\text{H}]\)thymidine incorporation for 8–10 h. Data (mean ± SE) are representative of three independent experiments.

IL-6 and IFN-\(\beta\) have been shown to induce phosphorylation of STAT3 and STAT1 in T lymphocytes (50–52). However, IL-6 was found to be more efficient than IFN-\(\beta\) in inducing STAT3 phosphorylation, whereas IFN-\(\beta\) induced a strong STAT1 activation (Fig. 1B).

Whereas IL-6 promotes survival of resting T cells (38–41, 50), IFN-\(\beta\) augments the survival of activated T cells (53). Therefore, we examined whether IL-6 and IFN-\(\beta\) stimulated proliferation of CD8\(^+\) T cells in synergy with IL-7 and IL-15. We observed that IL-6, but not IFN-\(\beta\), stimulated proliferation of purified CD8\(^+\) T lymphocytes in the presence of IL-7 or IL-15 (Fig. 1C). These results supported the idea that simultaneous activation of STAT3 and STAT5 might underlie the ability of IL-21 and IL-6 to synergize with IL-7 or IL-15 in stimulating proliferation of CD8\(^+\) T lymphocytes.

To compare the TCR-independent cytokine-driven responses with stimulation induced via the TCR, we used CD8\(^+\) T cells expressing the P14 tg TCR. P14 TCR\(^+\) CD8\(^+\) T cells express a clonotypic TCR that recognizes a peptide Ag (gp33–41) of lymphocytic choriomeningitis virus glycoprotein (54, 55). As shown in Fig. 1D, proliferation stimulated by cytokines alone was modest compared with that induced by the antigenic peptide gp33 presented by irradiated APC.
IL-21 elicited a slightly stronger response than IL-6 in terms of the CFSE-based proliferation assay using total lymph node cells, cytokine stimulations also failed to induce any significant expansion of NK cells (data not shown). These results show that IL-6 can substitute for IL-21 in stimulating Ag-independent proliferation of CD8+ T cells in the presence of IL-7 or IL-15.

**Synergistic stimulation of CD8+ T cells by IL-6 in the presence of IL-7 or IL-15 occurs in both naive and memory cell compartments**

IL-15 stimulates proliferation of memory, but not naive CD8+ T cells (19). Concomitant stimulation with IL-21 not only augments the proliferation of memory CD8+ T cells, but also stimulates naive CD8+ T cells to proliferate in response to IL-15 (19, 20). To determine whether IL-6 displays a similar synergy as IL-21 in stimulating naive and memory CD8+ T cells, we used CD8+ T cells expressing the tg H-Y TCR (43). The H-Y TCR is specific to the KCSRNRQYL peptide of the male-specific H-Y Ag and displays minimal reactivity to environmental or self Ags (56). CD8+ T cells bearing the H-Y TCR can be identified using the mAb T3.70 (57). All CD8+ T cells harbor the H-Y TCR by immunizing Rag1−/− females with naive H-Y Ag (45) and exhibit CD44low phenotype (Fig. 3A). We have generated memory H-Y TCRtg T cells by immunizing Rag1−/− females harboring adoptively transferred naive H-Y TCRtg T cells with male cells. Memory H-Y TCRtg CD8+ T cells uniformly displayed CD44low phenotype and exhibited higher level of CD44, Ly6C, CD127 (IL-7Rα), and CD122 (IL-2/15R) (Fig. 3A).

Nonetheless, the significant level of proliferation induced by cytokines raised the possibility that such cytokine-driven proliferation may potentially contribute to the overall T cell response during infections. Therefore, we conducted a detailed characterization Ag-independent proliferation of CD8 T cells driven by cytokines.

Evaluation of the synergistic effects of different concentrations of IL-6 or IL-21 in presence of IL-15 showed that IL-6 stimulated proliferation of purified CD8+ T cells as efficiently as IL-21 (Fig. 2A). Because the molecular masses of IL-6 and IL-21 are 30 and 15 kDa, respectively, IL-6 appeared to be more potent than IL-21. However, when proliferation of CD8+ T cells was assessed by the CFSE-based proliferation assay using total lymph node cells, IL-21 elicited a slightly stronger response than IL-6 in terms of the proportion of cells in successive divisions of the cell cycle (Fig. 2B, upper and lower panels). This variation between assays using purified CD8+ T cells and total lymph node cell population may arise from depletion of IL-6 by other cell types present in the total lymph node cell cultures. Furthermore, differences in receptor expression and cytokine concentrations in the tissue microenvironment could also influence the relative contribution of IL-6 and IL-21 in an inflammatory setting in vivo. Like IL-21 (19, 20), IL-6 did not stimulate proliferation of CD4+ T cells either alone or in combination with IL-2, IL-7, or IL-15 (data not shown). These cytokine stimulations also failed to induce any significant expansion of NK cells (data not shown). These results show that IL-6 can substitute for IL-21 in stimulating Ag-independent proliferation of CD8+ T cells in the presence of IL-7 or IL-15.

**Synergistic stimulation of CD8+ T cells by IL-6 in the presence of IL-7 or IL-15 occurs in both naive and memory cell compartments**

IL-15 stimulates proliferation of memory, but not naive CD8+ T cells (19). Concomitant stimulation with IL-21 not only augments the proliferation of memory CD8+ T cells, but also stimulates naive CD8+ T cells to proliferate in response to IL-15 (19, 20). To determine whether IL-6 displays a similar synergy as IL-21 in stimulating naive and memory CD8+ T cells, we used CD8+ T cells expressing the tg H-Y TCR (43). The H-Y TCR is specific to the KCSRNRQYL peptide of the male-specific H-Y Ag and displays minimal reactivity to environmental or self Ags (56). CD8+ T cells bearing the H-Y TCR can be identified using the mAb T3.70 (57). All CD8+ T cells harbor the H-Y TCR by immunizing Rag1−/− females with naive H-Y Ag (45) and exhibit CD44low phenotype (Fig. 3A). We have generated memory H-Y TCRtg T cells by immunizing Rag1−/− females harboring adoptively transferred naive H-Y TCRtg T cells with male cells. Memory H-Y TCRtg CD8+ T cells uniformly displayed CD44low phenotype and exhibited higher level of CD44, Ly6C, CD127 (IL-7Rα), and CD122 (IL-2/15Rβ) (Fig. 3A).
We cultured purified naive or memory H-Y TCRtg CD8\(^+\) T cells with IL-7 or IL-15 in the presence or absence of IL-6 or IL-21. Three days later, naive H-Y TCRtg CD8\(^+\) T cells showed negligible proliferation in response to IL-7 or IL-15 (Fig. 3B). Concomitant stimulation with IL-6 or IL-21 induced proliferation of naive CD8\(^+\) T cells in response to IL-7 and to a lesser extent to IL-15. This
synergistic response was significantly higher in presence of IL-21 than IL-6. In contrast to naive cells, memory H-Y TCR\textsuperscript{tg} CD8\textsuperscript{T} T cells proliferated strongly to IL-15 and to a slightly lesser extent to IL-7. IL-21, but not IL-6, significantly augmented the IL-15- or IL-7-induced proliferation of memory CD8\textsuperscript{T} T cells.

The memory H-Y TCR\textsuperscript{tg} CD8\textsuperscript{T} T cells were generated in Rag\textsuperscript{1-/-} mice. To determine whether the lymphopenic environment of the host might have modulated the cytokine responses of memory H-Y TCR\textsuperscript{tg} CD8\textsuperscript{T} T cells, we evaluated their cytokine responses of CD44\textsuperscript{low} and CD44\textsuperscript{high} CD8\textsuperscript{T} T cells purified from C57BL/6 mice (Fig. 3C). Compared with CD44\textsuperscript{low} cells, the CD44\textsuperscript{high} population showed elevated expression of Ly-6C, CD122, and CD127 memory markers (Fig. 3D). As shown for H-Y TCR\textsuperscript{tg} CD8\textsuperscript{T} T cells, CD44\textsuperscript{low} cells displayed increased synergy with IL-7 and CD44\textsuperscript{high} cells with IL-15 (Fig. 3E). Unlike memory H-Y TCR\textsuperscript{tg} CD8\textsuperscript{T} T cells, which did not respond to synergistic stimulation with IL-6 (Fig. 3B, right panel), CD44\textsuperscript{high} cells displayed strong proliferation in response to IL-15 plus IL-6 (Fig. 3E). Nonetheless, IL-21 provided a stronger stimulus than IL-6 in the presence of IL-7 or IL-15 to CD44\textsuperscript{high} cells. Collectively, these results show that IL-6 and IL-21 synergize strongly with IL-7 to stimulate proliferation of naive CD8\textsuperscript{T} T cells and with IL-15 to stimulate memory CD8\textsuperscript{T} T cells, and that these synergistic effects are more pronounced in the presence of IL-21 than IL-6.

IL-6 and IL-21 augment STAT5 activation induced by IL-7 or IL-15 in CD8\textsuperscript{T} lymphocytes

To investigate whether synergistic stimulation by IL-6 modulates the STAT signaling pathway triggered by IL-7 or IL-15, we examined phosphorylation of STAT5 and STAT3 proteins following cytokine stimulation. For this purpose, we used CD8\textsuperscript{T} T cells purified from P14 TCR\textsuperscript{tg} mice. More than 90% of these cells displayed a naive CD44\textsuperscript{low} phenotype (data not shown). P14 cells were stimulated by IL-7 or IL-15 in the presence or absence of IL-6 or IL-21, and phosphorylation of STAT proteins was evaluated by Western blot. Consistent with the fact that IL-7 is an essential survival cytokine for naive CD8\textsuperscript{+} T cells (1), IL-7 induced a strong phosphorylation of STAT5 on Tyr\textsuperscript{694}, whereas IL-15 elicited a weak phospho-STAT5 signal (Fig. 4A). Concomitant stimulation with IL-6 or IL-21 augmented the phospho-STAT5 signal induced by IL-7 or IL-15 by \textgreater;2-fold (Fig. 4, A and B). IL-6 and IL-21 induced strong phosphorylation of STAT3 on Tyr\textsuperscript{705}, and small, but significant phosphorylation on Ser\textsuperscript{727} residue (Fig. 4A). Neither IL-7 nor IL-15 caused an appreciable increase in phosphorylation of STAT3 induced by IL-6 or IL-21 (Fig. 4, A and B). Phosphorylation of STAT1 stimulated by IL-6 or IL-21 was also not significantly affected in the presence of IL-7 or IL-15 (Fig. 4A). These observations suggest that increased STAT5 signaling could be an important mechanism underlying the proliferation of CD8\textsuperscript{T} T cells stimulated by IL-7 or IL-15 in synergy with IL-21 or IL-6.

To determine whether the increased STAT5 phosphorylation was associated with enhanced DNA-binding activity, we conducted EMSA using the GRR probe, which has been previously used to reveal STAT5 activation in T cells by IL-15 (48). As shown in Fig. 4C, stimulation of P14 TCR\textsuperscript{tg} CD8\textsuperscript{T} cells with IL-7 in the presence of IL-6 or IL-21 augmented the amount of nuclear STAT proteins binding to the probe. The GRR sequence is known to bind STAT5-, STAT3-, and STAT1-containing complexes, and a comparison with the Western blot results (Fig. 4A) indicated that the IL-7-induced upper band contained STAT5, whereas the faster migrating band in IL-21- or IL-6-stimulated cells represented a...
STAT3-containing complex. This prediction was confirmed by supershift assays using STAT5- and STAT3-specific Abs. As shown in Fig. 4D, the anti-STAT5 Ab completely retarded the migration of STAT5-DNA complex, whereas the anti-STAT3 Ab reacted with the lower band. These observations show that concomitant stimulation by IL-7 and IL-6 or IL-21 increases phosphorylation and DNA-binding activity of STAT5.

Synergistic stimulation by IL-21 or IL-6, in addition to augmenting phosphorylation of STAT5 induced by IL-7 or IL-6 or IL-21, might modulate the kinetics of STAT5 activation. To address this question, we evaluated the decay kinetics of the phoso-STAT5 signal in P14 cells at different time points after a brief stimulation for 15 min by IL-7, either alone or in combination with IL-6 or IL-21 (Fig. 4E). IL-6 and IL-21 increased the magnitude of STAT5 phosphorylation as expected, but did not modulate the duration of the STAT5 signal, because the signal declined at a comparable rate under all three stimulatory conditions. These observations suggest that increased signal strength, but not an increase in the signal duration of STAT5 phosphorylation, contributes to the synergistic stimulation of CD8 T cells by cytokines.

Consistent with a critical role for the JAK-STAT pathway, an inhibitor of STAT3 activation, JSI-124 (58), almost completely inhibited the cytokine-induced proliferation (Fig. 4F). Because IL-7 and IL-6 also stimulate the PI3K and MAPK pathways (35, 59), we examined whether pharmacological inhibitors of these signaling pathways would modulate the proliferation of CD8 T cells induced by cytokines. Our results show that the PI3K inhibitor LY294002 and the p38 MAPK inhibitor SB 202190 severely compromised proliferation induced by IL-7 or IL-15 in the presence of IL-21 or IL-6 (Fig. 4F). These observations are consistent with a recent report that PI3K and MAPK pathways are important for IL-21-mediated activation of CD8 T cells (60). Interestingly, an inhibitor of the Src family protein tyrosine kinases (PTKs), PP2, also strongly inhibited the cytokine-induced proliferation (Fig. 4F). It is noteworthy that p56lck and p59fyn, PTKs that play important role in T cell activation via the TCR (61), also interact with

---

**FIGURE 6.** Cytokine-stimulated CD8 T cells display increased cytolytic activity following Ag restimulation. Total lymph node cells from P14 TCRtg mice were stimulated with gp33 peptide for 3 days or indicated cytokines for 5 days, and CTL activity was measured by 51Cr release assay using EL4 target cells loaded with gp33 peptide (A, upper panel). Cells stimulated by the control AV peptide displayed negligible CTL activity (data not shown). Cells cultured for 4 days with cytokines were washed and subsequently stimulated with a suboptimal concentration of gp33 peptide (0.01 μg/ml) for 2 days without cytokine addition. CTL activity was measured at lower E:T ratios (A, lower panel). For comparison, freshly isolated P14 cells were stimulated at the same concentration of gp33 peptide for 2 days (C). Cells from the above experiment were stained for intracellular granzyme B (B, upper panel). The IL-15+IL-21 group showed a strong increase in the mean fluorescence intensity (MFI) of granzyme B caused by prior cytokine stimulation was calculated based on the induction caused by gp33 alone (B, lower panel). Representative data from four separate experiments are shown. C, P14 TCRtg T cells were cultured with IL-6 or IL-21 in the presence of IL-15 for 4 days, washed, and stimulated with gp33 peptide for additional 2 days. CTL activity was measured, as described above. Data from one of the two independent experiments are shown. Cell recovery after culture with IL-21, IL-6, or IL-15 alone was too low to carry out CTL assay.
IL-6 and IL-21 on TCR signaling, we stimulated naive P14 TCRtg /H9255 cells following stimulation of the TCR by anti-CD3. IL-21 and IL-6 have been shown to augment proliferation of T cells via the TCR (22, 62, 63). Hence, in addition to the JAK-STAT pathway, Src family PTKs, PI3K, and p38 MAPK also seem to contribute to the synergistic stimulation of CD8 T cells by cytokines. Prior activation by cytokines augments proliferation and effector functions of CD8 T lymphocytes upon subsequent stimulation via the TCR.

IL-21 and IL-6 have been shown to augment proliferation of T cells following stimulation of the TCR by anti-CD3ε Ab or allogenic splenocytes (23, 42, 64). To compare the synergistic effect of IL-6 and IL-21 on TCR signaling, we stimulated naive P14 TCRε CD8 T cells with plate-bound anti-CD3ε mAb either alone or in the presence of IL-21 or IL-6. We observed that IL-6 augmented the proliferation of CD8 T cells induced via the TCR much more efficiently than IL-21 (Fig. 5A, left panel). IL-7 augmented the anti-CD3 response as efficiently as IL-6. However, neither IL-6 nor IL-21 increased the strong proliferation induced by the antigenic peptide, whereas IL-7 significantly augmented this response (Fig. 5A, right panel). These results suggested that the synergistic effect of IL-6 or IL-21 might be superfluous in the presence of costimulatory signals provided by APC, whereas the well-known prosurvival function of IL-7 (22) might have contributed to the increased response. Interestingly, naive P14 TCRε CD8 T cells that have been prestimulated with IL-7 in the presence of IL-6 or IL-21 showed heightened proliferation in response to very low concentrations of gp33 peptide or anti-TCR Ab in the absence of added cytokines (Fig. 5B, left and right panels). This priming effect was not observed with cells that were prestimulated with IL-7 alone. Similar results were obtained when CD8 T cells were prestimulated with IL-15 in the presence of IL-6 or IL-21 (Fig. 5C, left and right panels).

To determine whether cytokine stimulation of CD8 T cells also induced effector functions, we evaluated CTL response of P14 TCRε CD8 T cells stimulated with cytokines without and with subsequent stimulation by gp33 peptide-pulsed target cells (Fig. 6A, upper panel). However, the magnitude of these cytokine-induced responses was considerably lower than the CTL activity stimulated by the

**FIGURE 7.** Cytokine stimulation of P14 cells induces a distinct phenotypic differentiation program compared with stimulation via the TCR. A, Total splenocytes from P14 TCRtg /H9255 mice were stimulated with indicated cytokines, gp33 peptide in the presence of irradiated autologous APC, or a combination of anti-CD3 and anti-CD28 mAb for 3 days. Expression of indicated cell surface molecule was evaluated by flow cytometry (filled histograms). Freshly isolated P14 cells served as control (line histogram). The mean fluorescence intensity (geometric mean) of activated cells is given within each histogram plot, and the value for unstimulated P14 cells is given below each marker label. Data shown are representative of four independent experiments. B, Cells stimulated with IL-15 either alone or in the presence of IL-6 or IL-21 as in A displayed similar phenotypic changes as IL-7-stimulated CD8 T cells. Representative markers from one of the three separate experiments are shown.
antigenic peptide. Interestingly, the cytokine-activated cells showed dramatically higher CTL activity when subsequently stimulated by the antigenic peptide, which was evident at very low E:T ratio (Fig. 6A, lower panel). This increase in cytolytic activity was associated with an increase in the expression of granzyme B (Fig. 6B). P14 TCRγδ CD8+ T cells stimulated by cytokines alone did not up-regulate granzyme B; however, these cells showed a greater induction of granzyme B upon restimulation by antigenic peptide. CD8+ T cells prestimulated with IL-15 in the presence of IL-6 or IL-21 also caused a similar increase in CTL activity following subsequent Ag stimulation (Fig. 6C). Collectively, these results indicate that prior exposure of CD8+ T cells to inflammatory cytokines generated by the innate immune response can profoundly augment their responsiveness to Ag.

Synergistic stimulation by cytokines induces distinct phenotypic differentiation of CD8+ T lymphocytes

CD8+ T cells stimulated via the TCR either by antigenic peptide or by a combination of anti-CD3 and anti-CD28 mAb undergo proliferation accompanied by distinct alterations in the expression of the TCR, coreceptors, adhesion molecules, and cytokine receptors (65). Because cytokine stimulation of CD8+ T cells induced a 5- to 10-fold less proliferation compared with TCR stimulation (Fig. 1D), we examined whether synergistic stimulation by IL-7 and IL-6 or IL-21 induced a similar modulation of cell surface molecules as signaling via the TCR (Fig. 7A). Cells stimulated via the TCR showed significant up-regulation of all three chains of the IL-2R (CD25, CD122, and CD132) and CD69. In comparison, cytokine-induced proliferation was accompanied by only a modest increase in the expression of IL-2Rα, IL-2β, and CD69, and a strong up-regulation of γc. In contrast, the expression of IL-7Rα (CD127) was augmented by TCR signaling, but not by cytokine stimulation. TCR signaling increased the expression of CD44, but decreased those of CD62L and the TCR. In contrast, synergistic stimulation by cytokines strongly increased the expression of CD62L and TCR, with a modest increase in CD44 expression. As shown recently (66), cytokine stimulation markedly augmented the expression of the CD8 coreceptor. IL-15 also induced a similarly distinct phenotypic profile as IL-7 in the presence of IL-6 or IL-21 (Fig. 7B). For most markers, IL-7 or IL-15 alone was as efficient as the cytokine combinations, indicating that IL-6 and IL-21, which are necessary to induce proliferation (Fig. 1), do not contribute significantly to the distinct phenotypic changes associated with synergistic cytokine stimulation. These results indicate the following: 1) even though the TCR and cytokine stimuli induce proliferation of CD8+ T cells, they modulate the expression of cell surface molecules in a significantly different manner, and 2) cytokine-induced phenotypic differentiation occurs independently of cell proliferation.

Discussion

Activation of T lymphocytes via the TCR and concomitant delivery of costimulatory signals are the central requirements to mount an effective immune response against pathogens and to discourage activation of autoreactive cells. These requirements can be overcome under certain circumstances such as lymphopenia, in which cytokines such as IL-15 and IL-7 induce proliferation of T cells to rapidly restore homeostasis of T lymphocytes (17). The tg expression of IL-7, IL-15, or constitutively active STAT5, a key signaling molecule downstream of these cytokines, causes an accumulation of CD8+ T cells (67–70). These observations suggest that Ag-independent stimulation of CD8+ T cells may occur under conditions of cytokine abundance or increased cytokine receptor signaling. An earlier work has shown that bystander activation of CD8+ T cells can occur in vivo during immune response to a viral infection, presumably mediated by cytokines (71). The possibility that such bystander activation may contribute to the transition from innate immune responses to adaptive immunity cannot be ignored. In fact, circumstantial evidence suggests that Ag-nonspecific, cytokine-driven proliferation of autoreactive T cells might be involved in the initiation and exacerbation of autoimmune diseases (26, 72). This notion is further supported by recent findings that naive CD8+ T lymphocytes can be induced to proliferate following synergistic stimulation by IL-15 or IL-7 in the presence of IL-21 (19, 20). In this study, we have shown that IL-6, which is secreted by activated cells of the innate and adaptive immune system, including monocytes, macrophages, dendritic cells, and T and B lymphocytes (73, 74), also can synergize with IL-7 or IL-15 to stimulate activation of naive CD8+ T cells in an Ag-independent manner.

IL-7 and IL-15 belong to the IL-2 family of cytokines, which require γc to transduce cellular activation signals via the JAK-STAT pathway (29). The magnitude of STAT5 activation closely correlates with the TCR-independent proliferation of CD8+ T cells stimulated by cytokines. In fact, overexpression of constitutively active STAT5 results in the accumulation of CD8+ T cells, whereas STAT5-deficient mice display a paucity of CD8+ T cells (69, 70). IL-7, but not IL-15, induced a strong phosphorylation of STAT5 in naive CD8+ T cells (Fig. 4A), whereas both IL-7 and IL-15 strongly activated STAT5 in a polyclonal population of CD8+ T cells, which presumably contains a certain frequency of memory cells (20). It is likely that elevated expression of IL-2Rγc, γc, and IL-7Rα chains in memory CD8+ T cells (75) underlies the strong STAT5 phosphorylation induced by IL-15 or IL-7 in polyclonal CD8+ T cells. In naive CD8+ T cells, augmentation of the IL-7-induced STAT5 phosphorylation by IL-6 or IL-21 could be the primary signaling pathway that stimulates cell proliferation. Data shown in Fig. 4F indicate that other signaling molecules such as Src kinases, PI3K, and p38 MAPK also contribute to this synergistic response. Because IL-21 and IL-6 induce minimal or negligible STAT5 activation in naive CD8+ T cells, but induce strong STAT3 phosphorylation, which is not enough to induce proliferation, it is likely that cooperation between STAT3 and STAT5 pathways underlies the synergistic effect of IL-6 and IL-21 in the presence of IL-7. Further investigation is needed to determine how this cooperation rapidly translates into increased STAT5 activation (Fig. 4A).

A number of earlier studies have implicated IL-6 in the survival and activation of T cells. However, the underlying mechanisms have not been completely understood. IL-6 can promote proliferation of thymocytes and peripheral T cells induced by mitogens (76–79), which has been attributed to its antiapoptotic function through increased expression of Bcl-2 and inhibition of activation-induced cell death (38, 39, 50, 80). IL-6 has also been reported to promote differentiation of human and murine CTLs (81), which could account for its ability to confer increased resistance to intracellular bacterial infection (82). The present study has revealed a novel role for IL-6 in inducing TCR-independent proliferation in CD8+ T cells in synergy with IL-7 or IL-15. IL-6-mediated survival and increased proliferation of T cells have been shown to be dependent on STAT3 (39, 40). However, concomitant stimulation of naive CD8+ T cells by IL-7 or IL-15 did not augment the IL-6-induced STAT3 phosphorylation (Fig. 4). Therefore, the prosurvival function of STAT3 activated by IL-6 might only play a secondary role in the proliferation and functional differentiation of CD8+ T cells stimulated by the synergistic effect of IL-6 and IL-7 or IL-15.

IL-6 plays a critical role during transition from neutrophil-mediated acute inflammation to monocyte- and lymphocyte-dependent immune response (36, 37, 83). Important mechanisms underlying this transition could be the prosurvival function of IL-6 in T
creased proliferation and effector functions upon subsequent Ag presence of IL-7 and IL-15 could also contribute to the transition such as type 1 diabetes in which CD8 T cells by cytokines may function to bridge the innate and adaptive immune responses. For instance, the CD62Lhigh phenotype stimulated by cytokines (Fig. 7) may be useful in facilitating their responses to Ag. In contrast, the CD62Lhigh phenotype will facilitate the cytokine-primed CD8 T cells to re-enter peripheral lymph nodes in search of specific Ags (87, 88). Whereas IL-7 alone can induce this phenotype, the innate cytokines IL-6 and IL-21 are necessary to promote expansion of these cells, and may even help in the acquisition of this phenotype in vivo where IL-7 is limiting.

As much as the Ag-nonspecific activation of CD8 T cells by IL-6 in the presence of IL-7 or IL-15 would be beneficial to mount protective immune responses, it can also contribute to perpetuation of chronic inflammatory conditions and progression of autoimmune diseases (36, 37, 89). Cytolytic activity of cytokine-stimulated CD8 T cells, their reduced Ag threshold to undergo proliferation, and their distinct cell surface phenotype may have important implications in the pathogenesis of autoimmune diseases such as type I diabetes in which CD8 T cells significantly contribute to the disease (90).

Disclosures

The authors have no financial conflict of interest.

References


