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Inflammatory Cytokines Provide a Third Signal for Activation of Naive CD4\(^+\) and CD8\(^+\) T Cells\(^1\)

Julie M. Curtsinger, Clint S. Schmidt, Anna Mondino, Debra C. Lins, Ross M. Kedl, Marc K. Jenkins, and Matthew F. Mescher\(^2\)

The effects of inflammatory cytokines on naive T cells have been studied using MHC protein/peptide complexes on microspheres, thus avoiding the use of APCs whose functions may be affected by the cytokines. IL-1, but not IL-12, increased proliferation of CD4\(^+\) T cells in response to Ag and IL-2, which is consistent with effects on in vivo priming of CD4\(^+\) cells. In contrast, proliferation of CD8\(^+\) T cells to Ag and IL-2 required IL-12, and IL-12 replaced adjuvant in stimulating an in vivo response to peptide. These results support a model in which distinct inflammatory cytokines act directly on naive CD4\(^+\) and CD8\(^+\) T cells to provide a third signal, along with Ag and IL-2, to optimally activate differentiation and clonal expansion. *The Journal of Immunology*, 1999, 162: 3256–3262.

Infection of a T cell immune response that leads to full activation rather than tolerance appears to require that the T cell know that “danger” is present (1), and it has been argued that inflammatory cytokines produced by the innate immune system in response to pathogens provide the “danger signals” to T cells (2). Potent induction of inflammatory cytokines is likely to account, at least in part, for the adjuvant properties of CFA, LPS, and other adjuvants. Administration of Ag in the absence of adjuvant results in some clonal expansion of CD4\(^+\) T cells in draining lymph nodes, but the cells are rendered tolerant to subsequent challenge with Ag. Coadministration of Ag with CFA or LPS increases the extent of clonal expansion and prevents tolerance induction, and these effects can be mimicked by administration of either TNF-\(\alpha\) or IL-1 (3). The effects of these may be related, because TNF-\(\alpha\) induces IL-1 production (4). Thus, these inflammatory cytokines are necessary and sufficient to support the response in the absence of other adjuvants.

Where and how the inflammatory cytokines act to promote T cell responses is less clear. Studies of CD4\(^+\) T cell lines suggest that IL-1 may contribute to activation through direct interaction with the T cell (5–8). Alternatively, the cytokines may act indirectly by up-regulating, on APCs, the expression of ligands that costimulate T cell activation (9). TNF-\(\alpha\) and IL-1 can induce the expression of CD40 on dendritic cells (10) and CD40-dependent signaling can in turn increase B7 expression on APC (11–13), thus increasing the level of costimulation available to the T cells. Proliferation and differentiation of CD8\(^+\) T cells to generate cytotoxic T lymphocyte responses can also be enhanced by inflammatory cytokines produced by macrophages and/or dendritic cells, cytokines that include IL-1 (14), IL-6 (14, 15), IL-12 (16–18), and IFN-\(\gamma\) (19). Like CD4\(^+\) T cells, it is unclear whether these cytokines act directly on the CD8\(^+\) T cells, or indirectly by enhancing Ag presentation or costimulation by APC.

MHC protein/peptide Ag complexes immobilized on inert microspheres can be used to study T cell activation requirements in the absence of APC (20), thus making it possible to determine whether inflammatory cytokines have a direct effect on the T cells. Using this approach with highly purified naive T cells from TCR transgenic mice and signal 2 provided in the form of either exogenous IL-2 or coimmobilized B7.1 protein (21), we found that IL-1 significantly enhanced the response of CD4\(^+\) T cells, whereas IL-12 was required for a response by CD8\(^+\) T cells. The latter finding predicted that IL-12 may act as an effective adjuvant for in vivo induction of a CD8\(^+\) T cell response, and this prediction was confirmed. Many aspects of T cell activation can be accounted for by a two-signal model (22) with signal 1 provided by the TCR and signal 2 by costimulation leading to IL-2 production (23–25). The results described in this report suggest that a three signal model may more adequately describe T cell activation requirements, with the third signal being a “danger signal” provided by an inflammatory cytokine, IL-1 in the case of CD4\(^+\) T cells and IL-12 in the case of CD8\(^+\) T cells, that acts directly on the T cell.

**Materials and Methods**

**T cell purification**

Lymph node (LN)\(^3\) cells from OT-I mice (26) were harvested and passed over Cellect-plus CD8 enrichment columns (Biotex Laboratories, Edmonton, Alberta, Canada). The cells were then stained with anti-CD44-FITC and anti-CD8-phycocerythrin mAbs and sorted using a FACSVantage flow cytometer (Becton Dickinson, Mansfield, MA) to obtain a population of naive CD8\(^+\) CD44\(^{low}\) cells (>98% CD44\(^{low}\)). LN and spleen cells were harvested from DO11.10 mice (27), depleted of adherent cells by incubation on plastic petri dishes for 1 h, and passed over Cellect-plus CD4 enrichment columns. The cells were then stained with anti-CD45RB-FITC mAb and sorted to obtain naive CD4\(^+\) CD45RB\(^{high}\) cells (>98% CD45RB\(^{high}\)).

**Proliferation and cytotoxicity assays**

A total of 5 \(\times\) 10\(^4\) purified T cells and 10\(^3\) Ag-coated latex microspheres were placed in flat-bottom microtiter wells in 200 \(\mu\)l of RPMI 1640 medium supplemented with 10% FCS, 4 mM l-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin and streptomycin, 10 mM HEPES, and 5 \(\mu\)M 2-ME. Where indicated, cultures were

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3 Abbreviation used in this paper: LN, lymph node.
supplemented with human rIL-2 (R&D Systems, Minneapolis, MN), mouse rIL-12 (Genetics Institute, Cambridge, MA), or mouse rIL-1β (R&D Systems). Proliferation was measured after 3 days for CD8⁺ T cells and after 4 days for CD4⁺ T cells by addition of 1 μCi [3H]TdR per well for the last 8 h of culture. In some experiments, neutralizing antiserum against IL-12 (sheep anti-mouse IL-12; Genetics Institute) or mAb to IL-1R (PharMingen, San Diego, CA) were used. Triplicate determinations were done, and SDs are shown. Cytolytic activity was determined in a standard 4-h 51Cr release assay using E.G7 cells (EL4 cells transfected with OVA) as targets, with EL4 cells included as a control for specificity.

In Ags on microspheres

H-2Kb was purified, incorporated onto 5-micron-diameter latex microspheres and pulsed with OVA₂₅₇₋₂₆₄ as previously described (20) using 3.5 μg H-2Kb per 10⁶ beads for immobilization. Peptide pulsing was done using 2 μM peptide, unless otherwise indicated. IAα/OVA₂₅₇₋₂₆₉ fusion protein (28) was obtained from L cells transfected with a full-length construct that included the cytoplasmic and transmembrane regions of the class II protein. These cells were a kind gift from Dr. Andrea J. Sant (University of Chicago, Chicago, IL). Cells were solubilized using Triton X-100, and the IAα/OVA₂₅₇₋₂₆₉ protein was purified by affinity chromatography using M5/114 mAb specific for IAβ². The purified protein was incorporated onto latex microspheres exactly as for H-2Kb (20), and the extent of immobilization determined by flow cytometry using the M5/114 mAb. Naive B7.1 protein was purified by affinity chromatography and immobilized on microspheres as previously described (21, 29). For all microsphere preparations, the surface density of immobilized B7.1 was determined by flow cytometry to insure that it was in the range previously shown to provide effective costimulation to T cells (21).

Adoptive transfer and immunization

LN cells were harvested from 2C TCR transgenic mice (30), labeled with the fluorescent dye PKH26-GL (Sigma, St. Louis, MO) according to the supplier’s protocol and adoptively transferred into C57BL/6 mice (Charles River Laboratory, Wilmington, MA) by suspending 3–4 × 10⁶ CD8⁺ cells in 0.5 ml PBS and injecting into the tail vein. After transfer, mice were rested for 1 day and then immunized (day 0) by s.c. injection of a total of 0.3 μl of OVA in 0.5 ml PBS and injecting into the tail vein. After transfer, mice were rested for 1 day and then immunized (day 0) by s.c. injection of a total of 0.3 μl Ag in two sites on the back. Ag was SIYRYYGL peptide (31) in either PBS or emulsified in CFA. Cytokines were administered i.p. in 0.1 ml doses on days 0, 1, and 2. IL-12 was administered at 1 mg/day in PBS with 1% mouse serum, and IL-1β was administered at 0.5 μg/day in PBS with 0.1% BSA. On day 3, mice were sacrificed, and cells from draining LNs were harvested from 2C TCR transgenic mice (30), labeled with fluorescent dye and placed into flow cytometer to obtain a population of naive CD8⁺ T cells. CD44 low expressing cells (——) and a population of CD8⁺ CD44high expressing cells (—–) were identified by staining with 1B2 mAb specific for the TCR (Ref. 32; a gift from Dr. H. Eisen) and anti-CD8 mAb. These methods for identification, enumeration, and phenotypic characterization of adoptively transferred 2C cells, have been described in detail (33).

Results

IL-12 is needed along with IL-2 and Ag for in vitro stimulation of naive CD8⁺ T cells

Naive (CD44low) CD8⁺ T cells were purified by FACS from OT-I mice having a transgenic TCR specific for OVA₂₅₇₋₂₆₄ peptide and H-2Kb (26) to yield a population having >99% CD8⁺ CD44low T cells (Fig. 1) that are >99% positive for the β2 transgenic TCR. The purified cells are Ly-6C⁻, CD25⁻, and CD69⁻ and have the forward scatter profile of small resting lymphocytes, consistent with being naive cells (data not shown). Artificial APC were prepared by immobilizing H-2Kb on latex microspheres and pulsing with OVA₂₅₇₋₂₆₄ to form Ag complexes. Beads prepared in this way stimulate strong Ag-specific proliferation of CD44high memory cells from OT-I mice, provided that exogenous IL-2 is added (20). In contrast, naive CD44low cells respond only marginally to beads and IL-2 (Ref. 20 and Fig. 2A). However, Ag and IL-2 stimulate vigorous proliferation of naive cells when IL-12 is also added (Fig. 2A). The response depends on all three stimuli (Fig. 2A), and addition of anti-IL-2 mAb to the cultures blocks the response in the presence of IL-12 (data not shown).

Proliferation in response to Ag, IL-2, and IL-12 had essentially identical stimulation, enumeration, and phenotypic characterization of adoptively transfered 2C cells, have been described in detail (33).
the absence of IL-2, IL-12 alone did not support a response to Ag even at high concentrations (Fig. 3B) and IL-12 did not enhance survival of unstimulated CD8+ T cells (data not shown).

**IL-1, but not IL-12, supports optimal proliferation of naive CD4+ T cells in response to Ag and IL-2.**

Although IL-12 acts synergistically with Ag and IL-2 in stimulating CD8+ T cells, other inflammatory cytokines including IL-1, IL-6, and TNF-α did not support the response (data not shown). This finding was somewhat surprising because IL-1 can replace adjuvant in supporting Ag-dependent in vivo clonal expansion of CD4+ T cells (3), whereas IL-12 promotes differentiation to a Th1 phenotype (3, 34) but has little effect on the extent of clonal expansion. These results predicted that CD4+ cells may respond differently than CD8+ cells to inflammatory cytokines in vitro; therefore, experiments were done to compare the two cell types. The response of CD8+ T cells to Ag and IL-2 was not increased by the addition of IL-1β, whereas IL-12 supported vigorous proliferation, and this response was blocked by addition of anti-IL-12 Ab (Fig. 4A). The CD4+ response was examined using naive CD45R+B6 mice transgenic for CD4+ T cells from DO11.10 TCR and artificial APC made using I-Aα/OVA323–339 fusion protein (27). Ag alone induced a weak proliferative response that was increased about 2-fold when either IL-2 or IL-1β was added, and addition of both gave the strongest response (Fig. 4B). All three components were required for maximal response (Fig. 4B), and the response was completely blocked by anti-IL-2R mAb (data not shown). In contrast to IL-1, IL-12 had no effect on the CD4+ T cell response to Ag or Ag plus IL-2 (Fig. 4B). Thus, responses of naive CD4+ T cells are enhanced by IL-1 but not IL-12, whereas responses of naive CD8+ T cells require IL-12 but not IL-1.

**B7.1 ligand does not eliminate a requirement for inflammatory cytokines for optimal T cell responses**

To determine whether the B7.1 costimulatory ligand may alter the requirements for inflammatory cytokines, microspheres having Ag
and purified B7.1 coimmobilized on the same surface were prepared and used to stimulate cells. B7.1 was immobilized at a surface density previously shown to yield effective costimulation along with anti-TCR mAb (21), and this density was confirmed by flow cytometry (21, 29). The presence of the B7.1 on the same surface as the H-2K\(^ b \)/OVA\(_{257-264}\) Ag did not eliminate a requirement for IL-12 in stimulating OT-1 cells (Fig. 4C). CD8\(^ {+} \) cells make low levels of IL-2, and optimal proliferation required the addition of exogenous IL-2 even when B7.1 was present; biological activity of the B7.1 was confirmed in experiments examining CD4\(^ {+} \) cells. When Ag and B7.1 were coimmobilized and used to stimulate DO11.10 cells, B7.1-dependent costimulation caused sufficient IL-2 production to increase the response over that of Ag alone, and IL-1\(\beta\) doubled the response (Fig. 4D). Addition of anti-IL-1R mAb decreased the response to the same level obtained when just Ag/B7.1 beads were used, confirming that the effects are IL-1-dependent. Again, neither IL-1R (Fig. 4D) nor TNF-\(\alpha\) (data not shown) enhanced the CD4\(^ {+} \) T cell responses. Thus, when signal 2 is provided either in the form of IL-2 or IL-2 and B7.1 ligand, naive CD8\(^ {+} \) T cells remain dependent on IL-12 for a strong proliferative response, whereas IL-1\(\beta\) substantially increases the response of CD4\(^ {+} \) T cells when either IL-2 or B7.1 ligand are used to provide signal 2.

IL-12 is required for differentiation of naive CD8\(^ {+} \) T cells to lytic effector cells

In addition to proliferating in response to Ag-specific stimulation, naive CD8\(^ {+} \) T cells develop lytic effector function by day 3. Cytolytic activity of cells cultured with Ag alone or Ag and IL-12 could not be determined because too few viable cells remained to be assayed by day 3. A low level of proliferation of OT-1 cells does occur in response to just Ag and IL-2 (Figs. 2–4), and sufficient viable cells could be recovered from these cultures on day 3 to determine whether they had acquired lytic activity. Cells stimulated with Ag and IL-2, or with just IL-2 and IL-12 in the absence of Ag, did not develop detectable cytolytic activity (Fig. 5). In contrast, cells stimulated with Ag, IL-2, and IL-12 developed potent Ag-specific cytolytic activity. Thus, in addition to being required to support optimal proliferation, both IL-2 and IL-12 are required along with Ag to support acquisition of lytic effector function by naïve cells.

**IL-12 supports in vivo clonal expansion of CD8\(^ {+} \) T cells**

The in vitro results described above predicted that IL-12 may replace adjuvant in stimulating an in vivo response of CD8\(^ {+} \) cells to peptide. This finding was examined by adoptively transferring CD8\(^ {+} \) T cells from 2C TCR transgenic mice were labeled with PKH26 (see Fig. 7) and adoptively transferred into normal C57BL/6 recipients. SIYRYYGL peptide (50 \(\mu\)g) was injected s.c. in PBS alone (Peptide) or along with IL-12 (Peptide + IL-12), IL-1\(\beta\) (Peptide + IL-1) or CFA (Peptide + CFA). Controls included mice that received no challenge (Transfer Only) and mice that received just IL-12 (IL-12 only) or just CFA. Cytokines were delivered by i.p. injection on days 0, 1, and 2. On day 3, mice were sacrificed and the numbers of 2C cells in the draining lymph nodes, spleen, and peripheral blood were determined by flow cytometry using 1B2 anti-clonotypic mAb and anti-CD8 mAb to identify the cells. Groups included two mice each, and error bars indicate ranges. Comparable responses to peptide with CFA or IL-12 were obtained in six of six experiments, and nonresponsiveness to peptide and IL-1 in two of two experiments. A. Number of 2C cells in draining lymph nodes on day 3. 2C cells accounted for 0.16% of the total lymphocytes in transfer only LN, and 2.1% in IL-12 + peptide LN. B. Number of 2C cells in the spleens on day 3.

**FIGURE 6.** IL-12 replaces adjuvant in stimulating in vivo clonal expansion of CD8\(^ {+} \) 2C T cells in response to peptide Ag. Cells from 2C TCR transgenic mice were labeled with PKH26 (see Fig. 7) and adoptively transferred into normal C57BL/6 recipients. SIYRYYGL peptide (50 \(\mu\)g) was injected s.c. in PBS alone (Peptide) or along with IL-12 (Peptide + IL-12), IL-1\(\beta\) (Peptide + IL-1) or CFA (Peptide + CFA). Controls included mice that received no challenge (Transfer Only) and mice that received just IL-12 (IL-12 only) or just CFA. Cytokines were delivered by i.p. injection on days 0, 1, and 2. On day 3, mice were sacrificed and the numbers of 2C cells in the draining lymph nodes, spleen, and peripheral blood were determined by flow cytometry using 1B2 anti-clonotypic mAb and anti-CD8 mAb to identify the cells. Groups included two mice each, and error bars indicate ranges. Comparable responses to peptide with CFA or IL-12 were obtained in six of six experiments, and nonresponsiveness to peptide and IL-1 in two of two experiments. A. Number of 2C cells in draining lymph nodes on day 3. 2C cells accounted for 0.16% of the total lymphocytes in transfer only LN, and 2.1% in IL-12 + peptide LN. B. Number of 2C cells in the spleens on day 3.
and extent of cell death. Thus, the results obtained with PKH26 labeling do not provide a useful measure of the relative efficiencies of the different immunizations in expanding the 2C cell population. However, the results do support the conclusion that the majority of 2C cells proliferate in response to Ag, even when this is peptide alone that does not cause large clonal expansion (Fig. 6). Thus, the increased numbers of 2C cells in the draining LN and spleen are not simply due to migration.

**Discussion**

The ability of inflammatory cytokines to enhance T cell responses is becoming increasingly appreciated and is usually interpreted in terms of the ability of these cytokines to increase expression of costimulatory molecules on APC, thus enhancing the level of signal 2 available to the T cells (10–13). The results described in this study strongly suggest that instead, or in addition, the inflammatory cytokines can provide an essential third signal that acts directly on the T cell and is necessary to fully activate naive T cells. By using highly purified T cells from TCR transgenic mice and artificial APC in the form of Ag immobilized on microspheres, the potential effects of inflammatory cytokines on APC were eliminated, allowing their direct effects on T cells to be examined. Both CD4 and CD8 T cell responses in vitro were enhanced by inflammatory cytokines when artificial APC were used to present Ag and when signal 2 was provided in the form of either exogenously added IL-2 or coinmobilized B7.1. However, the effective cytokine and the extent to which it is required differ for the two T cell subsets. IL-12 is required along with Ag and IL-2 to stimulate a significant CD8 response (Figs. 2–5), whereas IL-1 augments a CD4 response to Ag and IL-2 (Fig. 4).

We recently showed that IL-2 and H-2Kb immobilized on microspheres and pulsed with OVA175–264 peptide were sufficient to stimulate proliferation and development of effector function by memory (CD44high) CD8+ T cells from OT-1 mice (20). In contrast, naive (CD44low) OT-1 cells showed only marginal proliferation and no development of lytic function in response to these stimuli despite the fact that the naive and memory cells expressed the same TCR and had almost identical surface densities of TCR and CD8. Those observations suggested that the signals needed to activate naive and memory CD8+ T cells differed either quantitatively or qualitatively.

It is clear from the present experiments that IL-12 can supply the missing signal to the naive cells, and that this is a direct effect of IL-12 on the T cells (Figs. 2–5). IL-12 has previously been shown to enhance CD8+ T cell responses (16–18), as have numerous other cytokines including IL-1 (14), IL-6 (14, 15), IFN-γ (19), and others. However, it has been unclear whether these reflect direct effects on the CD8+ T cell or effects on APC that enhance delivery of signal 2 to the T cells. Although IL-12 can supply the missing signal to naive CD8+ T cells in the absence of APC, this was not the case for IL-1 (Fig. 4), TNF-α, or IL-6 (data not shown). It also appears that IL-12 is not acting through its ability to stimulate T cell production of IFN-γ (35), because the addition of anti-IFN-γ Ab did not block the response to Ag, IL-2, and IL-12 (data not shown). Although only IL-12, of the cytokines tested, was able to support the CD8 T cell response, it is possible that there is some redundancy with other cytokines that are also able to support the response. Redundancy is seen with respect to signal 2 delivery; CD28/B7 seems to be the major pathway but a number of other receptor/ligand pairs have been implicated as providing costimulatory signals. It may also be the case that signal 3 is not always required. Signal 1, or signals 1 and 2, may be sufficient for activation at very high Ag levels or very high TCR affinity for Ag (37, 38).
that IL-12 will provide a means for effectively inducing CD8+ T cells when peptide pulsed spleen cells are used as APC (data not shown). Experiments are in progress to determine whether quantitative Ag effects, alternative cytokines, or both are involved in overcoming a requirement for IL-12 under some circumstances.

These in vitro results predicted that IL-12 may serve as an effective adjuvant in vivo and were confirmed using an adoptive transfer system to examine clonal expansion of TCR transgenic CD8+ T cells from 2C mice (Figs. 6 and 7). A striking parallel was found between the in vitro and in vivo responses. In the absence of IL-12, a low level of proliferation occurred in vitro in response to Ag and IL-2 (Figs. 2 and 3). In vivo, clonal expansion was minimal in response to just peptide, but the 2C cells had clearly recognized and responded to Ag, as evidenced by that fact that all of the cells recovered from the draining LN had undergone some proliferation (Fig. 7) and a substantial fraction (40–50%) had acquired a CD44high phenotype (data not shown). Addition of IL-12 in vitro resulted in a strong proliferative response, and in vivo administration along with peptide resulted in extensive clonal expansion, as good as or better than that obtained using peptide with CFA (Fig. 6). IL-12 will favor a cell-mediated response by promoting Th1 differentiation of CD4+ cells (3, 34, 41) and signaling for expansion (Fig. 6) and differentiation (Fig. 5) of CD8+ T cells. Differential effects of pathogens on the production of these two cytokines may have profound effects on the nature of the immune response that can be generated.

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References
