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Distinct Requirements for C-C Chemokine and IL-2 Production by Naive, Previously Activated, and Anergic T Cells

Cara G. Lerner,* Maureen R. Horton, † Ronald H. Schwartz,* and Jonathan D. Powell 1 *

Ag presented by activated APCs promote immunogenic responses whereas Ag presented by resting APCs leads to tolerance. In such a model, the regulation of cytokine release by the presence or absence of costimulation might potentially play a critical role in dictating the ultimate outcome of Ag recognition. C-C chemokines are a structurally defined family of chemoattractants that have diverse effects on inflammation. We were interested in determining the activation requirements for chemokine production by CD4+ T cells. Our data demonstrate for T cell clones and previously activated T cells from TCR-transgenic mice that stimulation with anti-TCR alone results in the production of copious amounts of macrophage-inflammatory protein-1α (MIP-1α) and other C-C chemokines, and that addition of anti-CD28 gives very little augmentation. Furthermore, MIP-1α production is nearly equivalent from both anergic and nonanergic cells. For naive T cells, anti-CD3 stimulation alone led to as much MIP-1α production as Ag + APC stimulation. The addition of costimulation gave a 3–10-fold enhancement, but this was 70-fold less than the effect of costimulation on IL-2 production. Thus, although C-C chemokines play a broad role in influencing inflammation, their production by signal 1 alone makes them unlikely to play a critical role in the decision between a tolerogenic and an immunogenic response. Furthermore, the production of MIP-1α by anergic T cells, as well as following signal 1 alone, raises the possibility that in vivo this chemokine serves to recruit activated T cells to become tolerant.

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T cells, signal 1 alone also proved to be sufficient to produce MIP-1α; however, the addition of anti-CD28 did lead to an enhancement of this response. In contrast, IL-2 production from the same cultures demonstrated a near absolute requirement for costimulation.

Materials and Methods

Cell lines and culture conditions

A.E7 is a CD4+ Th1 clone specific for the PCC peptide 81-104 and was grown and maintained as described previously (19). In general, cells were stimulated for 16 h with 10 ng/ml of rIL-2 (Genzyme, Cambridge, MA) at a final concentration of 0.25 ng/ml, ionomycin alone (Sigma), PMA alone (Sigma, St. Louis, MO) at a final concentration of 0.25 ng/ml anti-TCR. Cells were then harvested using a sterile cell scraper, washed, and rested in fresh medium. Anergic T cells demonstrated decreased proliferation to their cognate Ag upon restimulation as a result of their marked decrease in IL-2 production (3, 25). We have found that the decreased production of IL-2 is a more quantitative measure of anergy induction and thus throughout this paper anergy is assessed in terms of decreased IL-2 production.

Results

MIP-1α is produced by anergic T cells

Our laboratory has observed a consistent hierarchy of cytokine production by Th1 CD4+ T cell clones, whereby the more dependent a cytokine is on costimulation for production, the greater its inhibition in anergy. Previously, it was shown that MIP-1α production by T cells required costimulation in the form of CD28 signaling (16). According to the hierarchy then, we would predict that anergic cells would not produce MIP-1α. To test this hypothesis, anergic A.E7 T cells were stimulated for 16 h in fresh medium with anti-TCR + anti-CD28. Supernatant fluids were analyzed by ELISA for MIP-1α or IL-2 production. As seen in Table 1, there was a profound inhibition of IL-2 production (>100-fold) in the anergic A.E7 cells, indicating that they were indeed in an anergic state. Surprisingly, however, the cells produced similar amounts of MIP-1α as compared with their nonanergic counterparts. In six experiments, the anergic cells showed an average of only a 1.34-fold decrease in MIP-1α production as compared with nonanergic cells (paired Student’s t test, p = 0.11). Also, surprisingly, the unstimulated anergic clones continued to secrete a small yet consistent amount of MIP-1α after anergy induction. This expression, which appears to be due to newly transcribed protein, was not cumulative, but rather was freshly produced during the 16-h assay period. Small amounts of secretion were observed even up to 9 days after anergy induction. This continued expression of MIP-1α after anergy induction could be inhibited by the addition of fresh IL-2. In one experiment, anergic A.E7 cells produced 1333 pg/ml MIP-1α during a 16-h period on day 4 after anergy induction. This was decreased to 10.7 pg/ml by the addition of 100 U/ml IL-2. On the other hand, IL-2 does not inhibit MIP-1α production by cells that are actively being stimulated. For example, A.E7 cells produced 168,500 pg/ml MIP-1α in response to stimulation and

### Table 1. Anergic T cell clones produce MIP-1α

<table>
<thead>
<tr>
<th>Condition</th>
<th>IL-2 (U/ml)</th>
<th>MIP-1α (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.E7 unstimulated</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A.E7 anti-TCR + anti-CD28</td>
<td>1,843</td>
<td>276,000</td>
</tr>
<tr>
<td>Anergic A.E7 unstimulated</td>
<td>0</td>
<td>1,276</td>
</tr>
<tr>
<td>Anergic A.E7 anti-TCR + anti-CD28</td>
<td>15</td>
<td>148,000</td>
</tr>
</tbody>
</table>

* A.E7 and anergic A.E7 T cell clones were stimulated for 16 h and supernatant fluid was harvested and assayed for IL-2 and MIP-1α.
MIP-1α expression in CD4+ Th1 cells does not require costimulation. A, E7 cells were mock stimulated or stimulated with anti-TCR or anti-TCR and anti-CD28 for 8 h. The cells were then harvested and RNA was examined by Northern blot analysis.

280,000 pg/ml in response to stimulation in the presence of 100 U/ml IL-2. These findings are consistent with our observations that stimulation with signals 1 + 2 leads to the production of copious amounts of both IL-2 and MIP-1α (see below); the IL-2 produced under these conditions does not inhibit MIP-1α production.

C-C chemokine production in CD4+ Th1 T cell clones is not CD28 dependent

Since MIP-1α production was not affected by the anergic state, we decided to examine the costimulation dependence of this chemokine in our Th1 clone. As demonstrated by Northern blot analysis in Fig. 1, stimulation by anti-TCR for 8 h led to a dramatic increase in MIP-1α, MCP-1, and, to a lesser extent, RANTES mRNA. MCP-1 mRNA was undetectable (note different exposure times). In fact, preliminary data using microarray technology (26) (data not shown) has demonstrated that MIP-1α is one of the most abundant transcripts up-regulated in these T cell clones upon TCR engagement alone. The addition of anti-CD28 appeared to have minimal effect on all four chemokines, suggesting that A.E7 TCR engagement is sufficient for the maximum chemokine expression obtainable with this clone.

To confirm this conclusion, we next examined chemokine secretion at the protein level. As seen in Fig. 2, IL-2 production demonstrated a near absolute dependence on costimulation; in the presence of signal 1 alone, no measurable IL-2 was secreted. In contrast, analysis of the same supernatant fluid demonstrated production of both MIP-1α and RANTES in response to TCR engagement alone. The addition of anti-CD28 led to an average 1.2-fold increase in MIP-1α, MCP-1, and, to a lesser extent, RANTES mRNA. MCP-1 mRNA was undetectable (note different exposure times). In fact, preliminary data using microarray technology (26) (data not shown) has demonstrated that MIP-1α is one of the most abundant transcripts up-regulated in these T cell clones upon TCR engagement alone. The addition of anti-CD28 appeared to have minimal effect on all four chemokines, suggesting that A.E7 TCR engagement is sufficient for the maximum chemokine expression obtainable with this clone.

Because TCR stimulation alone resulted in the production of C-C chemokines, we were interested in determining which TCR-mediated signaling pathways were responsible. TCR engagement leads to activation of the Ca2+/calcineurin, NF-κB, and extracellular signal-related kinase (ERK) kinase pathways.

Because TCR stimulation alone resulted in the production of C-C chemokines, we were interested in determining which TCR-mediated signaling pathways were responsible. TCR engagement leads to activation of the Ca2+/calcineurin, ERK, NF-κB, and extracellular signal-related kinase (ERK) kinase pathways.

FIGURE 1. Up-regulation of C-C chemokine mRNA by CD4+ Th1 clones does not require costimulation. A, E7 cells were mock stimulated or stimulated with anti-TCR or anti-TCR and anti-CD28 for 8 h. The cells were then harvested and RNA was examined by Northern blot analysis.

FIGURE 2. MIP-1α production by CD4+ T cell clones does not require costimulation. A, E7 cells were mock stimulated, stimulated with anti-TCR or anti-TCR plus anti-CD28 for 16 h, and supernatant fluids were assayed by CTL-L for IL-2 production (A) or ELISA for MIP-1α production (B) and RANTES (C). All three cytokines were assayed from the same supernatant fluid.

Table II. Signal 1-induced MIP-1α production utilizes the Ca2+, ERK kinase, and NF-κB pathways

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No Drug</th>
<th>CSA, 1 μM</th>
<th>PS1, 2 μM</th>
<th>PD90859, 50 μM</th>
<th>PS1 + PD90859</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMA (0.25 ng)</td>
<td>19</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ionomycin (4 μM)</td>
<td>4,496</td>
<td>0</td>
<td>1,620</td>
<td>2,102</td>
<td>1,102</td>
</tr>
<tr>
<td>PMA + Ionomycin</td>
<td>192,000</td>
<td>27</td>
<td>30,858</td>
<td>18,306</td>
<td>6,034</td>
</tr>
<tr>
<td>Anti-TCR</td>
<td>135,500</td>
<td>59</td>
<td>45,734</td>
<td>9,775</td>
<td>4,135</td>
</tr>
</tbody>
</table>

* A.E7 cells were stimulated for 16 h as described above and supernatant fluid was assayed for MIP-1α production. All values are in pg/ml.
was consistently observed (3500 pg/ml ± 1400 in seven experiments) in spite of the fact that neither IL-2 nor RANTES (data not shown) was induced by ionomycin alone. In contrast, the addition of PMA and ionomycin together had a synergistic effect, virtually recapitulating the levels of MIP-1α secreted upon TCR engagement. These results were obtained with the optimal drug concentrations determined by dose-response curves for both reagents.

To further dissect the signaling pathways, specific inhibitors were utilized. Consistent with our observation that the Ca²⁺/CaM pathway is an important mediator of MIP-1α production, CSA completely inhibited MIP-1α production by either anti-TCR or PMA and ionomycin (Table II). This is in agreement with previous studies showing that human MIP-1α is CSA sensitive in Jurkat cells (28) and human CD4⁺ T cell clones (29) and FK506 sensitive in human CD4⁺ and CD8⁺ T cells (18). Interestingly, the MIP-1α gene has no characterized NF-AT sites in its proximal promoter (30, 31), and the expression of this gene is not CSA sensitive in macrophages (M. R. Horton, unpublished observations). The contribution of NF-κB proteins was assessed using PS1, an inhibitor of IκB degradation by blocking proteasome function (32), whereas the contribution of the mitogen-activated protein (MAP) kinase pathway was assessed using the drug PD90859, an inhibitor of mitogen-activated ERK kinase (MEK1/2) (33). Both PS1 and PD90859 markedly inhibited MIP-1α production (average, 69 and 75%, respectively, in 11 experiments; Student’s t test on log transformation, p < 0.001 and p < 0.002, respectively). Interestingly, the addition of both PS1 and PD90859 inhibited MIP-1α production by anti-TCR or PMA and ionomycin production down to levels comparable to that seen with ionomycin stimulation alone. The drugs, however, also significantly inhibited the production of MIP-1α in response to ionomycin alone (average, 69% inhibition in five experiments (p = 0.002) with PS1, and an average of 58% inhibition with PD90859 in four experiments (p = 0.06)). This suggests that basal levels of the NF-κB and ERK kinase pathways may be contributing to MIP-1α production in response to ionomycin alone. Thus, signal 1-induced MIP-1α production appears to be dependent on the Ca²⁺/calcineurin pathway as well as both ERK kinase signaling and NF-κB proteins for full stimulation.

**MIP-1α production in stimulated and anergized previously activated CD4⁺ T cells from TCR-transgenic mice mimics that of the T cell clones**

To generalize our findings to freshly isolated cells, CD4⁺ T cells were prepared by negative selection from splenocytes of B10.A 5CC7 TCR-transgenic Rag-2⁻/⁻ mice specific for the PCC peptide 81-104 in the context of I-Ek. As represented in Fig. 3A, negatively separated naive cells were 95% CD4⁺, CD44⁺, or CD44⁺. The small percentage of CD44⁺ cells were not CD4⁺ (data not shown). An aliquot of these cells was assayed as the naive population, while a portion was used for generating CD44⁺ cells in vitro (termed previously activated cells). The latter were generated by stimulating the naive cells with peptide and APC for 48 h. The cells were then expanded in 5 U/ml IL-2 and rested for 5–7 days before use. As seen in Fig. 3B, the activated population showed a shift in CD44 expression to mostly CD44⁺, confirming that they had been activated by Ag. Both the naive and previously activated cells were mock stimulated and stimulated with anti-TCR or anti-TCR and anti-CD28. Supernatant fluids were then analyzed for cytokine production.

As seen in Fig. 4, the CD44⁺ previously activated cells behaved similar to the A.E7 clones. Stimulation with anti-TCR alone resulted in minimal production of IL-2, whereas the addition of anti-CD28 greatly augmented this production (geometric mean, 48-fold increase in eight experiments; Student’s t test on paired log transformed data, p < 0.002). MIP-1α, however, was produced at comparable levels in the presence or absence of anti-CD28. The addition of costimulation had a minimal effect, increasing production by only 1.3-fold (geometric mean, eight experiments; p = 0.84). In addition, we examined the production of MIP-1α in previously activated 5CC7 cells induced into an anergic state. As seen in Fig. 4C, there is a profound decrease in IL-2 production upon restimulation, indicating that the cells are anergic. On the other hand, the anergic cells produced comparable levels of MIP-1α to their nonanergic counterparts (an average of only 1.6-fold decrease in four experiments; p = 0.59). Thus, previously activated TCR-transgenic cells and CD4⁺ memory cells in the form of Th1 clones have similar activation requirements for MIP-1α production.

**MIP-1α production by naive T cells**

The lack of a significant effect of CD28 costimulation on MIP-1α production by previously activated TCR-transgenic T cells and T
cell clones differs from the observations of Herold et al. (16). Their studies, however, were conducted using freshly isolated T cells and anti-CD3 or Ag as a stimulus. Therefore, to mimic their conditions, fresh naive T cells isolated from B10.A 5CC7 TCR-transgenic Rag-2−/− mice were stimulated as shown in Table III. Stimulation with PMA and ionomycin induced large amounts of MIP-1α as well as IL-2, demonstrating the ability of the naive T cells to produce both molecules. Likewise, stimulation of these cells by APC plus peptide resulted in significant production of MIP-1α and IL-2. Interestingly, the anti-CD3 alone induced as much MIP-1α as APC plus peptide. In this experiment, the addition of anti-CD28 led to a 2.9-fold increase. This ability of costimulation to enhance MIP-1α production was consistently observed, as seen by the kinetics experiment depicted in Fig. 5A and a third experiment shown in Fig. 5B. Overall, for six experiments, the mean fold augmentation produced by the addition of anti-CD28 was 5-fold (Student’s t test on log transformed data, p = 0.003). Even so, by contrast, the effect of costimulation on IL-2 production was much greater. For the experiment shown in Table III, the effect was 200-fold and for the experiment depicted in Fig. 5B the effect was 700-fold. For both experiments, the costimulatory effect of anti-CD28 was about 70-fold greater for IL-2 production than for MIP-1α production. Overall, these data demonstrate that under optimal stimulation conditions (48 h) anti-CD3 alone is sufficient to elicit large amounts of MIP-1α from naive T cells. In contrast to what is seen for T cell clones and previously activated cells, the addition of anti-CD28 can augment this production; however, this effect is small when compared with the costimulatory effect seen for IL-2 production.

**Discussion**

In emerging paradigms of immune activation, the decision as to whether a particular Ag will induce tolerance or immunity is not determined by the Ag itself, but rather the context in which the Ag is encountered (34, 35). In such models, activated APC will present Ag in the context of costimulation and promote immunogenic responses whereas resting APC will present Ag in the absence of costimulation, leading to tolerance. As such, cytokines that are strictly regulated by the presence or absence of costimulation might potentially play a critical role in dictating the ultimate outcome of Ag recognition. Thus far, based on our studies concerning IL-2, IFN-γ, IL-3, and TNF-α production for CD4+ Th1 clones, only IL-2 meets these requirements (36). In this paper, we found that for T cell clones and previously activated TCR-transgenic T cells, C-C chemokine production was not costimulation dependent. Indeed, anti-TCR stimulation alone resulted in large amounts of MIP-1α and MIP-1β production. In addition, MIP-1α was produced by both anergic and nonanergic cells in nearly equivalent amounts. Thus, MIP-1α follows the pattern of not requiring costimulation and not being inhibited in anergy. Although C-C chemokines clearly play a broad role in influencing the immune response, their production by signal 1 alone makes it unlikely that they play a critical role in the decision between a tolerogenic and an immunogenic response.

In our in vitro system, plate-bound anti-CD3 alone was able to induce substantial quantities of MIP-1α even in naive cells. This is in contrast to the observations of Herold et al. (16) who reported that anti-CD3 alone was not sufficient to induce MIP-1α production. In their experiments, however, MIP-1α production was only measured at 16 h. Although this time point provides optimal stimulation for T cell clones, we have found that naive T cells produce only one-tenth the amount of MIP-1α at 16 h that they produce at 48 h, even under optimal stimulation conditions (Fig. 5A). In addition, even at the early time point in our system, cells stimulated with anti-CD3 alone produced as much MIP-1α as cells stimulated with anti-CD3 + anti-CD28 in their system, perhaps indicating

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**Table III. MIP-1α production by naive T cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IL-2 (pg/ml)</th>
<th>MIP-1α (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>&lt;15.6</td>
<td>&lt;4.7</td>
</tr>
<tr>
<td>PMA + ionomycin</td>
<td>17,825</td>
<td>17,023</td>
</tr>
<tr>
<td>APC</td>
<td>&lt;15.6</td>
<td>26</td>
</tr>
<tr>
<td>APC + T cells</td>
<td>&lt;15.6</td>
<td>15</td>
</tr>
<tr>
<td>APC + T cells + peptide</td>
<td>13,021</td>
<td>3,049</td>
</tr>
<tr>
<td>Anti-CD3</td>
<td>286</td>
<td>5,040</td>
</tr>
<tr>
<td>Anti-CD3 + anti-CD28</td>
<td>57,605</td>
<td>14,534</td>
</tr>
</tbody>
</table>

* Freshly isolated 5CC7 T cells were stimulated for 48 h and supernatant fluid was harvested and assayed for IL-2 and MIP-1α.
that their stimulating conditions were not designed to induce maximal TCR stimulation. In this context, we would propose that in their experiments they were using a relatively naive population of T cells, and that under suboptimal stimulation conditions, the addition of anti-CD28 may have served to enhance the transduction of signal 1 as opposed to providing a distinct and necessary second signal. Likewise, when they added blocking Abs to CD28, they might have been decreasing ligand interactions involved in transducing signal 1. Such signaling could facilitate raft microdomain formation as has recently been described (37). We have also observed that the addition of anti-CD28 only enhances MIP-1α production in naive cells and not in previously activated T cells and T cell clones. Critically, the costimulatory enhancement of MIP-1α production by naive cells did not reflect an absolute requirement for costimulation. This is in contrast to IL-2 production which requires a distinct second signal, i.e., costimulation, as originally defined by LaFontoy and Cunningham (38). Notably, the two-signal requirement for the production of IL-2 holds for naive cells, previously activated cells, and T cell clones.

That MIP-1α, which does not require costimulation, is produced comparably by both anergic and nonanergic cells, fits with our previous observations on the similarities between the hierarchy of cytokine production with costimulation and in anergy. Anergy is characterized by a block in the MAP kinase pathway at the level of RAS (39, 40). It is presumed that this block leads to a decrease in the production and activation of AP-1 transcription factors, which in turn leads to a decrease in IL-2 production. In A.E7 T cell clones, however, TCR-induced MIP-1α production is PD90859 sensitive, suggesting that this transcription is dependent on the MAP kinase pathway. Thus, a paradox exists since MIP-1α production is minimally reduced in stimulated anergic T cells. One possible resolution is that the block in MEK1 activity in anergic cells is leaky such that the remaining activity of MEK1 is enough to drive transcription of MIP-1α but not IL-2. Another possibility is that PD90859 affects other unknown kinases that can lead to MIP-1α production but not IL-2 production. Finally, a third possibility, which we favor, is that the profound decrease in the production of IL-2 in anergic cells is not due solely to a block in the MAP kinase pathway, but the result of active repression at the level of the IL-2 promoter. Our laboratory has previously identified a site centered around the −180 region of the IL-2 promoter as a target for transcriptional repression (23). More recent studies showed that cAMP-responsive binding protein/cAMP-responsive modulator complexes bind to this site and promote the repression of transcription (25). Based on the observation that both IL-2 and MIP-1α are inhibited by PD90859, but only IL-2 is inhibited in anergy, we would argue that the mediators of transcriptional repression in anergy do not bind to the MIP-1α promoter.

The regulation of MIP-1α production is strikingly similar to the induction of anergy in T cells. Both T cell anergy and MIP-1α production are induced by TCR stimulation alone, as well as partially by stimulation with ionomycin alone. Both are inhibited by CSA. In addition, IL-2 prevents the induction of anergy and leads to its reversal, whereas IL-2 inhibits the low level of continuous production of MIP-1α seen by anergic cells. Finally, the long-lasting nature of T cell anergy suggests that the negative factors responsible for this state are constitutively expressed long after TCR stimulation is completed. Likewise, low levels of constitutive expression of MIP-1α are observed long after stimulation through the TCR. Although MIP-1α itself does not induce anergy (data not shown), delineating the pathways involved in the expression of MIP-1α may provide insight into the factors necessary for the maintenance of the anergic state.

A role for C-C chemokines is emerging in contributing to the pathogenesis of a number of autoimmune disorders. For example, murine experimental autoimmune encephalomyelitis is characterized by elevated levels of MIP-1α, MIP-1β, MCP-1, and RANTES in the CNS (41–43), whereas in autoimmune diabetes, secretion of C-C chemokines is thought to attract Th1 cells to pancreatic islets (44). Likewise, chemokine dysregulation has been found in patients with inflammatory bowel disease (45). Interestingly, inflammatory bowel disease is a prominent component of the autoimmunity seen in the IL-2 knockout mouse (46). In light of our observation that IL-2 shuts off the continuous expression of signal 1-induced MIP-1α, perhaps a component of the strong inflammation in the gut is due to dysregulation of chemokine production in the absence of IL-2. Finally, in light of the fact that anergic T cells produce MIP-1α, it may be that in the absence of costimulation, these T cells recruit other Ag-specific cells to become tolerized. In this case, to promote tolerance, a stimulated anergic T cell would attract Ag-activated T cells (which express C-C chemokine receptors) and/or APC to a particular site where they would be stimulated by signal 1 in the absence of costimulation. Such a mechanism might serve to promote linked suppression (47). It has recently been shown in a rat model that anergic T cells can suppress APC function (48). Thus, just as chemokine production by activated T cells might serve to attract cells to the sight of inflammation, chemokine secretion by anergic T cells may serve to suppress potential autoimmune disease.

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References

CHEMOKINE PRODUCTION BY NAIVE, ACTIVATED, AND ANERGIC T CELLS


