Here we show that CD40L (ligand for CD40) failed to induce the production of tumour necrosis factor α (TNF-α), interleukin (IL)-1β, IL-10 and IL-12 in macrophages matured in vitro in the absence of growth factors or in the presence of macrophage colony-stimulating factor (M-CSF). In contrast, enzyme-linked immunoabsorbent assay (ELISA) testing and cytofluorimetric (FACS) analysis demonstrated significant production of TNF-α and IL-1β, but not of IL-10 and IL-12 in macrophages matured in the presence of CD40L and re-stimulated with CD40L. The priming effect of CD40L on TNF-α and IL-1β production was related to induction of CD40 expression. Finally, CD40L priming did not modify the cytokine response of macrophages to lipopolysaccharide. In conclusion, our results suggest that CD40/CD40L interactions are important for the activation of macrophages as effector cells that mediate inflammation and tissue damage in T cell-mediated inflammatory processes.

CD40L-induced cytokine production

Figure 1 shows that at day 10 of culture no significant cytokine production could be induced by CD40L over the constitutive background level in macrophages cultured in the absence of growth factors (control macrophages) or in the presence of macrophage colony-stimulating factor (M-CSF) (M-CSF-primed macrophages). In contrast, significant release of tumour necrosis factor α (TNF-α) and interleukin (IL)-1β, but not of IL-10 and IL-12, was observed in macrophages cultured with CD40L (CD40L-primed macrophages).

The cytokine response of CD40L-primed macrophages to CD40L re-stimulation could be markedly inhibited by treatment from day 0 to day 9 with a neutralizing anti-human-CD40L antibody, but not by neutralizing anti-human granulocyte-macrophage colony-stimulating factor (GM-CSF) or anti-human...
Interestingly, CD40L-priming did not modify the pro-inflammatory cytokine response of macrophages to lipopolysaccharide (LPS) (data not shown).

FACS analysis of CD40L-induced TNF-α and IL-1β intracellular production

Analysis of cell supernatants demonstrated efficient secretion of TNF-α and IL-1β by CD40L-primed macrophages. Then, a time course FACS analysis was carried out to determine whether intracellular cytokine synthesis also took place in these cells. As shown in Figure 3, no TNF-α and IL-1β were found inside the cells before CD40L stimulation. Both the cytokines began to accumulate at 4 h and their intracellular levels increased at 24 h. Figure 3 also shows that the majority of the cells that stained positive for TNF-α and IL-1β after CD40L stimulation also expressed CD40.

Effect of CD40L on the expression of surface antigens

Finally, we addressed the issue of whether CD40L could induce the development of dendritic cells from blood monocytes. As shown in Table 1, after 10 days of culture, the percentage of CD40+ cells increased significantly in the CD40L-containing cultures compared to the control cultures. Additionally, the percentages of CD80+ and CD86+ cells also increased in the CD40L-containing cultures, indicating the maturation of dendritic cells.

Figure 2. Effect of neutralizing anti-human CD40L antibody on the cytokine response of CD40L-primed macrophages to CD40L stimulation.

CD40L was pre-incubated with an antagonistic mouse monoclonal anti-CD40L in a weight ratio of 1:1 for 30 min at 37°C and then used to stimulate the cultures. As alternative, 1 μg/ml antagonistic mouse monoclonal anti-human GM-CSF or 1 μg/ml anti-human IFN-γ were added to the cultures at day 0. The effect of these antagonistic antibodies on the cytokine response of macrophages to re-stimulation with CD40L was compared with that of CD40L alone. The data represent the mean of three experiments carried out with cells from three different donors. The error bars represent the standard errors.
of culture with CD40L, cells were CD14+, CD1a− and CD83+. In addition, cells had enhanced expression of CD40 as compared with control- and M-CSF-primed macrophages. Phenotypically, these cells were not consistent with dendritic cells.5,6

**TABLE 1. Expression of surface antigens on macrophages**

<table>
<thead>
<tr>
<th>Macrophage priming</th>
<th>CD14</th>
<th>CD40</th>
<th>CD1a</th>
<th>CD83</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>81 ± 18</td>
<td>&lt;2</td>
<td>3 ± 3</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>M-CSF</td>
<td>96 ± 11</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>CD40L</td>
<td>83 ± 7</td>
<td>34 ± 11</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

Note: The percentage of positive cells was assessed by FACS analysis at day 10 of culture. The data reported represent the arithmetic mean (±SD) of cells prepared from three to five different donors.

**DISCUSSION**

Here we show that CD40L is not able to induce a cytokine response in mature macrophages maturated in vitro in the absence of growth factors or in the presence of M-CSF. On the other hand, pro-inflammatory cytokine production could be obtained in macrophages cultured in the presence of CD40L and re-stimulated with CD40L. This is different from LPS activation which is known to induce a state of tolerance, where monocyctic cells do not adequately express cytokines when re-exposed to LPS.7 The possibility that the priming effect of CD40L on TNF-α and IL-1β production was not specifically due to the activating and trophic effect of CD40L is improbable since no modulation of cytokine production was observed in macrophages cultured in the presence of M-CSF, a very potent macrophage trophic and activating factor.8,9 Rather, the pro-inflammatory cytokine response of CD40L-primed macrophages appears to be related to the ability of CD40L to induce the expression of CD40 on macrophages. Indeed, in contrast with a previous report, we did not detect CD40 expression on unstimulated macrophages during in vitro maturation.2 However, in that study macrophages were cultured under non-adhering conditions, which may promote differentiation of monocytes into dendritic cells and consequently up-modulate CD40 expression.10

**Figure 3. FACS analysis of intracellular TNF-α and IL-1β production by CD40L-primed macrophages.**

The figure demonstrates a representative flow cytometric analysis of CD40L-primed macrophages stimulated by CD40L and analysed for the frequency of intracellular TNF-α and IL-1β. Macrophages were cultured in the presence or in the absence of 0.5 μg/ml CD40L. At day 9, the cultures were washed and re-fed with complete medium without CD40L, cultured for another 24 h and subsequently stimulated with 5 μg/ml CD40L. Thirty minutes after CD40L stimulation, 1 μg/ml of the protein transport inhibitor Brefeldin A was added. At different time points after Brefeldin A addition (0, 6 and 24 h), the cells were collected, stained and analysed by FACS. The data are displayed as dot plots. Macrophages were differentiated from lymphocytes and dead cells on the basis of forward angle and 90° scatter. Paired isotype-specific control antibodies were run with each sample. The quadrants were set according to the negative controls (less than 1% of the isotype control cells appeared positive). Five thousand cells were gated and analysed for each sample. The data refer to a typical experiment out of three performed with similar results.
Activation of macrophages by ligation of CD40 may represent a mechanism for potentiating the inflammatory response where the inflammation takes place. Indeed, repeated non-specific signalling to macrophages by activated T cells would result in both increased expression of CD40 and secretion of large amounts of pro-inflammatory cytokines. This, together with continuous antigen presentation, such as in autoimmune or chronic infectious diseases, may be in part responsible for chronic inflammatory lesions. On the other hand, the absence of a cytokine response of mature macrophages to CD40L could be a means to avoid recirculating activated T cells to cause inflammation and immune activation at sites distant from that of antigen presentation.

In contrast to TNF-α and IL-1β, production of IL-10 and IL-12 could not be induced in CD40L-primed macrophages. IL-10 has been described to act in a negatively autoregulatory fashion to limit the activation-induced pro-inflammatory state. In addition, IL-12 is known to benefit T-helper (Th)1 effector responses. This dichotomous effect suggests that CD40L selectively activates macrophages as effector cells that mediate inflammation and tissue damage in T cell-mediated inflammatory processes. Moreover, our findings indicate that macrophages may not play a critical role in the activation of Th1 T cells and are consistent with studies that have found that these cells have weak accessory cell activity.

### MATERIALS AND METHODS

**Compounds**

Soluble trimeric recombinant CD40L was provided by Immunex (Seattle, WA, USA). Recombinant M-CSF was kindly provided by Genetic Institute (Cambridge, MA, USA). LPS from *Escherichia coli* 0111B4 was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

**Cells**

Monocytes (>90% pure) were obtained by elutriation as previously described. The cells were cultured in RPMI-1640 medium supplemented with 20% heat-inactivated fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, with or without 0.5 μg/ml CD40L or 500 U/ml M-CSF, at 37°C in a humidified atmosphere of 5% CO₂ in air, in 48-well plates at a concentration of 5 x 10⁵ cells/well/ml.

**Limulus amoebocyte lysate test**

All the samples analysed were found free of endotoxin contamination (less than 0.1 EU/ml) by the limulus amoebocyte lysate test (QCL-1000, BioWhittaker, Inc., Walkersville, MD, USA).

### Assessment of cell viability

Propidium iodide staining and flow cytometric analysis was employed to quantify the percentage of live and necrotic cells, as previously described.

### Enzyme-linked immunoabsorbent assay (ELISA)

Commercially available sandwich ELISA kits (R&D Systems, Minneapolis, MN, USA) were used to determine the concentration of TNF-α, IL-1β, IL-10 and IL-12.

### FACS analysis

The procedure to quantify surface-antigen expression and intracellular cytokine production by FACS have been extensively described. The cells were stained with the following mouse anti-human monoclonal antibodies: fluorescein-conjugated (FITC) anti-CD40, anti-CD14 or phycoerythrin-conjugated (PE) anti-CD1a, anti-CD83, anti-human TNF-α and anti-human IL-1β (all from Immunotech, Marseille, France).

### Statistics

Student’s t-test was used to analyse data.

### REFERENCES


