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MONOKINE PRODUCTION BY HUMAN T CELLS; IL-1α PRODUCTION RESTRICTED TO MEMORY T CELLS[1]

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The production of cytokines is a key event of inflammation. In this report we demonstrate that normal human T cells are capable to produce IL-1α, IL-6, and TNF-α, cytokines formerly considered to be monokines. This production was optimal after stimulation with a combination of anti-CD2, PMA, and anti-CD28. All three cytokines were produced in a bioactive form. Both naive (CD4$^+$CD45RA$^+$) and memory (CD4$^+$CD45RO$^+$) subsets of T cells were shown to produce similar amounts of IL-6 and TNF-α. In contrast the production of IL-1α was found to be completely restricted to the CD4$^+$CD45RO$^+$ subset. The finding that T cells are such potent producers of these important mediators of the inflammatory response might be a key observation in the appreciation of the role of T cells in chronic inflammation.

TNF-α, IL-6, and IL-1 are important mediators of the inflammatory response. Monocytes stimulated by bacterial products are considered to be the main producers of these monokines, but nowadays a long list of potential cell sources has been reported (1–4). Concerning monokine production by human T cells, some reports indicate that human peripheral T cells can produce TNF-α (5, 6) or IL-6 (only in the presence of monocytes) (7).

Stimulation via CD28 gives a strong enhancement of IL-2 production by activated human T cells (5, 8). The effect of this CD28 stimulation, which has been reported to be due to stabilization of mRNA, is not restricted to the IL-2 gene, but has also been shown for cytokine genes like IFN-γ, granulocyte-macrophage-CSF, and TNF-α (9). Using a recently described anti-CD28 antibody (10), we investigated the effect of this antibody on the production of IL-1, IL-6, and TNF-α by highly purified T cells.

Furthermore we investigated whether the IL-1, IL-6, or TNF-α production by T cells could be attributed to a subset of human T cells. In human T cells a functional subdivision within the CD4$^+$ helper subset has been suggested (11). We investigated whether the CD4$^+$CD45RA$^+$ (naive) and CD4$^+$CD45RO$^+$ (memory) T cells differed in their ability to produce IL-1, IL-6, or TNF-α.

MATERIALS AND METHODS

Lymphocyte purification. Monocytes and lymphocytes were isolated from peripheral blood using counterflow elutriation techniques (12). Monocyte fractions contained >90% CD14$^+$ cells. T cells, B cells, and T cell subsets were isolated from the lymphocyte fraction by depletion with magnetic beads (Dyna-beads M450, Dynal, Oslo, Norway), using an appropriate cocktail of mAb. In all cases NK cells and residual monocytes were removed using CLB-FcR granl and CLB-CD14 respectively. T cells (CD2$^+$) were depleted using CLB-T11.1/1 resulting in a B cell population with >90% CD19$^+$ cells. B cells (CD19$^+$) were depleted using CLB-CD19 yielding >97% CD3$^+$ cells and <1% CD14$^+$ cells as determined by flow microfluorimetry analysis. For T cell subsets additional antibodies were CLB-T8/4 (anti-CD8) and UCHL1 (anti-CD45RO; a kind gift of P. Beverley, The Courtland Institute of Biochemistry, London, UK) for the CD4$^+$CD45RA$^+$ cells, or CLB-T8/4 and 2H4 (anti-CD45RA; Coulter Immunology, Hialeah, FL) for the CD4$^+$CD45RO$^+$ cells. Both populations were >95% pure and contained no detectable CD14$^+$ cells.

Lymphocyte cultures. All cells were cultured in Iscove's modified Dulbecco's medium supplemented with 5% FCS, penicillin, streptomycin, and 5×10^{-5} M 2-ME at a concentration of 2×10^5 cells/ml in 0.5 ml wells (Costar, Cambridge, MA). Supernatants were harvested after 65 h of culturing and tested for cytokine production. For stimulation of the cells we used combinations of anti-CD2 antibodies (CLB-T11.1/1 and CLB-T11.2/1 (8), both at a concentration of 1/2000 diluted ascites), PMA (Sigma Chemical Co., St. Louis, MO) at a final concentration of 2 ng/ml and anti-CD28 antibodies (CLB-CD28/1 at a final concentration of 5 μg/ml).

Detection of IL-1, IL-2, IL-6, and TNF-α in culture supernatants. IL-2 production was measured as previously described in detail by Gillis et al. (13) using the IL-2-dependent T cell line CTLL-2 and hrIL[3]-2 (a gift of J. Farrar, Hoffman-La Roche, Nutley, NJ) as a reference curve. IL-1 production was measured using the T cell line D10(N4)M as described in detail by Helle et al. (14). This assay is specific for IL1 and can detect both IL-1α and IL-1β. RhIL-1β (Hoffman-La Roche) was used as a standard curve. IL-6 production was measured using the hybridoma growth factor assay B9 as described in detail before (3) and with hrIL-6 (15) as a reference curve. TNF-α production was determined using a TNF-α specific ELISA. For this ELISA mAb 18b, anti-hrTNF-α was used as a coating and a rabbit polyclonal anti-TNF-α antibody as a second step (both were a gift of M. Brockhaus, Hoffman-La Roche, Basel, Switzerland). Horseradish peroxidase labeled horse anti-rabbit Ig (CLB-PK-17E) was used as a conjugate. Some supernatants were tested for biological activity using the TNF sensitive WEHI assay (16). This assay is sensitive for both TNF-α and TNF-β. For both assays rhTNF-α (provided by A. Creasy, Cetus Corp., Oakland, CA) was used as a standard. Specificity controls in the cytokine assays mentioned above were performed using an anti-hrIL-1α mAb (28G(3B1); provided by T. Kasahara (Jichi Medical school, Tochigi-ken, Japan) (17); 1/1000 diluted ascites fluid), a polyclonal anti-rhIL-1β antiserum (provided by J. van Damme (Rega Institute, Heuven, Belgium) (18); 1/2000 dilution), affinity purified sheep anti-hrIL-6 antibodies (final concentration 1 μg/ml), anti-hrTNF-β antibodies (a gift of A. Meager (National Biological Standards Board, Hertfordshire, UK)) and the mAb 18b, anti-

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[3] Abbreviation used in this paper: hrIL, human rIL.

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hrTNF-α respectively. Identity of IL-1α was confirmed in an ELISA specific for human IL-1α, using anti-hrIL-1α mAb as a coating and polyclonal rabbit anti-hrIL-1α antiserum as a second antibody (both were a kind gift of C. Rordorf-Adam, Ciba-Geigy, Basel, Switzerland) (19).

RESULTS

Effect of anti-CD2, PMA, and anti-CD28 on production of biologically active IL-2, IL-1, IL-6, and TNF-α by human T cells. No IL-2 production was found when either CD2, PMA, or CD28 were used as the only stimulus for T cells (Fig. 1*A*). As reported before, IL-2 production was induced when two stimuli were combined (5). Even stronger IL-2 production was found using a combination of CD2, PMA, and CD28. Instead of anti-CD2, also anti-CD3 antibodies could be used, although this resulted in a somewhat lower response (data not shown). In addition to the effects on IL-2 production we also determined the production of IL-1 (Fig. 1*B*), IL-6 (Fig. 1*C*), and TNF-α (Fig. 1*D*) in these T cell supernatants. Our experiments show that T cells can be stimulated to produce high levels of IL-1, IL-6, and TNF-α. Interestingly the production seemed to be differentially regulated, because IL-1 and IL-6 production were less dependent of the CD2 signal than the production of IL-2 and TNF-α. Furthermore after 24 h of culture the IL-1 and IL-6 production was only 2% of the production found after 3 days, whereas the IL-2 and TNF-α production was already 20% (data not shown).

Because IL-6 and IL-1 were tested in a bioassay, we confirmed the identity of these factors by inhibition with specific antibodies. T cell-derived IL-6 activity in the B9 hybridoma growth factor assay could be completely inhibited by polyclonal anti-IL-6 antibodies (data not shown). Inasmuch as two forms of IL-1 exist, the activity in the D10 assay was tested in the presence of both anti-IL-1α and anti-IL-1β antibodies. T cell-derived IL-1 could only be inhibited by anti-IL-1α antibodies (Fig. 2*A*), whereas these antibodies did not cross-react with human IL-1β (Fig. 2*B*) or with mouse IL-1α (17). A strong neutralizing antiserum against IL-1β (Fig. 2*B*) showed no inhibitory effect on T cell-derived IL-1 activity (Fig. 2*A*). This indicates that human T cells specifically produce IL-1α. Inasmuch as it is reported that D10 cells may contain intracellular mouse IL-1α after stimulation with Ag-presenting B cells (20, 21), we confirmed the IL-1α data using a human IL-1α-specific ELISA. As shown in Figure 3, similar amounts of IL-1α were detected in both D10 assay and human-IL-1α ELISA.

The TNF-α produced, as measured by ELISA, was found to be biologically active as a cytotoxic factor for WEHI 164-13 cells (data not shown). The TNF activity in T cell supernatants could only be inhibited by a combination of anti-TNF-α and anti-TNF-β antibodies, indicating that both TNF-α and TNF-β are produced in a bioactive form by activated human T cells.

Monokine production by human T cells is not due to contaminating non-T cells. To exclude that the cytokines measured were produced by contaminating cells, we compared the regulation of IL-1, IL-6, and TNF-α production among purified monocytes, B cells, and T cells. In the culture medium used, monocytes showed a high IL-1, IL-6, and TNF-α production without any additional stimulus (Fig. 4). This "spontaneous" production is caused by contaminating LPS or LPS-like substances in both FCS and Iscove's modified Dulbecco's medium (22). Inasmuch as T cells showed no spontaneous response (Fig. 4), we performed all experiments under conditions that were stimulatory for monocytes. Control experiments showed that the T cell responses were not influenced by the presence of LPS (data not shown).

Stimulation with a combination of CD2, PMA, and CD28 induced IL-1, IL-6, and TNF-α production by T cells. IL-1 and IL-6 production by monocytes was inhibited by this combined stimulation. Finally, monocytes produced both IL-1α and IL-1β, whereas T cells only produced IL-1α. Under no condition tested any significant cytokine production by B cells was found (data not shown).

IL-1α, IL-6, TNF-α, and IL-2 production by $CD4^+CD45RA^+$ and $CD4^+CD45RO^+$ subsets of human T cells. We tested the production of IL-1α, IL-6, TNF-α, and IL-2 by $CD4^+CD45RA^+$ (naive) and $CD4^+CD45RO^+$ (memory)

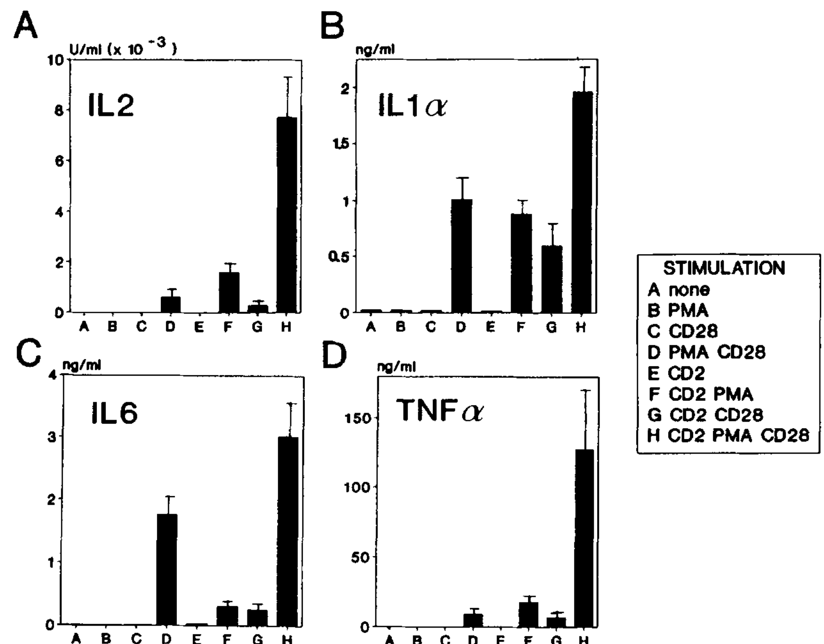

Figure 1. The effect of addition of anti-CD2, PMA, and anti-CD28 (for concentrations see *Materials and Methods*) on the production of IL-2, IL-1α, IL-6, and TNF-α by purified human T cells. Additions were as indicated. Supernatants were harvested after 3 days of culture and tested in the CTLL, D10, B9, and TNF-α ELISA, respectively. Results shown are the mean (± SE) of five experiments.

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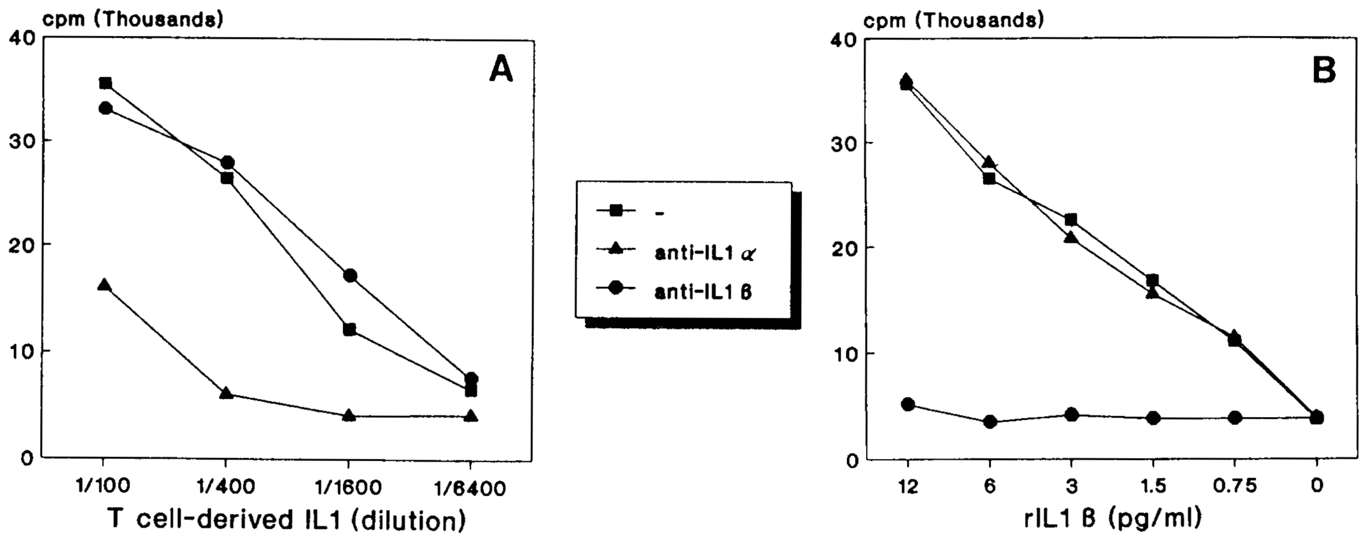

Figure 2. Inhibition of T cell-derived IL-1 in the D10 assay. *A*, T cell supernatant of 40000 peripheral T cells, stimulated during 72 h with anti-CD2, PMA, and anti-CD28, as indicated in *Material and Methods*, was tested in the D10 assay. To determine the identity of this T cell IL-1, we added mAb against human IL-1α, or a polyclonal antiserum against human IL-1β. *B*, The same antibodies were tested in combination with rIL-1β to show the specificity of the anti-IL-1α antibodies and the neutralizing effect of the anti-IL-1β antiserum.

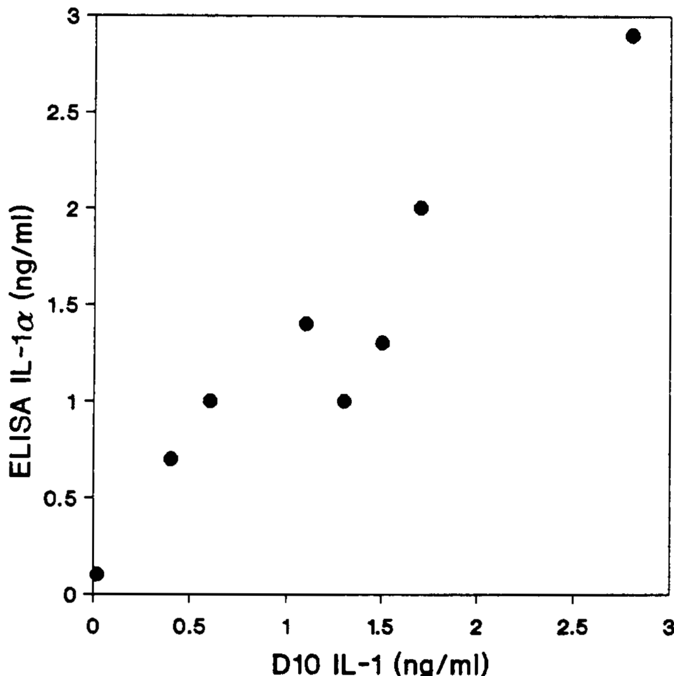

Figure 3. Comparison of T cell-derived IL-1α as measured by D10 assay or by a human IL-1α-specific ELISA. Eight representative supernatants, which were included in the results of Figure 1*B* or Figure 5, were tested in the D10 assay and the IL-1α-specific ELISA. Results were related to an IL-1α standard curve. rIL-1β showed no reactivity in the IL-1α ELISA.

subsets of human T cells. T cells were stimulated with combinations of CD2, PMA, and CD28. The result is shown in Figure 5. Under these conditions IL-2 production was induced in both subpopulations, although more efficient in $CD4^+CD45RA^+$ cells. These subsets showed no difference in IL-6 or TNF-α production. A striking difference was found for the production of IL-1α. Whereas $CD4^+CD45RO^+$ T cells were potent producers of IL-1α, we were not able to induce significant IL-1 production by $CD4^+CD45RA^+$ T cells.

DISCUSSION

In this study we described the production of IL-1α, IL-6, and TNF-α by normal human T cells. All cytokines were found to be biologically active. There are several arguments that rule out the possibility that these monokines were produced by contaminating monocytes. 1) The low amount of contaminating monocytes as shown by flow microfluorimetry analysis (<1%) and the absence of a LPS-driven spontaneous cytokine production. 2) The fact that a combination of CD2, PMA, and CD28 inhibits the IL-1 and IL-6 production by monocytes, whereas stimulating the production by T cells. 3) Kinetic studies showing that monokine production by monocytes is complete within 24 h of culturing (3) and therefore faster than production by T cells. 4) The amount of TNF-α produced by T cells (200 ng/ml) compared with the production by monocytes (5 ng/ml). 5) The production of IL-1β by monocytes vs the production of IL-1α by a subset of T cells. It could be excluded that the IL-1α was an artefact of the IL-1 measurement (D10 assay), because the activity could be blocked by monoclonal antibodies against human IL-1α, which not cross-reacts with mouse IL-1α (17). Furthermore similar amounts of IL-1α were detected using a specific ELISA (Fig. 3). Recently, another group observed specific IL-1α mRNA in peripheral T cells after stimulation with a combination of anti-CD2 and anti-CD28 antibodies (22a). The specific IL-1α production is in accordance with a previous report showing IL-1α mRNA in a series of T cell leukemic cell lines (23). A major question concerns the significance of monokine production by T cells. IL-1, IL-6, and TNF-α are important mediators of the inflammatory response. Production by monocytes is induced by bacterial products such as LPS and muramyl dipeptide (1, 2, 4). We would like to hypothesise that monokine production by T cells is important in chronic diseases, such as rheumatoid arthritis, and not during acute inflammation. This hypothesis is supported by the prolonged IL-1α mRNA expression in rheumatoid arthritis (24), whereas no IL-1α (but high levels of IL-1β) was detected in a model of acute inflammation (25). The reg-

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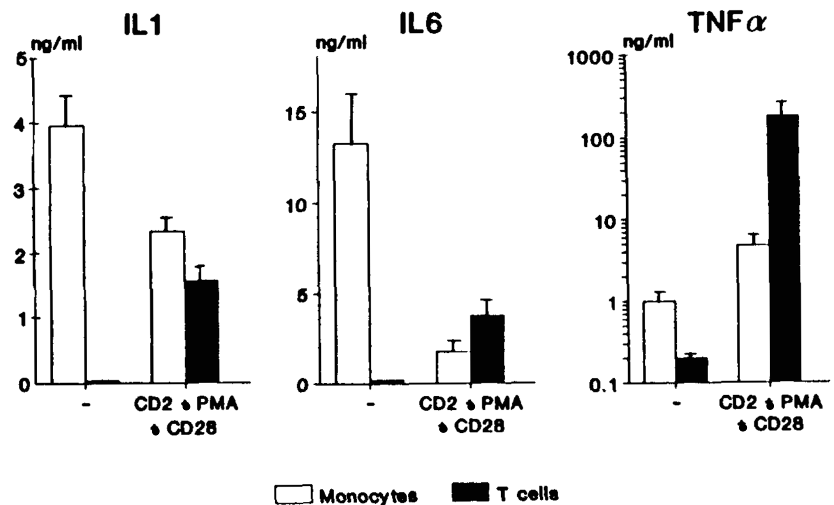

Figure 4. A comparison of IL-1, IL-6, and TNF-α production by purified monocytes and T cells. Supernatants were harvested after 3 days of culture and tested as described before. Results shown are the mean (± SE) of three experiments.

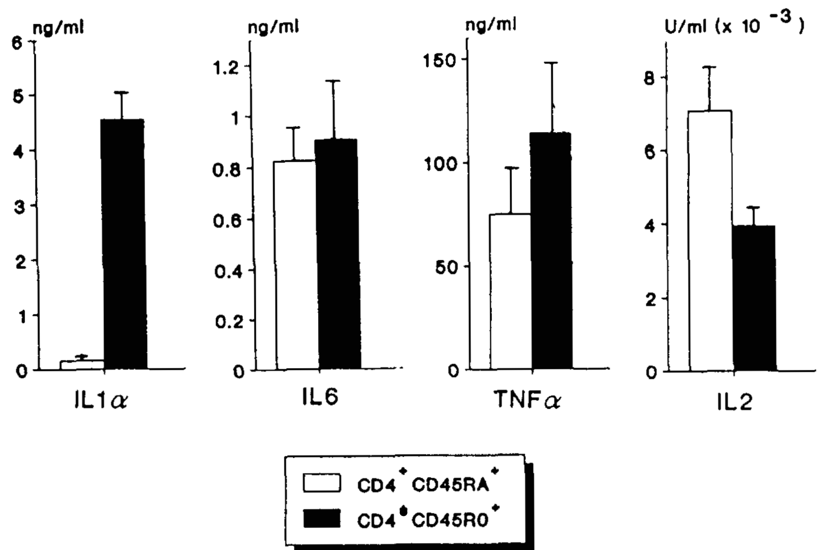

Figure 5. Production of IL-1α, IL-6, TNF-α, and IL-2 by $CD4^+CD45RA^+$ and $CD4^+CD45RO^+$ subsets of T cells. T cell subsets were purified as described in *Materials and Methods*. T cells were activated with a combination of CD2, PMA, and CD28 and supernatants were harvested at day 3 of culture. Results shown are the mean (± SE) of four experiments.

ulation of IL-1 and IL-6 production is different from the regulation of IL-2 and TNF-α production. IL-1 and IL-6 showed a delayed production compared to IL-2 and TNF-α. Furthermore the production of IL-1 and IL-6 is much less dependent of a Ca^{2+} signal, such as CD2 antibodies, because the combination of PMA and CD28 antibodies induce almost maximal levels of IL-1α and IL-6. CD28 stimulation is reported to cause stabilization of some mRNA (9). However, recent studies in our laboratory, using the 5′ flanking region of the IL-2 gene, indicate that CD28 also acts by a direct activation of transcription via a NF-κB-like site (C. L. Verwey, et al., manuscript in preparation). Most interestingly, recently functional NF-κB sites have been described in the regulating sequences of both the IL-6 and TNF-α gene (26, 27).

An intriguing observation is that IL-1α production is restricted to the $CD45RO^+$ memory subset of $CD4^+$ T cells, whereas both memory and naive cells produce similar amounts of IL-2, IL-6, and TNF-α. IL-1 production by memory T cells might lead to a lower stimulation threshold by APC or might allow non-IL-1-producing cells such as B cells to become efficient stimuli. It remains to be investigated how this relates to the observed differences in functional capacity between naive and memory T cells (11). That CD28 may play an important role in immune responses in vivo has become evident by the finding that BB1, an activation Ag of B cells, is the ligand for CD28 (28). This means that in a local immune response, activated B cells can provide a strong costimulus to surrounding T cells to produce a wide variety of cytokines, including monokines.

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