

## Influence of CD28 co-stimulation on cytokine production is mainly regulated via interleukin-2

H. M. KUIPER, R. DE JONG, M. BROUWER, K. LAMMERS, J. WIJDENES\* & R. A. W. VAN LIER  
Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology of the University of Amsterdam, the Netherlands, and \*Innotherapie laboratoires, Besançon, France

### SUMMARY

Interaction of CD28 with its ligand B7 plays an important role in the initiation of immune responses. The co-stimulatory signal generated by cross-linking of CD28 molecules results in enhanced T-cell proliferation and augmentation of cytokine production. In particular, mRNA levels of T-helper 1 (Th1)-type cytokines, such as interleukin-2 (IL-2) and interferon- $\gamma$  (IFN- $\gamma$ ) are reported to be strongly increased. We investigated the effect of CD28 co-stimulation on the production of Th2-type cytokines. CD28 mAb induced a strong augmentation of IL-2 secretion in activated T-cell clones. Production of IFN- $\gamma$  was also enhanced, but the increase in IL-4 secretion was generally moderate. Augmentation of IL-4 production by CD28 was most pronounced in clones that produced low amounts of IL-2, compared to clones producing high levels of IL-2. It was found that the up-regulation of IL-4 by CD28 co-stimulation was mainly controlled indirectly via an increase of IL-2. Some clones could produce IL-4 in an IL-2-independent manner; in these situations CD28 co-stimulation had no augmenting effect on the production of IL-4. The secretion of IL-4 by peripheral blood CD4<sup>+</sup> T cells, that were activated with B7-expressing transfectants, was also found to be dependent on IL-2. Finally, Northern blot analysis confirmed that co-stimulation of CD28 primarily affected IL-2 production, and that inhibition of IL-2/IL-2 receptor interaction abolished the augmenting action of CD28 monoclonal antibody on the production of the Th2-type cytokines IL-4, IL-5 and IL-10 and of the Th1 cytokine IFN- $\gamma$ .

### INTRODUCTION

During specific recognition of antigen, T lymphocytes are stimulated through the interaction with antigen-presenting cells (APC). At least two different signals are required for activation of T cells.<sup>1,2</sup> The first signal, which is provided by ligation of the T-cell receptor (TCR)/CD3 complex by binding to major histocompatibility complex (MHC)/peptide, warrants antigen specificity. The second signal is induced by interaction of non-polymorphic co-stimulatory molecules on APC cell surfaces with T-cell surface molecules. Such a co-stimulatory signal can be generated by interaction of B7 (CD80) or the recently cloned B7-2 with their ligand CD28.<sup>3–6</sup> Expression of B7 or B7-2 can be induced on several APC, such as B cells and monocytes, and is constitutively expressed on dendritic cells.<sup>5–10</sup> Both molecules are also expressed on activated T lymphocytes.<sup>5,11</sup> *In vivo* studies have demonstrated the importance of B7/CD28 interactions in immune responses. It was reported that melanoma cell lines transfected with the B7 gene were able to

induce an efficient immune response *in vivo*, resulting in tumour rejection, whereas the non-transfected cells did not lead to appropriate activation of the immune system.<sup>12,13</sup> In addition, disruption of the interaction between B7 and CD28 during allograft transplantation was shown to suppress the immune response, or even resulted in peripheral tolerance.<sup>14,15</sup> Until now the most prominent effect of CD28 co-stimulation documented has been the potent augmentation of cytokine secretion. Thompson *et al.*<sup>16,17</sup> reported that ligation of CD28 molecules enhances, by stabilization, the levels of mRNA of interleukin-2 (IL-2), tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), lymphotoxin (LT), interferon- $\gamma$  (IFN- $\gamma$ ) and granulocyte-macrophage colony-stimulating factor (GM-CSF). At the level of transcription, regulation by CD28 has been described for the IL-2 gene.<sup>18,19</sup>

At least two major T-helper subsets have been described in mice, Th1 cells, producing IL-2 and IFN- $\gamma$  and Th2 cells, producing IL-4, IL-5, IL-6 and IL-10.<sup>20</sup> For the human system the division between Th1 and Th2 cells is only found in certain immunopathological conditions.<sup>21,22</sup> Increase of IL-2 production, induced by CD28/B7 interaction, has been described for freshly isolated murine as well as human lymphocytes.<sup>23–26</sup> Whether co-stimulation of CD28 also influences the production of Th2-type cytokines (IL-4, IL-5 and IL-10) in these cells is not very clear, since production of IL-4 is more difficult to detect in bulk populations.<sup>23–25</sup> Nevertheless it has been shown

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Correspondence: Dr R. A. W. van Lier, Department of Clinical Viro-Immunology, Central Laboratory of the Netherlands Red Cross Blood Transfusion Services, Plesmanlaan 125, 1066 CX Amsterdam, the Netherlands.

that stimulation of peripheral blood lymphocytes (PBL) via CD2 in combination with CD28 monoclonal antibody (mAb) is one of the most efficient activation methods for induction of IL-4. However, this may be an indirect effect since replacement of the CD28 mAb by rIL-2 also resulted in high amounts of IL-4 secretion.<sup>27</sup>

Here we investigate whether CD28 co-stimulation can directly regulate the production of IL-4, or whether, indirectly, the increase of IL-2 is responsible for efficient IL-4 production.

## MATERIALS AND METHODS

### Monoclonal antibodies

CLB-T11.1/1 (IgG1), CLB-T11.2/1 (IgG1) and CLB-Hik27 (IgG1), directed against CD2, form a mitogenic triplet in the absence of accessory cells.<sup>28,29</sup> These mAb, and the mAb specific for CLB-CD28 (CD28, IgG1), CLB-IL-2R/1 (CD25, IgG2b), 2F2 (CD70, IgG1) and 5B5 (IL-4), were produced in the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, the Netherlands). BG-5, which recognizes IL-2, was generated at Innotherapie laboratoires (Besançon, France), while B7-24 (IgG2a) was generously provided by M. de Boer.<sup>30</sup> For the purification of CD4<sup>+</sup> memory cells, the following mAb were used: CD16 (CLB-FcR gran1, IgG2a), CD14 (CLB-CD14, IgG2a), CD19 (CLB-CD19, IgG1), HLA-DR (E1, IgG1), CD8 (CLB-T8/4, IgG2b) and CD45RA (2H4) (purchased from Coulter Immunology, Hialeah, FL).

### Cell lines

3T6-FcγRII and 3T6-FcγRII/B7 were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% heat-inactivated fetal calf serum (FCS) and antibiotics, thymidine (5 μg/ml), xanthine (10 μg/ml), hypoxanthine (15 μg/ml), mycophenolic acid (20 μg/ml) and deoxycytidine (2.3 μg/ml). Selection for the B7 gene was performed by addition of 400 μg/ml G418 (Gibco, Grand Island, NY).<sup>30</sup> Cells were collected weekly by incubation with 8 mM EDTA in phosphate-buffered saline (PBS), and diluted for further culture.

### Cell separations

Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Isopaque (d = 1.078) density centrifugation of buffy coats from healthy blood bank donors. Lymphocytes were isolated by counterflow centrifugation elutriation. Lymphocytes were incubated for 30 min at 4° with saturating concentrations of mAb directed against CD16, CD14, CD19, HLA class II, CD8 and CD45RA. After depletion of labelled cells by immunomagnetic beads (Dynabeads-M450; Dynal AS, Oslo, Norway), a population of >92% CD4<sup>+</sup> CD45RO<sup>+</sup> was obtained.

### Culture of T-cell clones

T-cell clones were derived from purified CD4<sup>+</sup> CD45RO<sup>+</sup> cells from one donor. Cells were cultured under limiting dilution conditions (0.3 cells/well) in the presence of phytohaemagglutinin (PHA; 1 μg/ml; Wellcome, Beckenham, UK) and irradiated (30 Gy) allogeneic PBMC (10<sup>6</sup>/ml) in IMDM supplemented with 10% human pooled serum (HPS), 20% IL-2-containing medium (concanavalin A supernatant) and antibiotics.<sup>31</sup> Clones were maintained by weekly passage. Cells (0.25 × 10<sup>6</sup>/ml) were stimulated with irradiated (30 Gy)

allogeneic human PBMC (10<sup>6</sup>/ml), PHA (1 μg/ml), 20% IL-2-containing medium, antibiotics and 10% HPS in IMDM. For studying cytokine production, clones were harvested 7 days after restimulation. At this time of culturing no feeder cells were present anymore and no proliferation could be measured.

### Proliferation assays

T-cell clones (5 × 10<sup>4</sup>/0.2 ml) were cultured in flat-bottomed 96-well microtitre plates (Nunc, Roskilde, Denmark) in IMDM supplemented with 10% HPS and antibiotics. Clones were stimulated with phorbol myristate acetate (PMA; CMC Cancer Research, Katonah, NY; 1 ng/ml) and the triplet of CD2 mAb (CLB-T11.1/1, CLB-T11.2/1, CLB-Hik27) in the presence or absence of CD28 mAb (CLB-CD28). All mAb were used at a final concentration of 5 μg/ml. Where indicated, human recombinant (r)IL-2 (50 'Cetus' U/ml, equivalent to 60 pg/ml; generously provided by Sandoz, Vienna, Austria) was added. Proliferation and production of CD4<sup>+</sup> memory T cells was performed in IMDM containing 10% inactivated FCS and antibiotics. For activation, the CD2 triplet and irradiated (6000 rads) mouse fibroblasts transfected with CD32 (3T6-FcγRII) or with CD32 and B7 (3T6-FcγRII/B7) were added (5000 cells/well). B7-24 was used to block specifically the interaction between CD28 and B7, as irrelevant mAb 2F2, directed against CD70, was applied. Proliferation was measured by [<sup>3</sup>H]thymidine ([<sup>3</sup>H]TdR) incorporation. For this purpose 0.2 μCi, 7.4 kBq, [<sup>3</sup>H]TdR was added on day 4. After 4 hr [<sup>3</sup>H]TdR incorporation was measured. Results are given as the mean of triplicate cultures. SE of the mean was less than 10%.

### Cytokine production

Triplicate cultures of T-cell clones were stimulated as described in the proliferation assay. IL-2 production was measured 20 hr after stimulation, by induction of proliferation of the IL-2-dependent murine CTLL-2 cell line.<sup>32</sup> One unit is defined as the amount of IL-2 needed to induce half maximal proliferation of these cells. An ELISA was used for determination of IL-4 produced after 3 days of stimulation.<sup>27</sup> Production of IFN-γ was measured by an ELISA purchased from Endogen (Boston, MA).

### Northern blot analysis

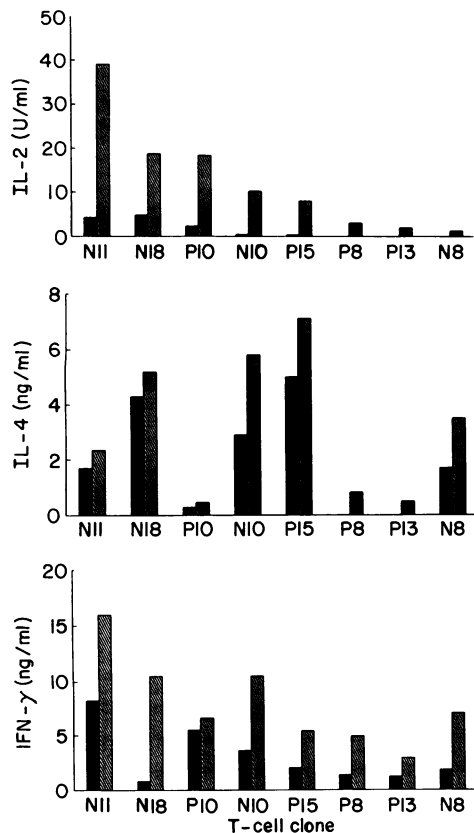
Total RNA of stimulated and unstimulated T-cell clones (10 × 10<sup>6</sup>) was isolated with the RNazol<sup>TM</sup> Method (Cinna/Biotex, Friendswood, TX). Of each sample, 10 μg RNA was subjected to electrophoresis on a formaldehyde agarose (1.2%) gel and transferred to nylon filters (GeneScreen, Dupont, Boston, MA).<sup>33</sup> Blots were sequentially hybridized with cytokine-specific probes. Labelling of probes was done with a random primer kit (Boehringer Mannheim, Mannheim, Germany). A 0.8 kb *Pst*I fragment of the human IL-2 gene, kindly provided by H. Pannekoek, (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service), was used for detection of IL-2 mRNA. cDNA clones of the other cytokines were generously given by Rob Kastelein (DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA). The following fragments were used for hybridization: IL-4 *Nhe*I-*Eco*RI (0.3 kb), IL-5 *Bam*HI-*Bam*HI (1.0 kb), IFN-γ *Bam*HI-*Bam*HI (1.2 kb), IL-10 *Bgl*II-*Hind*III (0.8 kb).<sup>34-36</sup> Autoradiography took place at -70°, by using Kodak XAR-5 film in combination with intensifier screens.

## RESULTS

**Ligation of CD28 molecules results in enhanced cytokine secretion**

We investigated the influence of ligation of CD28 molecules on the production of different (Th1- and Th2-related) cytokines by a panel of human T-cell clones. Figure 1 shows the production of IL-2, IFN- $\gamma$  and IL-4 by these clones, which were activated via CD2 and PMA in presence or absence of CD28 co-stimulation. The T-cell clones were ranked according to their capacity to produce IL-2, starting with the highest producers. Co-stimulation of CD28 molecules greatly enhanced IL-2 production (up to 10 times) in all clones, whereas IL-4 production was only moderately influenced (two to three times). CD28 ligation had an intermediate effect on the production of IFN- $\gamma$ .

In all experiments, clones that had a high capacity to produce IL-2 (N11, N18 and P10) showed hardly any augmentation of IL-4 secretion after CD28 mAb stimulation. However, clones that produced undetectable or low levels of IL-2 displayed a more pronounced induction of IL-4 secretion after addition of CD28 mAb. These findings suggested to us



**Figure 1.** Enhancement of interleukin production of T-cell clones induced by CD28 co-stimulation. Clones were stimulated with PMA (1 ng/ml) and a mitogenic combination of CD2 mAb (CLB-T11.1/1, CLB-T11.2/1, CLB-Hik27) in the absence (black bars) and presence (hatched bars) of CD28 mAb (CLB-CD28). IL-2 production was measured 20 hr after stimulation; IL-4 and IFN- $\gamma$  were determined 72 hr after stimulation. Data are shown as the mean of four experiments. SEM was <10%.

**Table 1.** Effect of addition of rIL-2 on IL-4 production

Clone	IL-2 production (U/ml)	% increase IL-4 (50 U/ml rIL-2 added)	
		CD2/PMA	CD2/PMA CD28
N18	28	150	90
P10	18	180	251
N10	12	323	125
P15	10	281	175
P8	3	ND	400
P13	2	ND	305
N8	2	330	200

T-cell clones were activated by PMA and the triplet of CD2 mAb or PMA/CD2 with co-stimulation of CD28, both in the presence or absence of rIL-2 (50 U/ml). Indicated are the percentage increases of IL-4 production when rIL-2 was added. The amount of IL-4 produced by clones stimulated in the absence of rIL-2 was defined as 100%.

ND means that IL-4 production was not detectable (<150 pg/ml) when clones were stimulated via CD2 and PMA.

IL-2 production of each clone stimulated with CD2/PMA/CD28 is indicated in the second column.

that the increase of IL-4 production that followed ligation of CD28 molecules may have been caused by elevated levels of IL-2, which indirectly influenced IL-4 synthesis. Indeed, for both mice and humans it has been described that IL-2 is involved in the regulation of IL-4 production.<sup>37-39</sup>

**Increase of IL-4 production by CD28 co-stimulation is regulated via IL-2**

Two different approaches were chosen to examine the role of IL-2 in the enhancement of IL-4 production by activated T-cell clones as a consequence of ligation of CD28 molecules. First, IL-4 production was determined in the presence of 50 U/ml rIL-2. The percentage increase in IL-4 secretion that resulted from addition of rIL-2 is depicted in Table 1. As expected, addition of rIL-2 enhanced the production of IL-4. Clones that secreted low levels of IL-2 secreted relatively more IL-4 in the presence of rIL-2, whereas clones that produced high amounts of IL-2 showed hardly any effect upon addition of rIL-2 on IL-4 production.

In the second approach, IL-2 effects were blocked by addition of mAb against CD25 and the  $\alpha$  chain of the IL-2 receptor (IL-2R), together with mAb directed against IL-2. Inhibition of production of IL-4 by this treatment, as shown in Table 2, again implicated involvement of IL-2 in the regulation of IL-4 synthesis. Interestingly, distinct effects were found between clones that differed in their capacity to produce IL-4. Clones producing low levels of IL-4 showed a total reduction of IL-4 secretion when IL-2/IL-2R interaction was blocked (P10, P8, P13). In contrast, marginal inhibition of IL-4 production was found in clones with a high capacity to produce IL-4.

When IL-4 was measured over a period of 7 days, we found that addition of mAb against IL-2 together with CD25 mAb did not influence the kinetics of IL-4 secretion, as is shown for clone P15 in Fig. 2. In the absence of these mAb, CD28

**Table 2.** Effect of blockage of IL-2 on IL-4 production

Clone	IL-4 production (pg/ml)	% inhibition (IL-2 blocked)	
		CD2/PMA	CD2/PMA CD28
N18	4000	24	0
P10	350	100	100
N10	7000	60	46
P15	6000	46	46
P8	650	ND	100
P13	550	ND	100
N8	4500	24	31

IL-4 production of clones stimulated with PMA and CD2 (CLB-T11.1/1, CLB-T11.2/1, CLB-Hik27) or PMA/CD2 with CD28 (CLB-CD28) co-stimulation, was measured, and the percentage decrease determined when IL-2/IL-2R (BG-5/CLB-IL-2R) interaction was blocked.

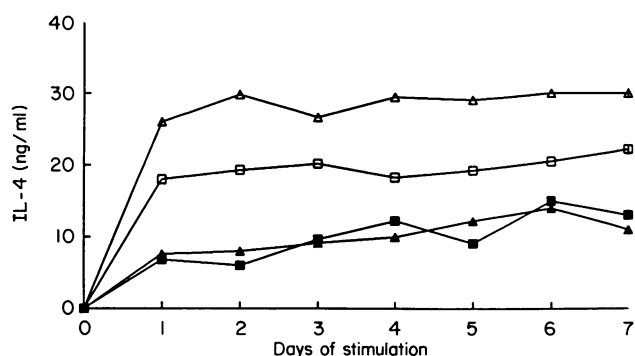
The percentage decrease could not be determined when the activated clones produced undetectable levels of IL-4 (ND).

The capacity to produce IL-4 when activated with CD2/PMA/CD28 is indicated in the second column.

co-stimulation enhanced production of IL-4 at every time-point. However, when IL-2 activity was eliminated by these antibodies, residual IL-4 production was found to be independent of co-stimulation via CD28. Similar results were found for four other clones tested (N18, P10, N10, N8; data not shown).

### CD28-induced IL-4 production by peripheral blood lymphocytes is dependent on IL-2

To investigate whether the indirect effect of CD28 triggering on IL-4 secretion was not only observed in T-cell clones but also in



**Figure 2.** Kinetics of IL-4 production of T-cell clone P15 induced by a combination of PMA (1 ng/ml) and CD2 (CLB-T11.1/1, CLB-T11.2/1, CLB-Hik27) stimulation and by stimulation with PMA/CD2 and CD28 (CLB-CD28), under normal conditions (open symbols), and while mAb directed against IL-2 (BG-5) and CD25 (CLB-IL-2R) were present (closed symbols). Supernatant of T-cell clones stimulated for 1–7 days was collected and IL-4 production determined. IL-4 production induced by CD2 and PMA stimulation is indicated by squares, whereas triangles describe the kinetics of IL-4 production when co-stimulation via CD28 molecules was given.

peripheral blood lymphocytes, CD4<sup>+</sup> memory cells were stimulated in a B7-dependent system. This purified subset was used since it has been shown that only CD4<sup>+</sup> CD45RO<sup>+</sup> cells are able to produce IL-4 efficiently.<sup>40</sup> Activation by a combination of CD2 mAb in the presence of B7 transfectants<sup>30</sup> resulted in both efficient proliferation and IL-4 production (Table 3). This response was dependent on the interaction between CD28 and B7, since addition of B7-24, which prevents interaction between CD28 and B7, completely inhibited the response of the T cells. Moreover, in the presence of control mouse fibroblasts (3T6-FcγRII) no proliferation was induced (data not shown). When effects of IL-2 were blocked, proliferation of the memory cells was only partially inhibited (50%), whereas no IL-4 was detectable in these cultures. These data strongly suggest that the influence of CD28 co-stimulation on the production of IL-4 by CD4<sup>+</sup> memory T cells is also indirectly regulated via IL-2.

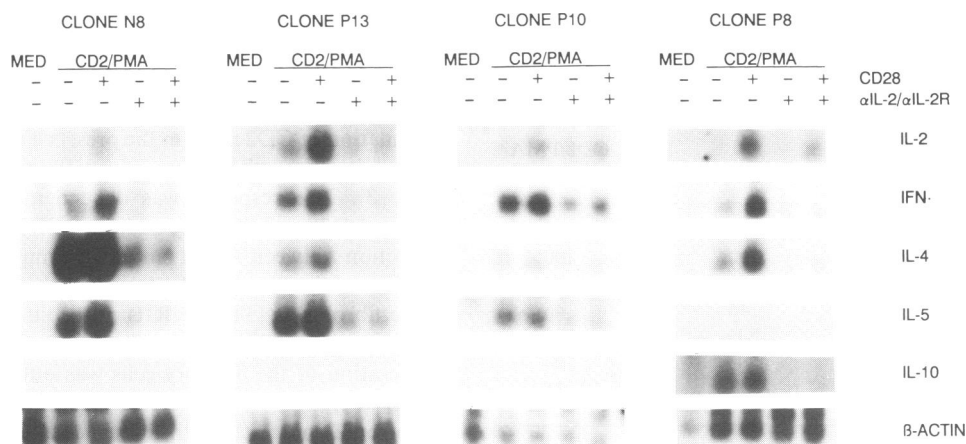
### CD28 co-stimulation regulates cytokine production at the mRNA level

Total mRNA of T-cell clones stimulated with CD2-specific mAb and PMA in the presence or absence of CD28 co-stimulation was isolated. The amount of specific cytokine mRNA in these samples was compared to mRNA derived from cells stimulated via CD2/PMA with or without CD28 co-stimulation, in the presence of mAb against IL-2 and CD25. IL-2, IL-4, IL-5, IL-10 and IFN-γ mRNA levels were determined, and the results obtained for four T-cell clones are depicted in Fig. 3. All clones showed a strong increase in the level of IL-2 mRNA when CD28 co-stimulation was given. Enhancements of mRNA levels of the other interleukins tested were less pronounced. Blocking of IL-2 resulted in a strong reduction of the mRNA levels of all cytokines. Augmentation of IL-2 mRNA of some clones (P10 and P8) by CD28 co-stimulation was still found when mAb against IL-2 and IL-2R were present, whereas others (N8 and P13) showed such a strong decrease of IL-2 mRNA under these circumstances that enhancement of IL-2 mRNA by CD28 co-stimulation could barely be detected. Any positive effect of CD28 co-stimulation on IL-4, IL-5, IL-10

**Table 3.** IL-4 production by CD4<sup>+</sup> memory cells is dependent on IL-2

	Proliferation (c.p.m.) anti-IL-2/IL-2R mAb		IL-4 production (pg/ml) anti-IL-2/IL-2R mAb	
	-	+	-	+
Med	2500	1750	< 150	< 150
CD2	65 400	31 350	10 100	< 150
CD2/2F2	77 300	25 750	8900	< 150
CD2/B7-24	2400	2300	< 150	< 150

CD4<sup>+</sup> memory T cells were stimulated by CD2 (CLB-T11.1/1, CLB-T11.2/1, CLB-Hik27) and B7-transfected fibroblasts (3T6-FcγRII/B7) in the presence and absence of IL-2 (BG-5) and IL-2R (CLB-IL-2R) mAb. Proliferation and IL-4 production on day 3 are indicated. Addition of mAb directed against B7 (B7-24) completely inhibited proliferation, demonstrating the B7/CD28 specificity of this system, while a control mAb (2F2), directed against CD70, had no effect.



**Figure 3.** Effect of CD28 co-stimulation and IL-2 on mRNA expression of cytokines. RNA of clones P10, N8 and P13, stimulated for 5 hr with PMA (1 ng/ml) and CD2 (CLB-T11.1/1, CLB T11.2/1, CLB-Hik27) or PMA/CD2 and CD28 (CLB-CD28), in the absence and presence of a combination of mAb specific for IL-2 (BG-5) and IL-2R (CLB-IL-2R), was isolated, and the amount of mRNA specific for the cytokines IL-2, IL-4, IL-5 and IFN- $\gamma$  was compared by Northern blot analysis. Hybridization with a  $\beta$ -actin-specific probe was used as a control to match the amount of RNA in each sample.

and IFN- $\gamma$  mRNA levels was strongly reduced when binding of IL-2 to its receptor was inhibited.

## DISCUSSION

Ligation of CD28 molecules via specific mAb, or its ligands B7 or B7-2, gives rise to enhanced proliferation and interleukin production. Here we studied the mechanism by which CD28 co-stimulation influences production of Th2-like cytokines.

We show that co-stimulation via CD28 efficiently augments secretion of IL-2 by human T-lymphocyte clones, and in this way has an indirect effect on the production of other cytokines.

Wierenga *et al.*<sup>41</sup> also reported a significant CD28-induced enhancement of IL-2 production by human Th1 clones, thereby stressing their Th1 phenotype. IL-2 production of Th2-type clones, however, was less affected. Others have reported a less restricted division between human Th1- and Th2-type clones.<sup>42-44</sup> Also, in our study clear distinctions between individual clones were apparent, but clones that secreted IL-4 or IFN- $\gamma$  exclusively were not tested. In all clones, IL-2 production was remarkably enhanced after CD28 co-stimulation, at the protein as well as mRNA level. Of notice, CD28 co-stimulation did not affect the ranking of T-cell clones with concern to their IL-2 production potential.

Northern blot analyses demonstrated that after 5 hr of activation a significant increase of IL-2 mRNA by CD28 co-stimulation was induced, whereas IFN- $\gamma$  as well as IL-4, IL-5 and IL-10, were less strongly enhanced. Blocking of IL-2 resulted not only in diminished levels of IL-4, but also mRNA encoding for IL-5, IFN- $\gamma$ , IL-10, and IL-2 itself, was found to be strongly reduced. This latter finding indicates that production of all these cytokines was positively influenced by IL-2. There are indications in the literature that IL-2 can play a role in the regulation of IL-2 itself and of both IFN- $\gamma$  and IL-5.<sup>38,45,46</sup> Another possible mechanism for the reduction of IL-5 and IFN- $\gamma$  production may be the strong decrease of IL-4 secretion. Experiments with both IL-4 knockout mice and IL-4 transgenic mice suggest a positive influence of IL-4 on IL-5, IL-10 and IFN- $\gamma$  production by lymphocytes.<sup>47,48</sup> So far nothing is known about the regulation

of IL-10 production by IL-2. IL-10 mRNA was only detectable in one of four clones. However, it has been reported by Yssel *et al.*<sup>49</sup> that IL-10 mRNA was detectable in the majority of their clones, after activation for 8 hr. A longer stimulation than 5 hr might have been necessary for detection of IL-10 mRNA in the other clones.

When mRNA levels are reduced by addition of mAb directed against IL-2 and IL-2R, enhancement of mRNA levels of IL-2, IL-4, IL-5, IL-10 and IFN- $\gamma$  by CD28 co-stimulation was strongly decreased or absent, implying that induction of these cytokines by CD28 co-stimulation was controlled mainly via IL-2. Therefore, it is possible that stabilization of mRNA, found after CD28 co-stimulation,<sup>16,17</sup> may be regulated by IL-2. Remarkably, when IL-2 effects were blocked, levels of IL-2 mRNA of clones P10 and P8 were still increased upon co-stimulation via CD28. Whether this phenomena is solely due to the IL-2 mRNA stabilization or is regulated at the level of transcription is not clear. Several groups have described the induction of protein binding to a  $\kappa$ B-like enhancer motif (CD28RE) in the up-stream region of the IL-2 gene following CD28 co-stimulation.<sup>18,19</sup> Recently, Ghosh *et al.* showed association of the rel family proteins c-Rel, p50 and p65 with CD28RE.<sup>50</sup> Interestingly, regulation of IL-4 by clones with a low capacity to produce IL-4 seemed to be completely dependent on IL-2, since inhibition of IL-2 by mAb in these clones reduced secretion of IL-4 to undetectable levels. IL-4 production of clones, which were differentiated to efficient IL-4 producers, could only be partially blocked by inhibition of IL-2/IL-2R interaction. For example, when IL-2 and CD25 mAb were present, IL-4 mRNA was still found in clone N8. Since this clone produced very low amounts of IL-2, elimination of IL-2 would be very efficient. In contrast, hardly any IL-4 mRNA was detectable in the other three clones tested (low IL-4 producers). It is possible that production of IL-4 in clones that produce high levels of IL-4 can be regulated via an additional, IL-2-independent mechanism. Insight into this mechanism might be valuable for understanding the pathogenesis of diseases with a Th2-type immune response, such as allergy or infection with *Leishmania major*. IL-4 production of peripheral

blood cells seemed to be completely dependent on IL-2 (Table 3). However, any IL-4 production regulated by a different mechanism might easily have been missed in this assay, since our data suggest that only highly differentiated, high IL-4-secreting cells, can produce IL-4 regulated in this IL-2-independent way. These cells will represent a very small subset of the CD4<sup>+</sup> cells. It would be interesting to test whether this phenomena can be found in atopic individuals, who have a greater subset of cells specialized in IL-4 production.

Our data suggest that increases in IL-2, IL-4, IL-5, IL-10 and IFN- $\gamma$  by CD28 co-stimulation is regulated mainly by an indirect mechanism via enhancement of IL-2 production. Production of IL-2, however, may also be regulated via a second more direct mechanism. This implies that activation of memory T cells by professional APC expressing B7, may induce, through ligation of CD28, high amounts of IL-2 and a strong augmentation of Th1 immune responses. On the other hand, it is reported that CD28 co-stimulation can induce responsiveness for IL-4 in mouse Th2-type T-cell clones and that IL-2 can function as a growth stimulator of lymphocytes of the Th2 subset.<sup>51,52</sup> We have shown that IL-2 by itself has a high, positive influence on the production of several cytokines, including Th2-type cytokines (IL-4, IL-5, IL-10). Co-stimulation of CD28 molecules may therefore augment both Th1 and Th2 immune responses.

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