Antigen-presenting cell-derived signals determine expression levels of CD70 on primed T cells

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SUMMARY

Interaction between CD27 and its ligand CD70 provides a second signal for T-cell proliferation and tumour necrosis factor-α (TNF-α) production. Whereas CD27 is broadly expressed during T-cell development, expression of CD70 in vivo is restricted. To determine when CD27–CD70 interactions can occur in immune reactions, we here analysed the regulation of CD70 expression on activated T cells. Mitogenic stimulation of purified T cells with either immobilized CD3 monoclonal antibody (mAb) or a combination of CD2 mAb induces only low levels of CD70 membrane expression. Markedly, expression of the CD27-ligand is strongly enhanced by antigen-presenting cells (APC) and APC-associated signals such as interleukin-1α (IL-1α), IL-12, TNF-α and CD28-ligation. In contrast, T-cell derived cytokines, such as IL-4, counteract CD70 up-regulation on activated T cells. Analysis of the small subset of circulating CD70+ T cells revealed that these cells have a primed phenotype as they express CD45RO and HLA-DR antigens and are in high frequency able to secrete interferon-γ (IFN-γ). We conclude that T–T interactions involving CD27 and CD70 are likely to occur relatively early in immune reactions, after productive T-cell priming by APC and that expression of CD70 on circulating T cells is a reflection of recent priming by antigen.

INTRODUCTION

Members of the tumour necrosis factor receptor (TNFR)/TNF family are generally believed to play important roles in the regulation of death, survival and differentiation of T and B lymphocytes.1 TNFR-related molecules are either constitutively expressed on subsets of human lymphocytes, e.g. CD27, CD40, CD952–6 or are induced on these cells after antigenic stimulation, e.g. CD120a (TNFR p55), CD120b (TNFR p75), CD30, OX40 and 4-1BB.7–10 Although most TNFR-family ligands are readily induced after cellular activation in vitro,11–20 demonstration of their expression in vivo has proven to be difficult. For instance, stimulation of peripheral blood T cells with phorbol 12-myristate 13-acetate (PMA) and ionomycin induces high levels of CD40L expression in the CD4+ subpopulation in vitro, however, in vivo, only a few T cells in mantle zone and germinal centres (GCs) of human tonsils are CD40L+13,14,21

Moreover, while CD70 can be induced at high levels on tonsillar and peripheral blood B cells activated via their B-cell receptor and/or CD40 ligation and at lower levels on CD3 monoclonal antibody (mAb)-stimulated T cells in vitro,11,12 the CD27-ligand has a very limited distribution in vivo: only B cells in occasional tonsillar GC and scattered T cells in tonsil, skin and gut express low levels of CD70.22,23 Thus far data on the membrane expression of CD30L, OX40L, 4-1BBL and CD95L in normal human lymphoid tissues are lacking.

In contrast to its ligand, CD27, the receptor for CD70, is constitutively expressed on the majority of peripheral blood T cells and on 25% of peripheral blood B cells.2–5 Based on the selected up-regulation of CD27 on unprimed T cells,24 a role for CD27/CD70 interactions in the expansion of naive T cells was suggested. Indeed, recombinant CD70, transfected into mouse fibroblasts, was shown to costimulate for T-cell proliferation and TNF-α production more vigorously in CD45RA+ cells than in CD45RO+ T cells.25,26 Interestingly,
professional antigen-presenting cells (APC) such as dendritic cells and macrophages do not express CD70, indicating that the initial priming of naive T cells occurs in the absence of CD27 ligation. Moreover, since only activated T and B cells have been found to express CD70, ligation of CD27 on T cells is likely to occur via T–B or T–T interactions after initial priming by antigen and APC. In accordance, we here show that accessory signals involved in early T-cell activation (e.g., costimulatory signals and APC-derived cytokines) could specifically enhance CD70 expression on activated T cells, whereas cytokines produced by primed T cells down-regulated CD70 expression. In addition, examination of the phenotypical and functional properties of circulating CD70+ T cells confirmed that these cells have recently encountered antigen.

**MATERIALS AND METHODS**

**Monoclonal antibodies and cytokines**
CLB-T11·1/1, CLB-T11·2/1, HIK27 (all CD2 mAb), CLB-T3/3, CLB-CD28/1, CLB-CD4/1, CLB-FeR gran1 (CD16), CLB-CD19/1, anti-human leucocyte antigen (HLA) class II (R3E2), biotinylated and unlabelled CLB-CD70/1, fluorescein isothiocyanate (FITC)-conjugated and unlabelled CLB-CD4/1, FITC-conjugated CLB-CD25/1, biotinylated CLB-CD27/3 and biotinylated and FITC-conjugated subclass control (anti-birch pollen) mAb were all produced at the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, the Netherlands).

CD80 mAb (B7-24) was generously provided by Dr M. de Boer (Innogenetics, Ghent, Belgium) and biotinylated according to standard procedures. Phycoerythrin (PE)-conjugated HLA-DR mAb was purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA), PE-labelled CD45RA mAb (UCHL1) from DAKO (Glostrup, Denmark) and PE-labelled CD45RA (2H4) mAb from Coulter Clone (Hialeah, FL). As a conjugate for biotinylated mAb either streptavidin–PE (SA–PE) obtained from Molecular Probes (Eugene, OR) or streptavidin RED670 from Life Technologies (Gaithersburg, MD) was used. Biotinylated CLB-IL-4/1 mAb anti-human IL-4 mouse IgG1, kindly provided by Dr T. van der Pouw-Kraan, CLB, Amsterdam, the Netherlands) and biotinylated MD-1 mAb anti-human INF-γ mouse IgG1, obtained from Dr P. van der Meide, TNO, Rijswijk, the Netherlands) were used for intracellular staining of cytokines. Biotin-conjugated mouse IgG1 (anti-birch pollen) mAb was used as a control reagent.

The following recombinant human cytokines were used: TNF-α, IL-1α, IL-4, INF-γ (all purchased from Genzyme Diagnostics, Cambridge MA), IL-2 (a kind gift of Sandoz, Vienna, Austria), IL-6 (generously provided by Prof. Dr L. Aarden, CLB, Amsterdam, the Netherlands), IL-10 (a generous gift of Dr J. de Vries, DNAX, Palo Alto CA) and IL-12 (kindly donated by Dr Stanley Wolf, Genetics Institute, Cambridge MA).

**Cell culture and activation**
All experiments were performed in Iscove’s Modified Dulbecco’s Medium (IMDM) containing 10% heat-inactivated human pooled serum (HPS), 5 × 10−5 M 2-β mercaptoethanol, penicillin and streptomycin. Purified T cells (5 × 10⁶/ml) were stimulated with either immobilized CD3 mAb (CLB-CD3/3, ascites final dilution 1:1000) or a mitogenic combination of three CD2 mAb (CLB-T11·1/1, CLB-T11·2/1, HIK27, ascites final dilution 1:1000) in the presence or absence of the following recombinant cytokines or mAb: TNF-α (50 ng/ml), IL-1β (5 ng/ml), IL-2 (50 U/ml), IL-4 (50 ng/ml), IL-6 (100 U/ml), IL-10 (100 U/ml), IL-12 (1 ng/ml), INF-γ (100 U/ml), CD28 mAb (ascites final dilution 1:1000). After the indicated incubation periods cells were harvested and prepared for FACS analysis. In parallel cultures purified T cells (1 × 10⁵/200 μl) were stimulated for 4 days in the same manner as described above. During the last 18 hr of culture [3H]-thymidine (2 Ci/mmol) was added.

Antigen-specific responses were initiated by incubating PBMC of 13 different donors with 15 μl/ml tetanus toxoid (TT, RIVM, Bilthoven, the Netherlands), 10 μg/ml purified protein derivative of *Mycobacterium tuberculosis* (PPD, Statens Seruminstitut, Copenhagen, Denmark) or 5 μg/ml *Toxocara canis* excretory/secretory antigen (TES, kindly provided by Dr J. Bujs, RIVM, Bilthoven, the Netherlands). After 8 days of incubation cells were harvested and prepared for FACS analysis.

**Flow cytometry**
Cells (2 × 10⁵) were incubated with saturating amounts of FITC-labelled CD25 mAb and biotinylated mAb for 30 min at 4°C in PBS containing 0.5% (v/v) bovine serum albumin (BSA). After removal of unbound mAbs SA-PE was added and incubated for another 20 min. Cells were washed three times with PBS/0.5% BSA and analysed on a FACScan (Becton Dickinson, San Jose, CA) using the Consort 30 program. In case of triple-staining experiments, 5 × 10⁶ PBMC were incubated with FITC-conjugated CD4 mAb, biotinylated CD70 mAb and different PE-labelled mAb as described above. SA-RED670 was used as a third fluorochrome. Cells were analysed on a FACScan using the FACScan program.

**Flow cytometric cytokine production measurement**
Flow cytometric measurement of cytokine production was based on the stimulation of cells in the presence of an inhibitor of protein secretion resulting in the cytoplasmic accumulation of the synthesized cytokines. After cell fixation and permeabil-
ization intracytoplasmic staining was performed according to a protocol originally described by Jung et al.31 Briefly, purified T cells were stimulated (1 × 10^6/ml) for 5 hr with PMA (5 ng/ml) and ionomycin (1 μM) in the presence of the protein-secretion inhibitor monensin (1 μM) (all from Sigma, St Louis, MO). Cells were extensively washed in PBS containing 0.5% BSA and incubated successively with the following reagents (all at 4°C for 20 min):

(1) CD70 mAb (5 μg/ml);
(2) PE-conjugated goat-anti mouse Immunoglobulin;
(3) 10% normal mouse serum to block free binding sites on the goat anti-mouse conjugate;
(4) FITC-conjugated CD4 mAb.

Subsequent intracellular staining was performed as follows. Cells were washed twice with cold PBS and fixed with PBS containing 4% paraformaldehyde for 10 min at 4°C. Fixation was followed by permeabilization with PBS containing 0.1% saponin (Sigma, St Louis, MO) supplemented with 10% human pooled serum for 10 min at 4°C. For all subsequent incubation and washing steps PBS containing 0.1% saponin and 0.5% BSA was used. Staining of the cytoplasm with a biotinylated cytokine mAb (5 μg/ml) was followed by incubation with streptavidin RED670 (both at 4°C, 20 min). Biotinylated mouse IgG1 was used to verify the staining specificity of the cytokine mAb. Analysis was performed on a FACScan.

### RESULTS

#### Regulation of CD70 expression on purified T cells

Purified T cells were stimulated with either immobilized CD3 mAb or a mitogenic combination of three CD2 mAb and the expression of CD70 was measured in time (Fig. 1a). Although both stimuli are potent inducers of T-cell proliferation and CD25 expression (refs 32,33 and data not shown) CD70 expression was induced in maximally 29 ± 6% of the T cells after 4 days of stimulation with CD2 mAb and in 36 ± 2% after 6 days of stimulation with CD3 mAb. In vivo, optimal activation and differentiation of T cells is conveyed by accessory signals derived from APC such as costimulatory molecules (e.g. B7-1 and B7-2) and cytokines (e.g. IL-1α, TNF-α, IL-12, IL-6 and IL-10) and is later on modulated by cytokines produced by activated T cells (IL-2, IL-4, INF-γ). Addition of IL-1α, TNF-α and IL-12 to CD2 mAb-stimulated T cells strongly enhanced the percentage of activated CD25 + T cells that expressed CD70 (Fig. 1b and 1c). Moreover, also ligation of CD28 with agonistic CD28 mAb resulted in a large number of activated T cells that co-express CD70 and CD25. This latter effect appeared to be independent from the amount of produced IL-2 since exogenous IL-2 did not have any effect on the expression of CD70, whereas this addition had a prominent effect on T-cell proliferation and CD80 expression (Table 1). Addition of IL-4, IL-6 and IL-10 lead to a decrease in the number of CD70 + T cells. In contrast to the effects of IL-6 and IL-10, in the case of IL-4, the diminishment of CD70 was not simply due to impaired T-cell activation as exemplified by the finding that addition of IL-4 enhanced T-cell proliferation more than two-fold (Table 1). Thus, it appears that APC-derived signals (IL-1α, TNF-α, IL-12, CD28 ligation) have a positive effect on CD70 expression whereas T cell-derived signals have either no effect (IL-2 and IFN-γ) or a negative effect (IL-4).

### T cells activated in an antigen-dependent manner express very high levels of CD70

To test regulation of CD70 membrane expression after antigen-specific (APC-dependent) T-cell activation, PBMC from 11 healthy individuals were stimulated with TT, PPD or TES. After 8 days of stimulation seven out of 11 individual donors responded to TT, 11 out of 11 responded to PPD and two out of 11 to TES (Table 2). Figure 2 shows the expression of CD70 and CD25 of a representative donor that responded to all three antigens and Table 2 summarizes the mean percentage of CD70-expressing cells (+ standard deviation) of the total population that was analysed. As a control the response of purified T cells to immobilized CD3 mAb (i.e. antigen-independent) is shown. In analogy with previous experiments (Fig. 1b), CD70 was only expressed on cells that are activated and

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**Table 1. Influence of cytokines and mAb on CD80 expression and proliferation of CD2 mAb-activated T cells**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>CD80 % gated (mean ± SD)</th>
<th>Proliferation (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD2 mAb+</td>
<td>20 ± 10†</td>
<td>11</td>
</tr>
<tr>
<td>TNF-α</td>
<td>67 ± 7</td>
<td>7</td>
</tr>
<tr>
<td>IL-1α</td>
<td>62 ± 10</td>
<td>11</td>
</tr>
<tr>
<td>IL-6</td>
<td>50 ± 4</td>
<td>2</td>
</tr>
</tbody>
</table>

*PBMC of 11 different donors were stimulated with the indicated stimuli for 4 days (CD3 mAb) or 8 days (TT, PPD and TES). Cells were harvested and stained with a combination of FITC-labelled CD25 mAb and streptavidin-PE. Markers were set using irrelevant FITC-labelled and biotinylated mAb.

† Percentage CD70-expressing cells within the CD25 + population.

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**Table 2. Induction of CD70 expression by specific antigens**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>% CD25 + (mean ± SD)</th>
<th>% CD70 + (mean ± SD)</th>
<th>Number of responding donors</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3 mAb</td>
<td>83 ± 8</td>
<td>20 ± 10†</td>
<td>11</td>
</tr>
<tr>
<td>TT</td>
<td>57 ± 8</td>
<td>67 ± 7</td>
<td>7</td>
</tr>
<tr>
<td>PPD</td>
<td>63 ± 6</td>
<td>62 ± 10</td>
<td>11</td>
</tr>
<tr>
<td>TES</td>
<td>67 ± 1</td>
<td>50 ± 4</td>
<td>2</td>
</tr>
</tbody>
</table>
Figure 1. (a) Kinetics of CD70 expression on purified T cells. Purified CD3+ T cells were stimulated with a combination of three CD2 mAb (filled circles) or immobilized CD3 mAb (open triangles). At different time-points cells were harvested and the expression of CD70 was measured using biotinylated CD70 mAb and FACS analysis. Results shown are mean ± SEM of the percentage positive cells, compared to an irrelevant control mAb, of three independent experiments. (b) Influence of cytokines and mAb on CD70 expression of CD2 mAb-stimulated T cells. Purified T cells (0·5 × 10^6/ml) were stimulated with a combination of three CD2 mAb (ascites dilution 1:1000) with or without the indicated cytokines or mAb. After 4 days of culture, cells were collected, double-stained with biotinylated CD70 mAb and FITC-labelled CD25 mAb and analysed on a FACScan. Percentages shown are the % CD70+ cells within the total CD25+ T-cell subset. The figure represents one experiment out of five. (c) Relative change in CD70 expression induced by cytokines and CD28 mAb compared to the expression of T cells that were stimulated with CD2 mAb only. Mean and standard deviation of five independent experiments are shown.

express CD25. In contrast to stimulation with immobilized CD3 mAb, all three antigens induced CD70 expression on a large fraction of CD25+ T cells. Moreover, it should be noted that CD70 expression can be induced by antigens that promote Th1-type of responses (PPD) as well as by those that induce Th0-type (TT) and Th2-type (TES) of responses.34

CD70+ T cells circulating in peripheral blood have a primed phenotype and are the main producers of INF-γ

From the above we conclude that T cells expressing CD70 may have been primed by antigen and APC. In peripheral blood a small subset of T cells (5-1% ± 1-9%, n = 7) expresses CD70.

To test whether these cells indeed show signs of antigenic encounter, we isolated PBMC from two healthy individuals and examined in triple-staining experiments the expression of a number of markers in the CD4+ CD70+ T-cell population. Table 3 shows that the majority of CD70+ T cells expressed CD45RO (see also Fig. 3) and CD27. Furthermore, compared to the total CD4+ T cell population the CD4+ CD70+ T-cell subset was enriched for HLA-DR+ cells (Table 3).

Stimulation of peripheral blood T cells of normal donors with PMA and ionomycin for 5 hr and subsequent intracellular staining for cytokines revealed that 19–26% of CD4+ T cells could produce IFN-γ and only 0-4–1-2% IL-4 (Fig. 3). Interestingly, the majority of the CD4+ T cells that expressed
CD70 also contained IFN-γ (62–70%) corroborating that these cells have been primed in vivo. Furthermore, upon isolation of the CD70+ T-cell subset from peripheral blood by FACS sorting and subsequent stimulation with CD2 and CD28 mAb we found that compared to the CD70−-sorted T cells, the CD70+-sorted T cells expressed much higher levels of CD70 and CD80 (Fig. 4). Previous studies have shown that memory (CD45RO+) T cells lose CD27 expression upon repeated stimulation.24 The fact that stimulated CD70+-sorted T cells expressed less CD27 than CD70−-sorted T cells fits with the idea that CD70+ T cells have previously been primed.

**DISCUSSION**

Based on the expression of CD27 during T-cell ontogeny, the regulated surface expression of CD70, and functional data we have proposed a model for the role of CD27–CD70 interactions in the immune response.35 Professional APC, such as dendritic cells and macrophages, express the costimulatory ligands CD80 and CD86 but do not express CD70.12,27,28,36–38 These types of APC are thought to be instrumental in the initial priming of T cells which is followed by the activation of adjacent antigen-specific B cells, probably via CD40L–CD40 interactions.39 Activated B cells express next to CD80 and CD70+ sorted T cells expressed much higher levels of CD70 and CD80 (Fig. 4). Previous studies have shown that memory CD86 also CD70 and are as such thought to be involved in CD27 ligation.12 In addition to the putative role for B cells in the ligation of CD27 on T cells, CD27 and CD70 also appear to be involved in T–T interactions.12,28 We here show that T cells that have been stimulated by antigen and APC can very efficiently be induced to express CD70. Particularly, cytokines, such as IL-12 and IL-12 which can be produced by activated macrophages39–42 are potent positive regulators of CD70 expression on T cells. Furthermore, ligation of CD28 also induces high levels of CD70. Interestingly, the presence of exogenous IL-4, while costimulating for T-cell proliferation, decreased the induction of CD70 expression by

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CD70 expression on human T cells

CD70 expression on human T cells

Figure 3. CD70+ T cells reside within the CD45RO+ subset and produce IFN-γ. Purified T cells of two healthy donors were triple stained with CD4, CD45RO and CD70 specific mAb. A life gate was set on the CD4+ T cells. In a parallel experiment T cells were stimulated for 5 hr with PMA and ionomycin and intracellular staining for cytokines was performed as described in the Materials and Methods section. The numbers shown are the percentage INF-γ or IL-4-containing cells within the different T-cell subsets.

CD2 mAb. Moreover, continuous presence of activating stimuli was necessary to maintain CD70 expression as removal of the activating mAb and cytokines resulted in a decrease in membrane CD70 expression (not shown). The inhibitory effect of IL-4 on T-cell CD70 expression was also found for IgM/CD40 mAb-stimulated tonsillar B cells and discriminates CD70 from CD80 regulation as expression of CD80 is enhanced by IL-4 on activated B cells and is not influenced by IL-4 on activated T cells (Lens et al., Eur J Immunol).

Although IL-1α, TNF-α, IL-12 and CD80 have been proposed to direct Th1 responses and IL-4 Th2 responses43 both Th1- (PPD) and Th2-type antigen (TES)44 could efficiently induce CD70 expression. Still, in both donors that responded to TES, the percentage of CD70-expressing cells was lower (46 and 54%) than the mean percentage of CD70+ cells induced by PPD (62 ± 10%, n = 11) or TT (Th0, 67 ± 7%, n = 7). Upon stimulation with TES, both monocytes/macrophages producing IL-12, TNF-α, IL-1α and expressing CD80 as well as T cells producing IL-4 will be present in culture. Apparently, in this situation IL-4 produced by the T cells is only partially able to counteract all the positive regulators of CD70 expression. In line with this, simultaneous addition of IL-12 and IL-4 to purified activated T cells gave rise to a percentage of CD70+ T cells that was lower than the percentage found in the presence of IL-12 alone, but higher than in the presence of IL-4 alone (data not shown), suggesting that the number of CD70+ T cells is a result of a delicate balance between positive and negative regulators of CD70 expression.

Efficient induction of CD70 on T cells after antigenic priming was reflected in vivo by the finding that the majority of the CD70+ T cells reside within the CD45RO+ subset, express CD27 and, compared to the total CD4+ population, are enriched for HLA-DR+ cells. Moreover, a large number of CD70+ T cells produce INF-γ and upon isolation and subsequent stimulation of these CD70+ T cells they gain high expression levels of CD70 and CD80 and rapidly lose CD27 expression. The latter findings could imply that the high percentage of CD70+ T cells we found upon stimulation with the recall antigens TT, PPD and TES is a result of selective outgrowth of CD70+ T cells. However, several lines of evidence argue against this possibility. First, high percentages of CD70+ T cells can also be found after allogeneic stimulation (Brugnoni et al., submitted for publication). Second, CD70 expression can be induced on CD70−sorted T cells both by polyclonal stimulation with CD2 and CD28 mAb and by antigen-specific stimulation with TT (data not shown). Finally, costimulation by CD28 mAb of purified CD45RA+ T cells induces CD70 expression on these naïve cells (our unpublished results).
Thus, relatively early in the immune response, when initial priming of naive T cells has taken place via antigenic peptides presented by APC, CD70 expression can be induced both on T cells and on adjacent, activated B cells. Previous studies have shown that signals transduced via CD27 costimulate for T-cell proliferation and may be involved in B-cell differentiation and diversification of the immune response.\(^{11,25,26,35,44}\) Furthermore, the fact that CD70 is associated with a phosphorylated molecule\(^{12}\) and that signalling via TNFR-family ligands has previously been shown\(^ {45,46} \) implicates that CD70 may function as a signal-transducer itself. If CD27/CD70 interactions are indeed instrumental in amplification and diversification of immune responses\(^ {47} \) the delivery of these signals has to be tightly controlled to prevent unwanted side-effects of immune-activation such as, lymphoproliferation and autoimmunity. As CD70 is strongly up-regulated by factors that can be produced by activated APC but is antagonized by cytokines (IL-4) that are produced by long-term stimulated T cells, specific regulation of CD70 expression may control the extent of CD27/CD70 interactions. In general, restricted ligand expression may be an important mechanism the immune system employs to dose TNFR/TNF family interactions.

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REFERENCES

17. Smith C.A., Greus H.J., Davis T. et al. (1993) CD30 antigen, a marker for Hodgkin’s lymphoma, is a receptor whose ligand defines an emerging superfamly of cytokines with homology to TNF. *Cell* 73, 1349.

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