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CD70 expression on T-cell subpopulations: study of normal individuals and patients with chronic immune activation

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Abstract

CD70, the ligand of CD27, is a member of the TNF family, which includes molecules essential in the regulation of lymphocyte growth and survival. It is absent on resting lymphocytes but can be induced in vitro with activating stimuli. To extend information about its expression by different T-cell subpopulations, and its regulation in normal and pathologic conditions, highly purified T-cell subpopulations were studied: CD70 expression depended both on the activating stimulus and on the T-cell subset analyzed. PMA + Ionomycin induced CD70 on the large majority of CD8^{+} cells, but only on a minority of CD4^{+} cells ($P < 0.002$), and among these, preferentially on the CD45R0^{+} subset compared with the CD45RA^{+}. The presence of CD4^{+} lymphocytes in cell cultures containing mixtures of T-cell subsets inhibited CD70 expression on CD8^{+} cells. On the contrary, stimulation with allogeneic cells induced CD70 expression also on CD4^{+} cells. Moreover, CD70 was found to be expressed by chronically in vivo activated T-cells, in conditions characterized by allogeneic and autoimmune reactions. These data suggest a possible role of CD70 in the pathogenesis of immune dysregulation; interestingly, this role may not be simply restricted to bind to, and signal through, CD27, since cross-linking of CD70 enhances the proliferative response induced by the stimuli used to elicit its expression. © 1997 Elsevier Science B.V.

Keywords: CD70; CD4^{+} cells; CD8^{+} cells; CD27

1. Introduction

CD70, the ligand of CD27 [1–3], is a member of the TNF family, a group of type II transmembrane proteins that, interacting with their counterreceptors, plays important roles in the regulation of activation, proliferation, differentiation and survival of a variety of cells [4,5].

In particular, CD27 ligation by CD70 amplifies T-cell [6] and B-cell [7] responses: CD70-transfected cells enhance T-cell proliferation induced by suboptimal concentrations of PHA [1,3,8] and can cooperate with T-cell activation signals delivered by the CD80/CD28 system [9], while a block of CD70-CD27 interactions with anti-CD70 mAb [10,11], or recombinant soluble CD27 [12], inhibits T-cell proliferation induced by various stimuli such as PHA, alloantigens, tetanus toxoid and anti-CD2. Not much is known about the physiological role of CD70 on T-cells, apart from binding to CD27: it has been suggested that CD70 itself can function as a transducer of cellular activation signals, since immobilized anti-CD70 mAb costimulates, in association with PMA, the proliferation of T-cells [3].

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CD70 is absent on resting lymphocytes but its expression can be induced on B and T-cells after in vitro activation [3,10,13–15]. Information about expression of CD70 by different T-cell subpopulations, as well as about requirements and regulation of CD70 expression in normal and pathologic conditions are still incomplete.

In this study, we analyzed the expression of CD70 by purified T-cell subpopulations from healthy individuals and from patients with clinical conditions characterized by in vivo ongoing immune activation.

2. Materials and methods

2.1. Cell preparation

CD4$^+$ or CD8$^+$ cells were purified after Ficoll–Hypaque density gradient centrifugation, using positive selection with anti-CD4- or anti-CD8-coated magnetic beads and Detach-a-beads (Dynal, Oslo, Norway), as described [16].

In some experiments, CD45RA$^+$ and CD45RO$^+$ populations were further purified from CD4$^+$ and CD8$^+$ cells using negative selection with anti-mouse IgG-conjugated magnetic beads (Dynal) precoated for 30 min at 4°C with CD45RA (2H4; Coulter Immunology, Hialeah, FL) or CD45RO (UCHL1; Beckton–Dickinson, Mountain View, CA) mAbs; < 2% of negatively selected CD45RA$^+$ or CD45RO$^+$ population expressed the opposite CD45 isoform after this procedure.

2.2. Cell culture

PBMC, 2×10^5, or purified cells from preparations of T-cell subsets were cultured for various times at 37°C in 5% CO_2 in flat-bottomed microtitre plates in 200 μl of RPMI-1640-10% fetal calf serum (FCS) with or without different activating agents: PHA (1.2 μg/ml; Irvine Scientific, Santa Ana, CA); PMA (5 ng/ml; Sigma, St. Louis, MO) plus Ionomycin (500 ng/ml; Sigma); *Staphylococcus Aureus* Enterotoxin B and E (SEB, 500 ng/ml, SEE, 250 ng/ml; Serva Feinbiochemica, Heidelberg, Germany); 2×10^5 irradiated (2000r) allogeneic PBMC from a pool of healthy donors.

2.3. CD70 expression

After activation, cells were washed twice with phosphate buffer saline (PBS)-5% FCS, incubated for 30 min at 4°C with 50 μl of the anti-human CD70 specific mAb 2F2 [11]. After washing, cells were incubated with fluorescine isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Ortho, Raritan, NJ).

For double immunofluorescence analysis, biotin-conjugated anti-CD70 mAb (2F2) was used, followed by incubation with PE-conjugated Streptavidin (Sigma), in combination with FITC-conjugated mAbs.

In some experiments, the cells were incubated with PBS of various acid strength (down to pH 4.1) for 3 min at room temperature and washed twice with PBS pH 7.4, 5% FCS before staining for CD70 in order to dissociate possible soluble form of CD27 bound to CD70.

Flow cytometer analysis was carried out with an argon-ion laser equipped instrument (488 nm; Cytoron Absolute; Ortho), as described [16]. FITC-CD3, -CD4 and -CD8 mAbs were purchased from Ortho, PE-CD28 and -CD45RO from Beckton–Dickinson and PE-CD45RA from Coulter immunology.

2.4. CD70 mAb costimulation assay

To assess costimulatory activity of CD70 mAb, flat-bottomed microtitre plates were coated for 3 h at room temperature with 1:5000 dilution of either anti-human CD70 (2F2) or an isotype-matched control antibody (anti-human CD40, mAb 89, kindly provided by J. Bancherau, Dardilly, France) before the addition of T-cell populations and mitogenic stimuli. The CD70 mAb concentration was chosen as optimal for costimulation in preliminary experiments.

Cultures were labelled for the final 18 h with 1 μCi/well of [^{3}H]Thymidine (Amersham, UK).

2.5. Statistical analysis

Data are presented as mean $\pm$ S.D. Wilcoxon's test was used to determine the significance of comparisons between means.

3. Results

3.1. CD70 expression on normal T CD4$^+$ and CD8$^+$ cells

CD70 was absent in fresh peripheral blood lymphocytes from normal individuals. In particular, only 3.5 + 1.5% of purified CD4$^+$ cells coexpressed CD70 ($n = 7$). As already reported [13,15], its expression could be induced on a proportion (35 $\pm$ 13%) of PBMC by stimulation with PHA for 4 days. A similar level of CD70 expression (39 $\pm$ 20%) was obtained by stimulating total PBMC with PMA + Ionomycin for 4 days.

The issue of the differential expression of CD70 by CD4$^+$ and CD8$^+$ T lymphocytes has been addressed in former studies analyzing by means of 2-color immunofluorescence bulk cultures of PBMC: no difference

was observed among the percentages of CD8 $^+$ and CD4 $^+$ cells gaining CD70 expression after stimulation with PHA [15] or anti-CD3 [11] in these conditions. Using a similar approach, we observed the same pattern in PBMC stimulated with PMA + Ionomycin: the percentage of CD70 $^+$ cells after stimulation was very similar in CD8 $^+$ (36 ± 24%) and in CD8- lymphocyte populations (34 ± 17%).

However, in these conditions, the ability to express CD70 by a T-cell subset might be influenced by the presence of other lymphocyte subsets (through cell-to-cell membrane interactions, soluble mediators, etc). To eliminate this interference, we analyzed CD70 expression on highly purified populations of CD4 $^+$ or CD8 $^+$ cells, obtained by positive selection with magnetic beads. To induce CD70 expression, we used stimuli acting indipendently from the presence of accessory cells.

The in vitro expression of the molecule depended both on the stimulus utilized for activation and on the T-cell subset analyzed. Stimulation with PMA + Ionomycin led to CD70 expression only on a minority of purified CD4 $^+$ cells (12 ± 6%, $n = 9$), as opposed to purified CD8 $^+$ cells (81 ± 7%, $P < 0.002$). A similar pattern was observed in four experiments in which stimulation with superantigens (SEB + SEE) was used: CD70 expression was detected on 29 ± 10% of purified CD4 $^+$ cells, as opposed to 64 ± 19% of CD8 $^+$ cells ($P = 0.03$). On the contrary, stimulation with allogeneic cells induced CD70 expression not only on CD8 $^+$ cells (85 ± 6%), but also on CD4 $^+$ cells (73 ± 10%; n = 3) (Fig. 1).

In all cases, CD4 $^+$ or CD8 $^+$ lymphocytes were able to express other activation markers (CD69, CD25) at similar levels (data not shown), thus ruling out the possibility of a different sensitivity to the activating stimuli used.

3.2. CD70 expression is inhibited by the presence of CD4 $^+$ T lymphocytes in cell culture

The observation that the large majority of CD8 $^+$ cells acquires CD70 expression with all the activating agents studied when purified populations are cultured, whereas, if bulk cultures of PBMC are evaluated, only a minority of CD8 $^+$ cells becomes CD70 $^+$, irrespective of the stimuli used ([10,15] and *supra*), indicates that other mononuclear cell populations interfere with the expression of CD70 by activated CD8 $^+$ cells. To understand better this phenomenon, we performed two experiments: First, we observed that the depletion of CD4 $^+$ cells from total PBMC populations is sufficient to allow CD8 $^+$ cells to acquire full CD70 expression, indicating that the population responsible for this phenomenon is CD4 $^+$ (data not shown). Second, we mixed different proportions of autologous purified CD8 $^+$ and

CD4 $^+$ cells and evaluated the expression of CD70 after stimulation with PMA + Ionomycin: our results confirmed a negative regulatory role of CD4 $^+$ cells, as the expression of CD70 on both activated CD8 $^+$ and CD4 $^+$ cells decreased inversely to the proportion of CD4 $^+$ cells in the mixture (Fig. 2).

A possibility to explain the inhibitory effect performed by CD4 $^+$ cells on CD70 expression is that this is masked by soluble CD27 (sCD27) able to bind CD70 [17,18], released by CD4 $^+$ cells after stimulation with PMA + Ionomycin. To address this possibility, two sets of experiments were performed before staining for CD70: First, purified CD8 $^+$ cells were incubated for 1h at 37°C with supernatant of PMA + Ionomycin-stimulated purified CD4 $^+$ cells, in order to reproduce the inhibitory effect performed by CD4 $^+$ cells. Second,
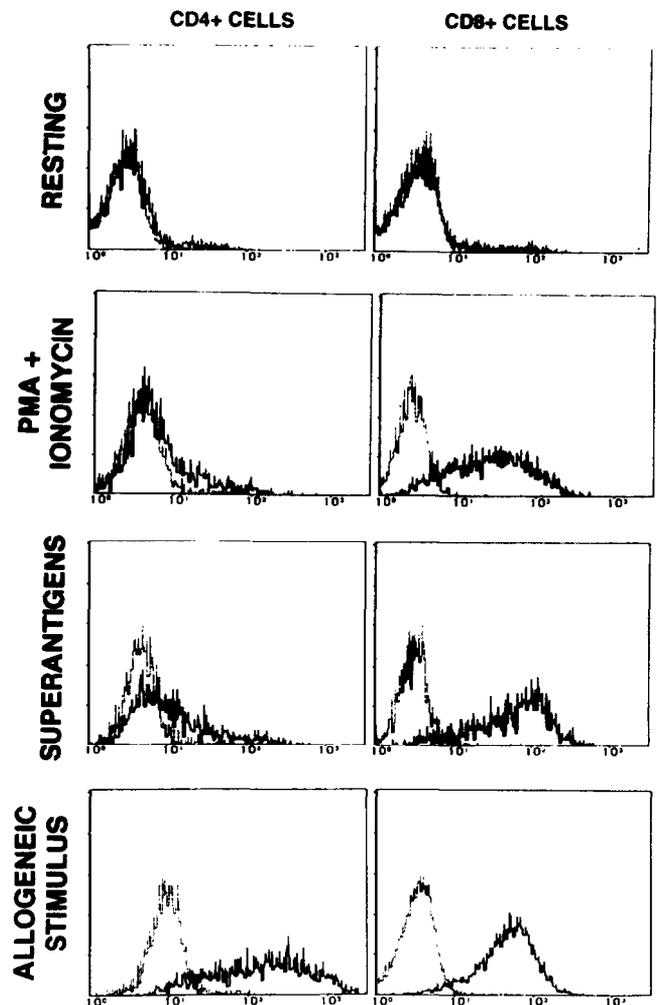

Fig. 1. CD70 expression on purified CD4 $^+$ or CD8 $^+$ lymphocytes, obtained by positive selection with magnetic beads, was evaluated on cells cultured in RPMI-1640-10% FCS for 4 days without activating stimuli, 4 days with PMA (5 ng/ml) + Ionomycin (500ng/ml), 4 days with SEB (500ng/ml) + SEE (250 ng/ml) or 6 days with a pool of irradiated allogeneic cells. Solid lines represent the specific mAb; dotted line the isotype-matched control. On the *x* axis: green-fluorescence; on the *y* axis: number of cells.
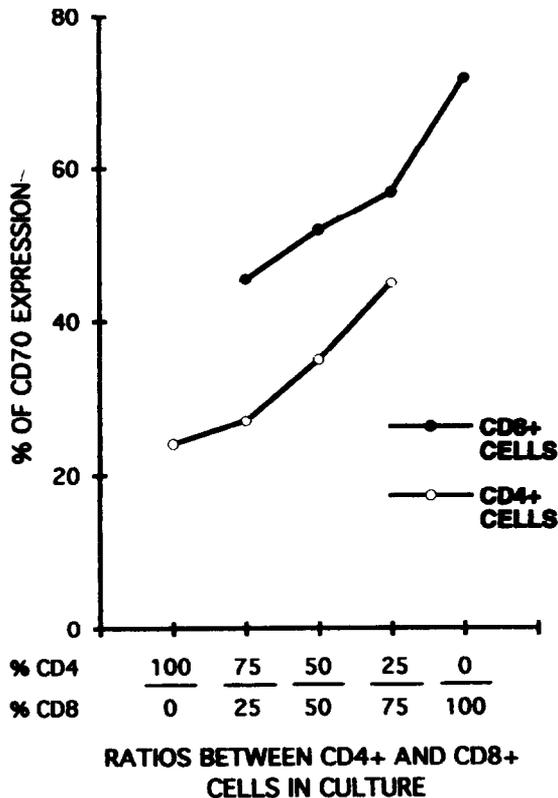
Fig. 2. Percentage of cells expressing CD70, after stimulation with PMA (5ng/ml) + Ionomycin (500ng/ml) for 4 days, as evaluated by two-colour immunofluorescence in cultures of purified CD4$^+$ or CD8$^+$ lymphocytes obtained by positive selection with magnetic beads, mixed at fixed ratios.

purified CD4$^+$ cells stimulated with PMA + Ionomycin were incubated for 3 min at room temperature with acid buffer (pH 4.1) in order to remove possible sCD27 bound to membrane CD70 (as described in the CD40L/sCD40 system; [19]). However, in neither experiments were we able to demonstrate an effect of sCD27 in reducing detection of CD70 molecule on activated cells (data not shown).

3.3. CD70 expression by different subsets of CD4$^+$ or CD8$^+$ lymphocytes separated according to the expression of different isoforms of CD45 antigen

Purified CD4$^+$ or CD8$^+$ were further divided into CD45RA$^+$ and CD45RO$^+$ by negative selection with magnetic beads and stimulated with PMA + Ionomycin as above. While no difference was observed among CD8$^+$ subsets, CD70 was preferentially expressed by the CD4$^+$CD45RO$^+$ subset as compared to the CD4$^+$CD45RA$^+$ subset (47 ± 26% vs. 21 ± 12%; $n = 5$; $P = 0.06$).

3.4. CD70 is expressed by T-cells chronically activated in vivo

CD70 was expressed in vivo by CD4$^+$ cells in conditions characterized by allogeneic reaction, such as engraftment of maternal T-cells in T$^-$B$^-$ Severe Combined Immune Deficiency (two cases: 31 and 23%, respectively; Fig. 3). In primary immunodeficiencies with residual circulating activated T-cells, as in Omenn's syndrome, a condition clinically resembling a Graft-Versus-Host reaction (five cases; Fig. 3) or combined immunodeficiency (four cases), expression of CD70 was observed on a large number of CD4$^+$ cells (35 ± 19%, $P < 0.01$ vs. healthy controls). In addition, CD70 was expressed also in the setting of autoimmune reactions by CD4$^+$ lymphocytes isolated from synovial fluid from patients with rheumatoid (four cases; Fig. 3) or psoriatic (two cases; Fig. 3) arthritis (41 ± 23%, $P = 0.01$ vs. controls).

In all cases, CD70$^+$ cells expressed activation markers (CD25, HLA-DR) and were of 'memory' phenotype (> 97% CD45RO$^+$).

3.5. Crosslinking of CD70 enhances T-cell proliferation

To assess the hypothesis that CD70 itself can transduce cellular activation signals, as suggested for other members of the TNF family [20–22], we investigated

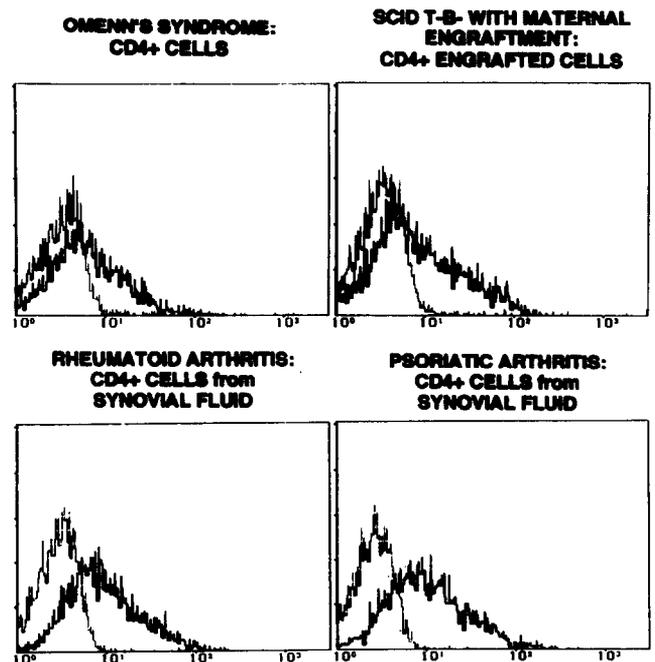Fig. 3. CD70 expression on fresh purified CD4$^+$ cells, obtained from peripheral blood or synovial fluid of patients with diseases characterized by chronic immune activation. Solid lines represent the specific mAb: dotted line the isotype-matched control. On the x axis: green-fluorescence; on the y axis: number of cells. The cases shown here are representative of a larger number of patients (see text).
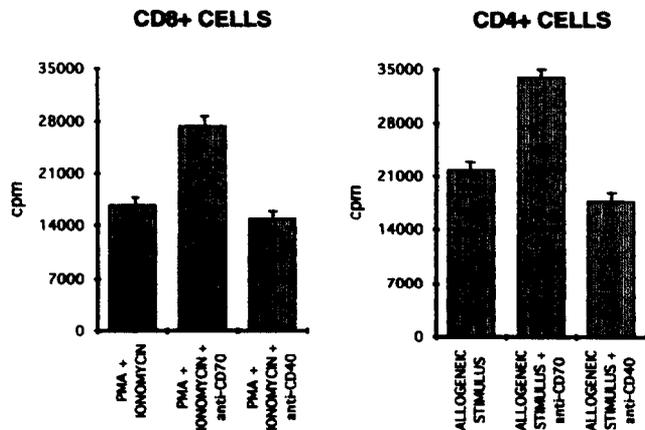
CD8+ CELLS

CD4+ CELLS

Fig. 4. Proliferative response of purified CD4+ or CD8+ lymphocytes, obtained by positive selection with magnetic beads, induced by stimuli used to elicite CD70 expression (for CD8+ cells: PMA 5 ng/ml + Ionomycin 500 ng/ml for 4 days, left; for CD4+ cells: pool of irradiated allogeneic cells for 6 days, right) was enhanced by crosslinking of CD70. One representative experiment of three, means of triplicate culture; horizontal bars represents ISD.

the proliferative capacity of purified T-cell populations exposed to stimuli able to induce CD70 expression on them. As shown in Fig. 4, proliferation of both PMA + Ionomycin-activated CD8+ cells and MLC-activated CD4+ cells was consistently enhanced by crosslinking of CD70 with an immobilized antibody, but not by an indifferent antibody.

4. Discussion

CD70 expression in vivo is demonstrable at a low density only in a few perifollicular lymphoid blasts [23]. We demonstrated that a large proportion of CD8+ cells, if purified and cultured in the absence of other populations, expresses CD70 after appropriate in vitro activation. This is at variance with data previously reported derived by studies of bulk cultures of PBMC. This difference is not explained by the use of different stimuli: in the present study CD70 expression was induced on highly purified CD8+ cells using stimuli indipendent on the effect of antigen-presenting cells (PMA + Ionomycin; Superantigens), that may be intrinsically more effective in the induction of CD70 expression than those used in previous studies (PHA, anti-CD3). However, when the same powerful stimuli were added to bulk cultures of PBMC, CD70 expression did not differ from those obtained with PHA, and this was similar to those previously reported [15]. An alternative explanation is that CD70 expression by CD8+ cells is down-modulated or masked when other cell populations are present. We have confirmed this hypothesis and defined the population responsible for this phenomenon showing that removal of CD4+ cells from cultures of total PBMC is sufficient to allow

activated CD8+ cells to fully acquire their CD70 expression and, vice versa, that the number of activated CD8+ gaining CD70 expression, if properly stimulated, depends on the proportion of CD4+ cells present in the culture.

One possible mechanism accounting for this phenomenon is the production of a soluble form of CD27 (sCD27), generated by proteolytic cleavage of the putative CD70-binding domain by activated T-cells [17,18]. sCD27 released in the culture supernantant could mask the binding site of CD70 for the 2F2 mAb [11] used in this study. We have challenged this hypothesis adding the supernatant of activated CD4+ lymphocytes to activated CD8+ cells before staining for CD70, to allow competition with the monoclonal antibody; in addition, we have attempted to remove the possible sCD27 bound to CD70 by acid treatment before the staining. Although both these attempts were unsuccessful, the possibility of interference by sCD27 cannot be definitively excluded. Alternatively, activated CD4+ cells may induce a down-modulation of CD70 expression through other mechanisms (e.g. cell-to cell contact, or cytokine signalling). The expression of CD70 by T-cells after an activating signal is transient [15], but the mechanisms operating in the down-modulation of this molecule are still unknown. In analogy with what happens for CD40L, another molecule of the TNF family, rapidly down-modulated after contact with its receptor [24], it can be suggested that CD70 down-modulation follows contact with CD27. The converse, i.e., CD27 down-modulation after CD27/CD70 interaction has already been clearly demonstrated [10,15].

The limited number of CD4+ cells that can express CD70 after stimulation with PMA + Ionomycin mainly belong to the CD45R0+ subset. This is in accordance with previous data showing that, among CD4+ cells, CD70 is expressed preferentially by CD45R0+ cells upon activation with PHA, and in long-term culture is mainly restricted to CD4+CD45R0+ clones [15]. The preferential expression of CD70 by CD4+CD45R0+ cells might play a functional role in the process of cooperation of this subset with other lymphoid cell populations. In particular, CD70 might play a role in 'helper' T-cell signalling with B-cells and cytotoxic T-cells, as the interaction of CD70 with its counterreceptor CD27, expressed on B-cells and CD8+ cells, drives B-cell growth and differentiation to produce IgG and IgM [7] and CD8+ alloantigen-induced proliferation and cytolytic activity [25], respectively. Moreover, since CD27 is preferentially expressed by CD45RA+ cells [26,27], and the costimulatory signals accepted by CD27 act more efficiently in these cells [8], CD27/CD70 interaction might play an important role in the communications between CD45RA+CD27+ and activated CD45R0+CD70+ CD4+ cell subsets. This interaction (or that with activated CD70+CD8+cells) may inter-

vene in the amplification of the immune response, reducing the threshold for activation of CD4$^+$ CD45RA$^+$ recruited in a 'second wave' of the response [6], acting sinergically with the CD28 ligands, CD80/CD86 [9]. Since the signals delivered to CD4$^+$ cells through the CD27 pathway are somehow qualitatively different from those acting through CD28 [9], it has been suggested that this might also contribute to the diversification of the immune response.

On the other hand, allogeneic cells can induce CD70 expression on both CD4$^+$ and CD8$^+$ cells in vitro. This finding is accounted for by the recent demonstration of the important role played by APC-derived signals in the induction of CD70 expression on primed T-cells (Lens SM et al., in press). The demonstration that in vivo, in conditions characterized by an allogeneic response or chronic immune activation, a large number of T lymphocytes (higher than that obtained in vitro by stimulation with mitogens on purified T-cells) express CD70 on the membrane of activated T lymphocytes provides support to data emerging from in vitro experiments, indicating the need for signals by APC in CD70 expression induction. Moreover, this observation suggests a relevant functional role of this molecule in the processes of lymphocyte activation and cell-to-cell communications. This role may not be simply restricted to bind to and signal through CD27 on other cell populations. In fact, we have shown that cross-linking of CD70 enhances the proliferative response induced by the stimuli used to elicit CD70 expression. Our results confirm preliminary observations [3] and demonstrate that CD70 itself can transduce activation signals. Interestingly, CD70 is associated with an undefined transmembrane protein, which can be the target of tyrosine phosphorylation [10]. These data therefore suggest that the effects of the interactions between CD27 and CD70 should be analyzed in both directions. This couple of counterreceptors adds to others identified in recent years operating in the regulation of T-cell clonal expansion and effector function, whose interrelationships have just started to be studied for their possible implications in the pathogenesis of immune dysregulation [6,15].

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