CD46-mediated costimulation induces a Th1-biased response and enhances early TCR/CD3 signaling in human CD4$^+$ T lymphocytes

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The role of membrane cofactor protein (MCP, CD46) on human T cell activation has been analyzed. Coligation of CD3 and CD46 in the presence of PMA or CD28 costimuli enhanced IL-2, IFN-γ, or IL-10 secretion by CD4$^+$ T lymphocytes. The effect of CD46 on IL-10 secretion did not require additional costimuli like anti-CD28 antibodies or phorbol esters. CD46 also enhanced IL-2 or IFN-γ secretion by CD4$^+$ blasts. In contrast, IL-5 secretion was inhibited upon CD46-CD3 coligation, in all the cells analyzed. These effects were independent of IL-12 and suggest that CD46 costimulation promotes a Th1-biased response in human CD4$^+$ T lymphocytes. CD46 enhanced TCR/CD3-induced tyrosine phosphorylation of CD3ζ and ZAP-70, as well as the activation of the ERK, JNK, and p38, but did not modify intracellular calcium. The effect of specific inhibitors shows that enhanced ERK activation contributes to augmented IFN-γ and lower IL-5 secretion and, consequently, to the Th1 bias. Cross-linking CD46 alone induced weak tyrosine phosphorylation of CD3ζ and ZAP-70. However, CD46 cross-linking by itself did not induce cell proliferation or lymphokine secretion, and pretreatment of CD4$^+$ T lymphocytes with anti-CD46 antibodies did not significantly alter TCR/CD3 activation.

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1 Introduction

Membrane cofactor protein (MCP, CD46) is a type 1 transmembrane glycoprotein widely expressed by nucleated cells [1–3]. CD46 is a complement regulatory protein (CRP) binding C3b and C4b deposited on the host cell, acting as a cofactor for their inactivation through proteolytic cleavage by factor I; thus protecting the cells from the attack by homologous complement [3, 4]. The extracellular portion of CD46 consists of four domains known as short consensus repeats (SCR) [also known as complement control protein repeats (CCP)], characteristic of proteins of the family of 'regulators of complement activation' (RCA), and an alternatively spliced serine-, threonine-, proline-rich region (STP region) [5–7]. In addition, alternative splicing produces two possible cytoplasmic domains (CYT-1 and CYT-2) of 16 and 23 amino acids, respectively [6, 7]. Consequently, polymorphisms of the STP and cytoplasmic domains produce multiple isoforms of CD46 [6, 7].

Like other members of the RCA family, MCP also serves as a cell receptor for human pathogens [8]. MCP binds the hemagglutinin of several strains of measles virus (MV) [9, 10], the pili of *Neisseria gonorrhoeae* and *Neisseria meningitidis* [11], the M protein of *Streptococcus pyogenes* [12, 13], and human herpesvirus 6 [14].

In addition to its functions as a regulator of complement and as a receptor of pathogenic microorganisms, CD46 possesses the potential of inducing cell signaling. In human macrophages, CD46 ligation by MV, antibodies or C3b can selectively inhibit IL-12 production [15], although certain antibodies and strains of MV can enhance IL-12 production with concomitant dissociation of the SHP-1 protein tyrosine phosphatase [16]. The cytoplasmic domains of CD46 associate with macrophage kinases and can be tyrosine phosphorylated [17], and recent data show that CYT-2, and not CYT-1, cytoplasmic domains are the targets for src tyrosine kinases like Lck in Jurkat or epithelial cells [18]. Furthermore, Wong et al. have observed that mouse macrophages transfected with

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Abbreviations: CRP: Complement regulatory protein(s) **CYT:** Cytoplasmic domain **ERK:** Extracellular signal-regulated kinase **HRP:** Horseradish peroxidase **JNK:** Jun N-terminal kinase **MAP kinase:** Mitogen-activated protein kinase **MCP:** Membrane cofactor protein **MEK:** MAP kinase/ERK kinase **MV:** Measles virus **p38:** p38/HOG, stress-activated protein kinase 2 **PDB:** Phorbol di-butyrate **RCA:** Regulators of complement activation **Tr1:** T regulatory cell 1

human CD46 possessing CYT-1, but not CYT-2, mediate the enhanced production of nitric oxide (NO) in response to MV infection in the presence of IFN-γ, through the production of IFN-α/β [19, 20]. These data show the signaling potential of CD46, which is also set forth by the CD46-dependent calcium flux induced by binding of *N. gonorrhoeae* pili to human epithelial cells [21].

We have described that the mouse CRP Crry/p65 is a costimulator for TCR/CD3-mediated activation of mouse T lymphocytes [22]. In view of these results, we performed the experiments described here, to analyze the costimulatory effect of human MCP, as a type I membrane protein with CRP function homologous to mouse Crry/p65. CD46 augmented CD3-induced tyrosine phosphorylation of several cell polypeptides basic to TCR/CD3 signaling, including the CD3 ζ chains and the ZAP-70 tyrosine kinase, and enhanced the activation of classical as well as the stress-activated mitogen-activated protein (MAP) kinases. Furthermore, CD46 by itself induces a low level of CD3ζ and ZAP-70 tyrosine phosphorylation. We also observed that CD46 costimulated human $CD4^+$ lymphocyte proliferation and IL-2, IFN-γ, and IL-10 secretion, but not IL-5 production, independently of IL-12 production by accessory cells. Thus, in addition to its possible effect as a T regulatory cell 1 (Tr1) differentiation promoter [23], CD46 might favor a Th1 type of response, increasing IL-2 and IFN-γ but not IL-5 secretion.

2 Results

2.1 CD46 costimulation of $CD4^+$ T cell proliferation and cytokine secretion

To analyze the potential of CD46 as a costimulator, $CD4^+$ T cells isolated from peripheral blood were cultured in the presence of anti-CD3 and anti-CD46 mAb. CD3 + CD46 ligation significantly enhanced IL-2, IFN-γ or IL-10 secretion when PMA (Fig. 1) or anti-CD28 antibodies (Fig. 2) were present. Furthermore, anti-CD46 mAb enhanced anti-CD3 mAb-induced proliferation, as determined by a colorimetric assay and by cell cycle analysis (data not show). Interestingly, CD46 clearly enhanced IL-10 secretion, even if additional costimuli like anti-CD28 antibodies or PMA were absent (Fig. 1, 2). In contrast, IL-5 secretion was inhibited by coligation of CD3 + CD46. Similar results were observed in isolated naïve $CD4^+$ T cells or by using C3b as a CD46 ligand (Sánchez et al., unpublished data).

CD46 ligands have been described to enhance or inhibit IL-12 secretion by accessory cells [15, 16]. To discard a role of IL-12 in the observed effects, the IL-12 p40 and

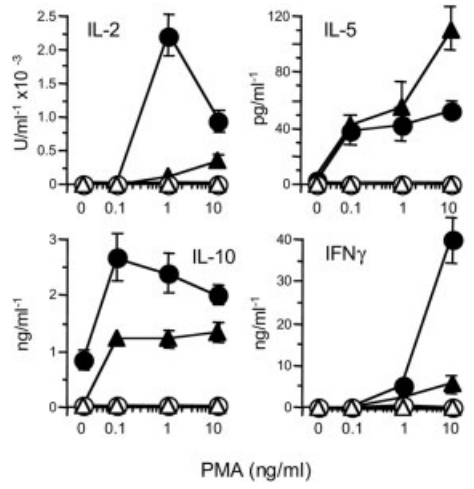

Fig. 1. CD46 ligation serves as a costimulus for CD3-induced activation of human $CD4^+$ T lymphocytes. Plate-bound anti-CD46 (158.2A5, 20 μg/ml, open circles), anti-CD3 (OKT-3, 10 μg/ml, closed triangles), anti-CD3 + anti-CD46 (closed circles), or control antibodies (open triangles) + PMA were used to activate the cells, as indicated in the figure. Costimulation was determined as IL-2, IL-5, IL-10, or IFN-γ secretion at 72 h of culture. Data represent the means ± SE of triplicate determinations of one representative experiment of five separate experiments.

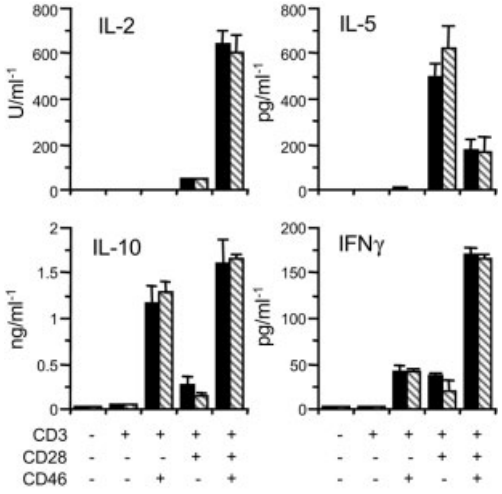

Fig. 2. CD46 costimulation of CD3 activation in human $CD4^+$ T lymphocytes: Effect of anti-CD28 and anti-IL-12 antibodies. Cells were activated with plate-bound anti-CD46, anti-CD3, anti-CD3 + anti-CD46, or control antibodies + anti-CD28 antibody, as indicated in the figure. Blocking anti-IL-12 mAb was added (▨) or not (■) to the cultures. IL-2, IL-5, IL-10, or IFN-γ secretion at 72 h of culture was determined. Data represent the means ± SE of triplicate cultures of one representative experiment of three separate experiments.

p70 content in culture supernatants from different experiments was analyzed by specific capture ELISA. In all the cases, the IL-12 content was below the detection limit of the assay (4 pg/ml, data not shown). In addition, we also used neutralizing anti-IL-12 antibodies under conditions previously used to inhibit IL-12-mediated effects on lymphocyte activation [23], showing no detectable effect of these antibodies in our system (Fig. 2 and data not shown).

Since $CD4^+$ T cell blasts were used to detect early activation events or the effect of metabolic inhibitors (see below), the effect of CD46 costimulation in these cells was also analyzed. We observed that IL-2 and IFN-γ secretion was enhanced by CD46 (Fig. 3), although IL-2 secretion was dependent on additional costimuli like PMA (Fig. 3) or CD28 (data not shown). Flow cytometry of intracellular cytokines showed that this was due to an enhanced number of cytokine-producing cells (data not shown). As in normal, resting peripheral $CD4^+$ T lymphocytes, IL-5 secretion was slightly inhibited upon CD46-CD3 coligation (Fig. 3). In blasts, IL-10 secretion was clearly lower than in $CD4^+$ T lymphocytes, and CD46 did not enhance IL-10 secretion under the experimental conditions tested (Fig. 3). Taken together, our results suggest that CD46 favors a Th1 lymphokine profile in human T lymphocytes.

2.2 Effect of CD46 on early TCR/CD3-mediated signals

Epithelial cells stimulated by *Neisseria* pili undergo a transient, CD46-dependent Ca^{2+} flux [21]. Since Ca^{2+} fluxes are an early signal of TCR/CD3 activation, we analyzed the potential of anti-CD46 antibodies to induce Ca^{2+} fluxes in human T cells or to enhance CD3-induced calcium fluxes. Surprisingly, we detected no Ca^{2+} flux induced by anti-CD46 mAb, even after cross-linking with an anti-mouse Ig antibody (Fig. 4). Furthermore, anti-CD46 mAb did not enhance anti-CD3 mAb-induced Ca^{2+} fluxes, before or after cross-linking. This experiment was repeated several times with Jurkat cells (shown in Fig. 4), but also with $CD4^+$ T cell blasts, using different ratios and concentrations of anti-CD3 and anti-CD46 antibodies as well as different anti-CD46 mAb; always with the same negative results (Fig. 4 and data not shown).

CD46 cross-linking has recently been shown to induce tyrosine phosphorylation of several cell substrates in Jurkat cells and T lymphocytes. These substrates include $p120^{CBL}$, LAT, Vav, and CD46 itself, and at least in Jurkat cells, the tyrosine phosphorylation depends on Lck [18, 24–26]. Since LAT and Vav are substrates for the ZAP-70 tyrosine kinase [27, 28] and ZAP-70 activation depends on its binding to phosphorylated immunoreceptor tyrosine-based activation motifs (ITAM) of CD3 ζ chains [29, 30], we set out to perform experiments to

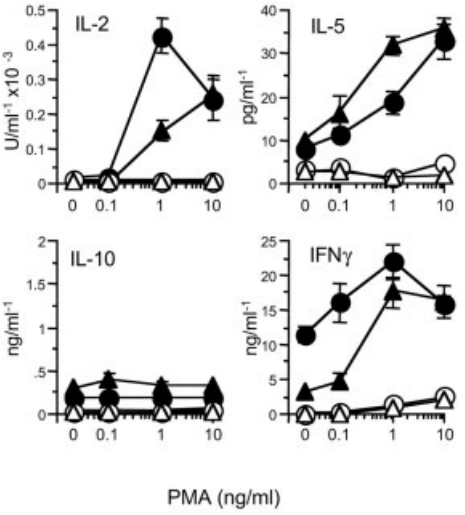

Fig. 3. Effect of plate-bound anti-CD46 mAb on the activation of $CD4^+$ T cell blasts by anti-CD3 mAb. Supernatants of cells activated for 24 h with antibodies + PMA were used to determine IL-2, IL-5, IL-10, or IFN-γ, as shown in the figure. Symbols as described in Fig. 1. Data represent the means $\pm$ SE of triplicate determinations of one representative experiment of four separate experiments.

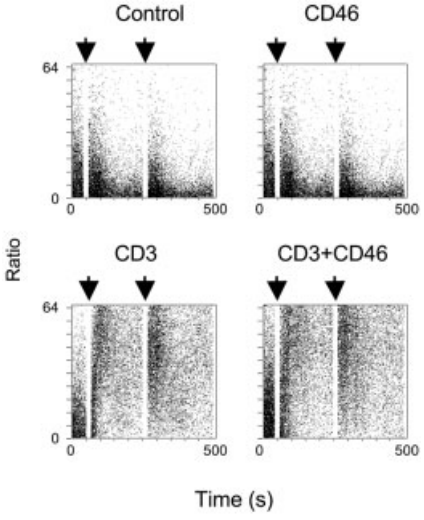

Fig. 4. CD46 cross-linking by antibodies does not change intracellular Ca^{2+}. Anti-CD46 (158.2A5, 20 μg/ml), anti-CD3 (OKT-3, 10 μg/ml) or control antibodies were added at the times indicated (open arrows); then a cross-linking rabbit anti-mouse Ig antibody (20 μg/ml) was added (closed arrows). Intracellular calcium was determined by flow cytometry in cells loaded with Fluo 3AM and Fura RedAM.

determine the effect of CD46 cross-linking on the phosphorylation of CD3 ζ chains and ZAP-70 (Fig. 5). As shown in this figure, cross-linking of CD46 induced low-level tyrosine phosphorylation of both CD3ζ and ZAP-70 (Fig. 5A, B; lane 2). The low-level activation of CD3ζ and ZAP-70 by CD46 cross-linking fit with previous data showing CD46-induced tyrosine phosphorylation of LAT [26], a major cell substrate of ZAP-70. However, CD46 had a stronger effect when cross-linked together with CD3. Under these conditions, CD46 clearly enhanced CD3-induced tyrosine phosphorylation of CD3ζ (Fig. 5A; lane 4) or ZAP-70 (Fig. 5B; lane 4).

2.3 Pre-incubation with CD46 ligands does not alter CD3-mediated activation

Our results and previous data show that CD46 ligands induce low-level tyrosine phosphorylation of different early activation substrates including TCRζ, ZAP-70, LAT, Vav, or $p120^{CBL}$ in T lymphocytes (Fig. 5, [25, 26]) and that they can also induce calcium fluxes [21]. Since these weak signals might lead to altered (*i.e.* lower) T lymphocyte activation [31, 32] contributing to pathogen-mediated immunosuppression, we analyzed the effect of pre-incubation of $CD4^+$ T lymphocytes with CD46 ligands on TCR/CD3 activation. To do this, the lymphocytes were cultured with anti-CD46 mAb-coated beads for 24 h, washed and then activated with different stimuli. Fig. 6 shows that pretreatment with anti-CD46 mAb did not significantly alter the activation of T cells, as measured by anti-CD3 antibody-induced T cell proliferation in the presence or absence of phorbol esters or by activation using combinations of ionomycin and phorbol esters. Similar results were obtained when IL-2, IL-5, IL-10, or IFN-γ secretion was determined (data not shown).

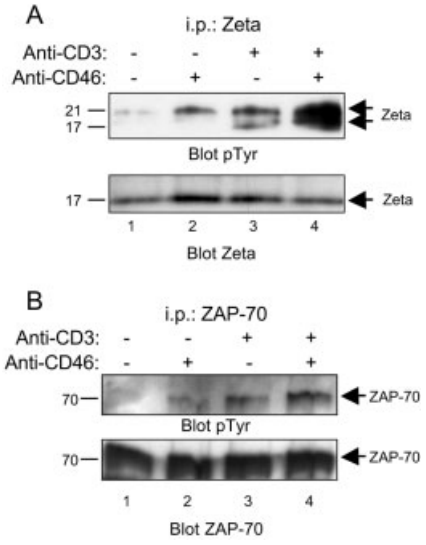

Fig. 5. Effect of CD46 and CD3 ligation on the tyrosine phosphorylation of ζ and ZAP-70. $CD4^+$ blast cells were activated for 5 min using latex microspheres coated with antibodies (20 μg/ml anti-CD46, 10 μg/ml anti-CD3, or control antibodies), as indicated. Post-nuclear cell lysates were immunoprecipitated with antibodies against ζ chains (A) or ZAP-70 (B). The immunoprecipitates were separated by electrophoresis and analyzed by immunoblot with anti-phosphotyrosine antibody and chemiluminescence, then stripped and reprobed with anti-ζ or anti-ZAP-70 antibodies and chemiluminescence, as shown in the figure. Figs. in the blots indicate molecular masses (M_r) in kDa.

2.4 CD46 costimulation of MAP kinase activation

Since we have observed that Crry, a protein functionally related to CD46, enhanced extracellular signal-regulated kinase (ERK) activation in mouse T lymphocytes [22], we next examined the effect of CD46 ligation on the activation of different MAP kinases. These included ERK-1 and ERK-2, as well as the stress-activated MAP kinases c-Jun N-terminal kinase (JNK) and p38/HOG (p38), which are implicated in the regulation of different transcription factors basic to cytokine secretion. Fig. 7A shows that ligation of CD3 + CD46 synergized for ERK activation, confirming recent results by Zaffran et al. [25].

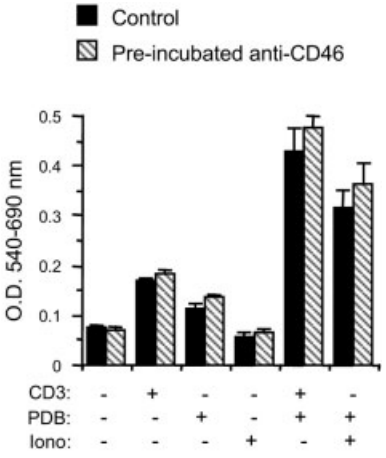

Fig. 6. Pre-incubation of peripheral blood $CD4^+$ T cells with anti-CD46 antibodies does not modify CD3-dependent or -independent activation. Cells (10^6/ml) were incubated for 24 h in the presence of latex microspheres (10^6/ml) pre-coated with anti-CD46 antibodies (10 μg/ml 158.2A5, ▨) or control antibodies (10 μg/ml 3D3, ■). Then, the cells were washed and cultured at 10^5 cells/well for a further 72 h in the presence of plate-bound OKT-3 (10 μg/ml), 10 ng/ml PDB, and ionomycin (1 μg/ml), as shown in the figure. Data represent the means ± SE of triplicate determinations of one representative experiment of two separate experiments.

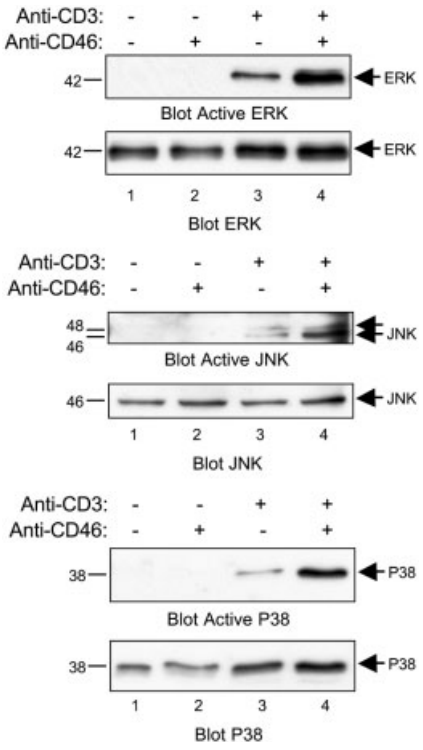

Fig. 7. Effect of CD3 and CD46 ligation by antibodies on the activation of the ERK, JNK and p38 MAP kinases. $CD4^+$ blast cells were activated for 5 min with anti-CD3 (OKT-3, 10 μg/ml) and anti-CD46 (158.2A5, 10 μg/ml) mAb adsorbed to latex beads. Post-nuclear lysates were separated by electrophoresis and analyzed by immunoblot with antibodies specific for active, dually phosphorylated forms of ERK (A), JNK (B), and p38 (C) and chemiluminescence. Then, the membranes were stripped and reprobed with antibodies to ERK, JNK, and p38, respectively, and chemiluminescence. Figs. in the blots indicate molecular masses (M_r) in kDa.

Furthermore, we observed that CD3 and CD46 signaling also synergized for the activation of JNK (Fig. 7B) and p38 (Fig. 7C).

2.5 Effect of metabolic inhibitors on lymphokine secretion

To confirm the role of MAP kinases and other activation cascades in CD46 costimulation, the effect of inhibitors blocking different pathways in cells activated by CD3 or by CD3 + CD46 was analyzed. Preliminary experiments using a 3-day culture of primary $CD4^+$ T lymphocytes showed that the p38 inhibitor SB203580 (10 μM) strongly suppressed CD3- or CD3 + CD46-mediated activation, as measured by proliferation or by IL-2 and IFN-γ secretion (data not shown). These results were in agreement with previous data showing that inhibitors of p38 blocked IL-2 secretion [33] or IL-2-induced proliferation of human T lymphocytes [34], and suggested that the inhibition of IFN-γ secretion in primary $CD4^+$ T cells was secondary to these effects.

To directly analyze the effect of the inhibitor, we used $CD4^+$ T cell blasts, since CD46 costimulated IL-2 and IFN-γ secretion in short-term (24 h) cultures of these cells (Fig. 3). In addition to the p38 inhibitor SB203580, we also used inhibitors of molecules involved in different activation cascades triggered by TCR/CD3 ligation, including PD98059, an inhibitor of MAP kinase/ERK kinase (MEK)1 blocking ERK activation; RO-31–8220, an inhibitor of Ca^{2+}-dependent and -independent PKC isoforms; or the calcineurin inhibitor cyclosporin.

Inhibition of MEK by PD98059 clearly inhibited IL-2 and IFN-γ synthesis, particularly in CD46-costimulated cultures (Fig. 8A, B). On the contrary, blocking ERK markedly fostered IL-5 secretion, suggesting that ERK activation is a strong negative regulator of IL-5 and favors IFN-γ production in this system (Fig. 8C). The p38 inhibitor SB203580 had negligible effects on IFN-γ, yet IL-2 secretion was clearly enhanced upon p38 inhibition, and IL-5 synthesis was unaffected (Fig. 8). Inhibition of PKC produced effects roughly similar to those of the MEK1 inhibitor PD98059, namely strong (IL-2) or partial (IFN-γ) inhibition of secretion and enhanced secretion of IL-5 (Fig. 8). The addition of the calcineurin inhibitor cyclosporin clearly inhibited the secretion of all cytokines analyzed, particularly IL-2 and IFN-γ (Fig. 8).

3 Discussion

In a previous publication, we observed that Crry/p65, the main CRP of mouse T lymphocytes, costimulated mouse $CD4^+$ T lymphocyte activation [22]. Here, we show that CD46, a type 1 human cell surface molecule that, like Crry, has cofactor effect for C3b and C4b inactivation by plasma factor H, costimulates CD3-induced proliferation and lymphokine secretion by $CD4^+$ T cells (Fig. 1–3 and data not shown). This confirms recent data showing the costimulatory effect of CD46 in human $CD4^+$ T lymphocytes [25, 26], extending the data to show that CD46 enhances proliferation and IL-2 secretion, but also IL-10 and IFN-γ secretion (Fig. 1–3). Interestingly, the effect of CD46 on IL-2 and IFN-γ needs additional costimuli delivered by PMA or CD28 (Fig. 1, 2), whereas costimulation of IL-10 secretion can be also observed without these costimuli (Fig. 1, 2), in agreement with recent data by Kemper et al. [23]. CD46 costimulation is selective, as it does not enhance but inhibits CD3-induced secretion of the IL-5 Th2 lymphokine (Fig. 1–3). Furthermore, IFN-γ and IL-2 secretion are enhanced by CD46 under conditions (*i.e.* in blast cells; Fig. 3) where it

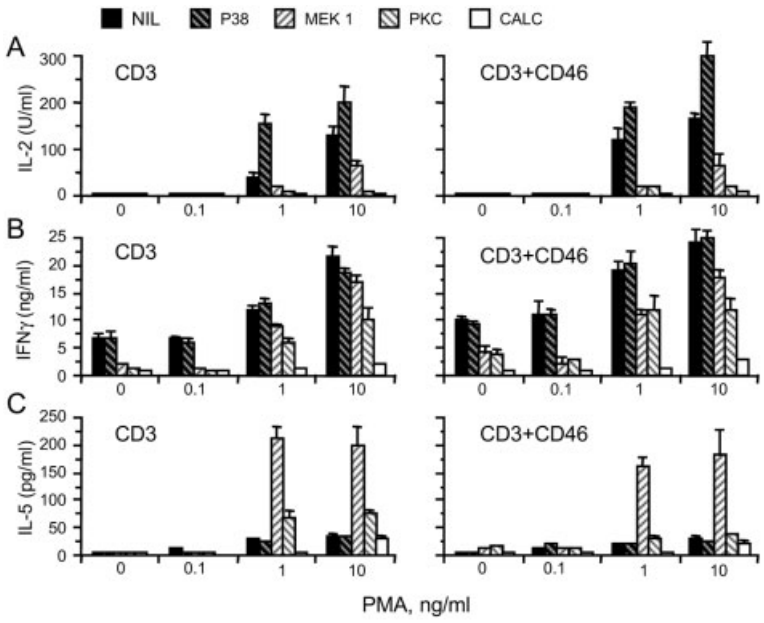

Fig. 8. Effect of enzyme inhibitors on CD3 activation and CD46 costimulation. $CD4^+$ T cell blasts were activated for 24 h with plate-bound anti-CD3 (10 μg/ml OKT-3 + 10 μg/ml control antibody, left panels) or anti-CD3 + anti-CD46 antibodies (10 μg/ml OKT-3 and 10 μg/ml 158.2A5) and PMA at the concentrations indicated. IL-2 (A), IFN-γ (B) and IL-5 (C) were determined in the supernatants by capture ELISA in the presence or absence of drugs, as follows: the p38 inhibitor SB203580 (10 μM; P38); the MEK1 inhibitor PD98059 (50 μM; MEK1); the PKC inhibitor RO-31–8220 (0.5 μM; PKC); or the calcineurin inhibitor cyclosporine A (1 μM; CALC). Symbols as indicated in the figure. Cytokine content in cultures activated with anti-CD46 mAb alone or with control, irrelevant antibodies was below the detection limits of the assay (2 U/ml IL-2, 0.6 ng/ml IFN-γ, 1 pg/ml IL-5) and are not shown.

does not enhance IL-10 secretion. This suggests that CD46 favors a Th1 differentiation profile in human $CD4^+$ T lymphocytes whenever TCR + CD28 or CD28-like costimuli are provided (Fig. 1–3).

Recent data indicate that CD46 costimulation of IFN-γ might be dependent on IL-12 produced by accessory cells and could be blocked by anti-IL-12 antibodies [23]. In this regard, previous results show that CD46 ligation of human monocytes may inhibit [15] or enhance [16] IL-12 production. However, the amounts of IL-12 in our cultures were undetectable, and blocking anti-IL-12 antibodies had no significant effect on CD46 costimulation under the conditions used (Fig. 2 and data not shown). Rather, our results indicate that in the presence of TCR and CD28 ligands, CD46 might directly promote Th1 differentiation, regardless of its effect on monocytes or other accessory cells. However, this does not preclude an IL-10-mediated effect of CD46 on Tr1 differentiation of distinct $CD4^+$ subpopulations, as recently observed by Kemper et al. [23], particularly when CD28 costimuli are absent (Fig. 1, 2). Thus, it is possible that antigen presentation by non-professional APC expressing CD46 ligands (*i.e.* MV-infected cells expressing viral hemagglutinin), but no CD28 ligands, might favor Tr1 differentiation and immune suppression, whereas professional APC expressing both CD46 and CD28 ligands might favor Th1 differentiation of effector lymphocytes and pathogen clearance.

Using human CD46-transgenic mice, Marie et al. have recently observed that $CD4^+$ T lymphocyte proliferation by anti-CD3 + anti-CD46 antibodies is enhanced in T cells expressing the short (CD46–1) but not the long (CD46–2) cytoplasmic isoform of CD46 [24]. Unlike our results in human lymphocytes (Fig. 1–3), in these transgenic mice CD46–1 inhibited and CD46–2 had no effect on IL-2 secretion, and IFN-γ secretion was significantly inhibited. Furthermore, IL-4 secretion was not changed, and CD46–2 inhibited IL-10 secretion [24]. These differences are difficult to explain in terms of the preferential expression of the Cyt2 CD46 isoforms by human T cells, even if the dominant effect of Cyt1 is taken into account [18]. Rather, they suggest differences between human and mouse T lymphocytes regarding their responses to CD46 costimulation.

One clear base for CD46 costimulation is its enhancement of TCR/CD3-mediated signals, particularly tyrosine phosphorylation of numerous protein substrates (Fig. 5, [25, 26], and unpublished data). Our results showing CD46-mediated enhancement of CD3ζ and ZAP-70 tyrosine phosphorylation (Fig. 5) provide a clear basis

to previously observed effects, since the substrates ($p120^{Cbl}$, LAT, and Vav) identified so far as being phosphorylated upon CD46 cross-linking or CD3-CD46 co-cross-linking [25, 26] are substrates of ZAP-70 [27, 28].

Our results show that CD46 costimulation enhances the stress-activated kinases JNK and p38 (Fig. 7) and confirm previous data showing that CD46 enhances the activation of the MAP kinase ERK (Fig. 7, [25]). The effect of the MEK inhibitor PD98059 on lymphokine secretion (Fig. 8) shows that ERK activation is important to the enhanced IL-2 and IFN-γ secretion induced by CD46, but also to the lower secretion of IL-5. Thus, ERK activation is a major contribution to the Th1-biased secretion pattern observed upon CD46 costimulation.

Intriguingly, p38 activation has no significant effect on IFN-γ (Fig. 8). This is in contrast with data from mouse T cells showing p38 dependence of IFN-γ activation [35]. Since IL-12-induced IFN-γ production is dependent on p38 [36], the differences might stem from the IL-12 independence of the effect of CD46 in our system (Fig. 2). Unlike primary T cells, where p38 inhibitors inhibited proliferation and IL-2 secretion in a 3-day activation assay ([33] and our own unpublished data), inhibition of p38 clearly enhanced IL-2 secretion in a short-term culture of CD4 T cell blasts, pointing to major differences in activation between these experimental systems.

As in other leukocytes, CD46 cross-linking itself can induce some signaling in T lymphocytes. Thus, CD46 induces low-level tyrosine phosphorylation or activation of different substrates including CD3ζ and ZAP-70 (Fig. 5), as well as LAT [26], Vav, Rac, and ERK [25], an effect that might produce lower responses upon TCR/CD3 activation. However, our results show that pre-incubation of T lymphocytes with anti-CD46 antibodies does not significantly inhibit subsequent CD3-mediated activation of T cells, even under suboptimal activation conditions (Fig. 6 and data not shown).

Unlike data obtained in epithelial cells using *Neisseria* pili as CD46 ligands [21], we have not detected a calcium rise upon CD46 cross-linking by antibodies in human T cells (Fig. 4 and data not shown). The reason for these different results is not clear, although *Neisseria* pili might bind CD46 as well as other cell surface molecules contributing to the CD46-dependent calcium rise, an effect that is difficult to conceive when specific anti-CD46 mAb are used as ligands.

In summary, CD46 is a costimulatory molecule with the potential to bias T cell differentiation towards a Th1 (this paper) or a Tr1 phenotype [23]. These different capabilities might underlie the success or failure in mounting an effective immune response to pathogens directly or indirectly binding CD46.

4 Materials and methods

4.1 Antibodies and other reagents

The following mouse mAb were used: OKT-3 (anti-CD3), OKT-4 (anti-CD4), OKT-8 (anti-CD8), OKM1 (anti-CD11b), L243DR (anti-human HLA-DR), and 158–2A5 (mouse IgG1 anti-human CD46, a kind gift of Dr. J. Vives, Hospital Clínic i Provincial, Barcelona, Spain). 158–2A5 bound a site in CD46 overlapping or close to the sites of C3 and C4 binding, as judged by flow cytometry assays showing enhanced C' deposition on cell surfaces (data not shown). 3D3, a clonotypic mouse IgG1 mAb, was used as filling antibody. All antibodies were used as protein A- or protein G-purified preparations of hybridoma supernatants. Anti-CD28 (CD28.2) and a neutralizing anti-human IL-12 p40/p70 (C8.6) antibody were from Becton Dickinson. Horseradish peroxidase (HRP)-coupled anti-phosphotyrosine mAb PY-20 was obtained from Amersham Pharmacia Biotech (Little Chalfont, GB).

Rabbit anti-ZAP-70 antibodies were obtained by immunization with a fusion protein of glutathione-S-transferase (GST) and a fragment coding the sequence between residues 266 and 344 of mouse ZAP-70. Rabbit anti-ζ antibodies were obtained by immunization with a synthetic peptide corresponding to residues 132–144 of mouse ζ chains coupled to ovalbumin. Anti-ZAP-70 or anti-ζ antibodies were purified by affinity chromatography over the immunizing protein or peptide coupled to Sepharose. For immunoprecipitation, the anti-ZAP-70 or anti-ζ antibodies were coupled to CNBr-Sepharose. Rabbit anti-active-ERK antibodies were from New England Biolabs (Beverly, MA). Rabbit anti-active-JNK, and anti-active-p38 antibodies were from Promega (Madison, WI). Rabbit anti-ERK, anti-JNK1 and anti-p38 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). FITC-conjugated goat anti-mouse IgG was from Calbiochem (San Diego, CA). HRP-conjugated anti-rabbit Ig antibodies were from Sigma Chemical Co. (St. Louis, MO).

The calcineurin inhibitor cyclosporin A, PMA and phorbol dibutyrate (PDB) were from Sigma. The MEK1 inhibitor PD98059, the p38 inhibitor SB203580, the PKC inhibitor RO-31–8220, and ionomycin were from Calbiochem.

4.2 Cells and cell lines

The Jurkat T cell lymphoma was maintained in RPMI 1640 medium supplemented with 200 mM L-glutamine, 5×10^{-5} M 2-ME, 10 % heat-inactivated FBS and antibiotics (culture medium).

$CD4^+$ T lymphocytes were isolated from peripheral blood of normal, healthy donors that had been informed of the purposes of the study and after their written consent, or from buffy coats of anonymous normal donors. The study followed institutional and national guidelines and had been approved by the Ethics Review Board of the institution. Mononuclear cells were obtained by Ficoll-Hypaque centrifugation, washed and incubated with anti-CD8 (OKT-8), anti-CD11b (OKM1), and anti-DR antibodies (L243DR) in the cold. After washing, $CD4^+$ cells were obtained by negative selection by passing the cell suspension over mouse Ig-anti-Ig columns [37]. The cells in the eluate were >95% $CD3^+$ $CD4^+$, as determined by flow cytometry. To obtain $CD4^+$ T cell blasts, $CD4^+$ lymphocytes (10^6/ml) were activated with PHA (1 μg/ml) in the presence of syngeneic, mitomycin C-treated peripheral blood mononuclear cells (2×10^5/ml) and human recombinant IL-2 (100 U/ml). After 48 h of culture, the cells were washed and expanded for a further 3 days in the presence of 10 U/ml IL-2, washed and used for activation. The cells obtained were >98% $CD4^+$, as determined by flow cytometry.

4.3 Cell culture and activation

For short-term activation, the antibodies were adsorbed to polystyrene beads (5 μm diameter, Polysciences) by overnight incubation at 4°C in PBS. Adsorption to beads was performed by incubating 4×10^7 beads/ml with 10 μg/ml anti-CD3 mAb and 10 μg/ml anti-CD46 mAb, with 3D3 being used as filling antibody. The cells to be activated were washed twice in serum-free culture medium, resuspended in the same medium at 10^8 cells/ml and mixed 1:1 with washed antibody-coated polystyrene beads. After incubation for 5 min at 37°C, 1 ml of ice-cold PBS, 500 μM EDTA, 200 μM sodium orthovanadate was added, and the cells were spun 2 min at 1,000×g before lysis. Where indicated, cells (10^7/ml in culture medium) were incubated with metabolic inhibitors for 30 min at 37°C, washed and then activated as described.

Activation of $CD4^+$ T lymphocytes or $CD4^+$ T cell blasts for proliferation or lymphokine secretion was performed by incubation (10^5 cells/well in 0.2 ml of culture medium) in flat-bottom 96-well culture plates previously coated with anti-CD3 and anti-CD46 antibodies, for the times specified in Sect. 2. To determine lymphokine secretion, 0.1 ml of supernatant was taken after 72 h or 24 h of culture for lymphocytes and blasts, respectively. Proliferation was determined by a colorimetric method, as described [22]. The data of one representative experiment out of at least three are shown.

4.4 Intracellular calcium

Intracellular calcium was determined by flow cytometry. Cells (2.5×10^6–5×10^6/ml) were loaded with Fluo 3AM (4 μM, Molecular Probes) and Fura RedAM (10 μM, Molecular Probes) in 0.1% Pluronic F127 by incubation for 45 min at 37°C and washed with HBSS + 1% FCS. The cells (5×10^6/ml) were analyzed by flow cytometry, and anti-CD3 (5 μg/ml), anti-CD46 (50 μg/ml), or control antibodies were added as indicated. Then, affinity-purified rabbit anti-mouse Ig antibodies (20 μg/ml) were added as cross-linkers, as shown in Sect. 2. The data was expressed as ratio of green fluorescence (FL1) to red fluorescence (FL2).

4.5 Cell lysis and immunoprecipitation

Cells were lysed on ice for 30 min at 2×10^7 cells/ml with 50 mM Tris/HCl pH 7.6, 10 mM 3-[(3-cholamido propyl)-dimethyl ammonium]-1-propane sulfonate (CHAPS), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, and 1 mM $NaVO_4$. Post-nuclear lysates were used for immunoblot or precleared by rotation with Sepharose-coupled normal rabbit IgG (1 h at 4°C, 20 μl of packed beads/10^7-cell lysate). Precleared lysates from 5×10^6–10×10^6 cells were immunoprecipitated with 15 μl of Sepharose-conjugated anti-ζ or anti-ZAP-70 antibody (2 h rotation at 4°C). Immunoprecipitates were washed five times with 50 mM Tris/HCl pH 7.6, 2 mM CHAPS, 150 mM NaCl, 200 μM $NaVO_4$, and used for immunoblot.

4.6 Immunoblot

For immunoblot of cell lysates, 10 μl (equivalent to 2×10^5 cells) were mixed 1:1 (vol/vol) with 2× reducing SDS Laemmli sample buffer. Immunoprecipitates were extracted with 1× reducing sample buffer. The samples were boiled for 5 min and separated by SDS-PAGE in 10% acrylamide gels. Proteins were transferred to PVDF membranes (Immobilon-P, Millipore), and the membranes were blocked for 2 h at room temperature with 1% BSA in TBST containing 200 μM sodium orthovanadate for phosphotyrosine blot or 3% non-fat dry milk in TBST, 200 μM sodium orthovanadate. The membranes were washed with TBST, incubated overnight in the cold with anti-active-MAP kinase antibodies or HRP-coupled anti-phosphotyrosine antibodies in 1% BSA in TBST, washed with TBST and visualized using the supersignal West Pico chemiluminiscent substrate (Pierce) for phosphotyrosine determination. For detection of anti-active-MAP kinase antibodies, the membranes were incubated for a further 2 h at room temperature with HRP-conjugated anti-rabbit IgG in 1% non-fat dry milk in TBST, washed and developed with chemoluminiscent substrate as above. Then, the membranes were washed, stripped and reprobed with antibodies specific for MAP kinases, ZAP-70, or ζ, and then with HRP-conjugated anti-rabbit or anti-mouse IgG in 1% non-fat dry milk in TBST.

4.7 Cytokine detection by ELISA

IL-2, IL-5, and IFN-γ were detected in culture supernatants by specific capture ELISA. For capture of IL-2, 534.411 (mouse IgG1, Becton Dickinson) was used, and biotinylated B33-2 (Mouse IgG1, Becton Dickinson) was used for detection of IL-2. The IL-5 ELIpair kit (Diaclone Research, Besançon, France) was used for IL-5 capture ELISA. NIB42 and biotinylated 4S.B3 (mouse IgG1, Becton Dickinson) were used as capture and detection antibodies, respectively, in IFN-γ assays. For IL-10, the antibodies JES3-19F1 and JES3-12G8 (rat IgG2a, Becton Dickinson) were used for capture and detection. The assays were performed according to the instructions of the supplier and developed using streptavidin-HRP (Amersham) and Sigma-fast *o*-phenylenediamine substrate (Sigma). Content was calculated from reference standard curves set up with recombinant cytokines.

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