The EMBO Journal vol.14 no.15 pp.3731–3740, 1995

IL-2 gene expression and NF-κB activation through CD28 requires reactive oxygen production by 5-lipoxygenase

Marek Los[1,2], Heike Schenk[2], Klaus Hexel[2], Patrick A.Baeuerle[1], Wulf Dröge[2] and Klaus Schulze-Osthoff[1,2,3]

[1]Institute of Biochemistry, Albert-Ludwigs-University, D-79104 Freiburg, and [2]Division of Immunochemistry, German Cancer Research Center, D-69120 Heidelberg, Germany

[3]Corresponding author

Communicated by P.A.Baeuerle

Activation of the CD28 surface receptor provides a major costimulatory signal for T cell activation resulting in enhanced production of interleukin-2 (IL-2) and cell proliferation. In primary T lymphocytes we show that CD28 ligation leads to the rapid intracellular formation of reactive oxygen intermediates (ROIs) which are required for CD28-mediated activation of the NF-κB/CD28-responsive complex and IL-2 expression. Delineation of the CD28 signaling cascade was found to involve protein tyrosine kinase activity, followed by the activation of phospholipase A_2 and 5-lipoxygenase. Our data suggest that lipoxygenase metabolites activate ROI formation which then induce IL-2 expression via NF-κB activation. These findings should be useful for therapeutic strategies and the development of immunosuppressants targeting the CD28 costimulatory pathway.

Keywords: CD28/costimulation/IL-2/NF-κB/ROI

Introduction

T lymphocytes require distinct signals from antigen-presenting cells for activation (reviewed by Mueller *et al.*, 1989; Jenkins and Johnson, 1993; Crabtree and Clipstone, 1994). An antigen-specific stimulus is delivered through the T cell receptor (TCR) following the binding of TCR to antigenic peptides presented by MHC molecules. However, while engagement of the TCR triggers the initial steps of T cell activation, TCR stimulation is not sufficient to ensure a full immune response. Complete T cell activation requires a second costimulatory signal, since TCR signaling in the absence of an accessory cell-derived costimulus leads to anergy and a long-lasting state of unresponsiveness to antigenic stimulation (Harding *et al.*, 1992; Crabtree and Clipstone, 1994). TCR stimulation in the absence of costimulatory signals may also lead to cell death in previously activated T cells (Punt *et al.*, 1994).

An important costimulus which can reverse induction of anergy and programmed cell death is the CD28 surface receptor that is expressed on the majority of thymocytes and peripheral T lymphocytes (reviewed by Linsley and Ledbetter, 1993; June *et al.*, 1994). CD28 is a homodimeric molecule composed of two 44 kDa subunits. Its predicted sequence is that of a receptor of the Ig superfamily with a single V_H-like domain in the extracellular part, a transmembrane region and a short intracellular domain (Aruffo and Seed, 1987). The counter-receptor for CD28 is B7 which is expressed on activated B cells, dendritic cells and macrophages (reviewed by June *et al.*, 1994). At least two forms of B7 (B7-1/CD80 and B7-2/B70) exist and both are members of the Ig superfamily. B7 also binds to CTLA-4, a receptor that is structurally related to CD28 and expressed in low copy number following T cell activation (Linsley *et al.*, 1991).

Triggering of the CD28 receptor in conjunction with stimulation of the TCR–CD3 complex strongly enhances T cell proliferation and T cell cytotoxic activity (reviewed by June *et al.*, 1990). A major effect of the CD28 costimulus consists of the enhanced production of cytokines, such as IL-2, IFN-γ, GM-CSF, TNF and others, which are upregulated by both transcriptional and post-transcriptional processes (Martin *et al.*, 1986; Guba *et al.*, 1989; Thompson *et al.*, 1989; Cerdan *et al.*, 1991, 1992). It has been observed that CD28 stimulation leads to the coordinate stabilization of the mRNAs for these cytokines (Lindsten *et al.*, 1989). In addition to these post-transcriptional events, IL-2 mRNA levels are increased due to enhanced transcriptional activity. Studies on the IL-2 promoter have identified a CD28-responsive element (CD28RE) with enhancer activity (Fraser *et al.*, 1991; Verweij *et al.*, 1991). The nucleotide sequence of CD28RE is structurally related to the classical κB consensus sequence and was found to bind a CD28-responsive complex (CD28RC) consisting of protein subunits of the NF-κB family (Verweij *et al.*, 1991; Costello *et al.*, 1993). In accordance, stimulation of CD28 in lymphocytes pretreated with anti-CD3 or phorbolesters results in increased nuclear amounts of the NF-κB proteins p50, p65 and c-Rel (Ghosh *et al.*, 1993). In Jurkat T cells, it has been further observed that CD28 activates JNK, a protein kinase involved in post-transcriptional activation of AP-1, which is known to bind to the IL-2 gene enhancer (Su *et al.*, 1994).

Relatively little is known about the early signaling pathway triggered by CD28. A hallmark of the CD28 costimulus is its refractoriness to the immunosuppressants cyclosporin A and FK506 (June *et al.*, 1987; Bjorndahl *et al.*, 1989; van Lier *et al.*, 1991). Consequently, signals associated with TCR activation, such as a rise in cytosolic free calcium, formation of diacylglycerol (DAG) and activation of protein kinase C (PKC), are typically not observed in response to B7 or agonistic anti-CD28 antibodies. Recently, CD28 stimulation has been observed to lead to the rapid appearance of tyrosine-phosphorylated proteins, including the CD28 molecule itself (Lu *et al.*, 1992; Vandenberghe *et al.*, 1992; Hutchroft and Bierer, 1994; Nunès *et al.*, 1994). CD28-mediated enhancement

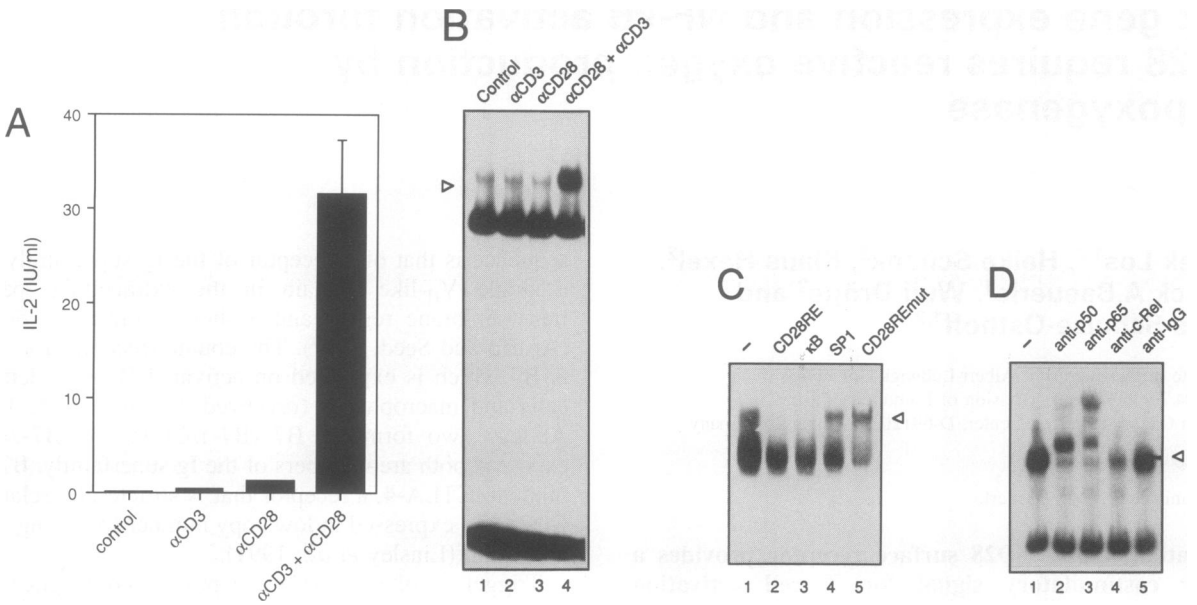

Fig. 1. Characterization of the CD28 costimulus in purified T lymphocytes. (**A**) Effects on IL-2 secretion. Cells (1×10^6 cells/ml) were stimulated for 48 h in the presence of saturating concentrations of either anti-CD28, anti-CD3 or anti-CD28 + anti-CD3 or left unstimulated (control). Supernatants were assayed for IL-2 using an ELISA system. Results are given in IU/ml ± SD using an IL-2 standard. (**B**) Effect of CD28 stimulation on the activation of CD28RC. Cells were stimulated with the indicated reagents and extracts were prepared after 2 h. Equal amounts of protein were incubated with the ^{32}P-labeled CD28RE oligonucleotide and analyzed by EMSA on a native polyacrylamide gel. The arrowhead indicates the position of the CD28RE-specific complex. (**C**) Competition analysis. Reaction mixtures containing extracts of anti-CD28 + anti-CD3-stimulated cells were incubated with a 100-fold excess of unlabeled CD28RE (lane 2), a κB-specific oligonucleotide from the mouse κ light chain enhancer (lane 3), an unrelated oligonucleotide containing the SP1-binding site (lane 4) or an oligonucleotide with a mutated CD28RE (lane 5). A section of the fluorogram is shown. (**D**) Analysis of CD28RC subunit composition. Reaction mixtures of an extract from anti-CD28 + anti-CD3-stimulated cells were incubated in the absence (lane 1) or in the presence of antibodies raised against the indicated NF-κB subunits (lanes 2–4). Lane 5 shows the results with an unrelated rabbit IgG antibody. A section of the fluorogram is shown.

of IL-2 production and cell proliferation was prevented by the tyrosine kinase inhibitor herbimycin A, suggesting that early signaling steps are controlled by still unknown protein kinases. In addition, following tyrosine phosphorylation of CD28, formation of a complex with phosphatidylinositol-3-kinase has been observed which binds to a Tyr-Met-X-Met motif in the intracellular part of CD28 (Pagés *et al.*, 1994; Prasad *et al.*, 1994; Truitt *et al.*, 1994). However, it is presently unknown whether 3′-phosphorylated phosphoinositides, the products of this lipid kinase, represent signaling molecules of the CD28 pathway (Lu *et al.*, 1995). Other messenger systems, such as a rise in cGMP (Ledbetter *et al.*, 1986) or phosphoinositol levels (Ledbetter *et al.*, 1990; Nunès *et al.*, 1993), appear to be affected by the degree of crosslinking of the CD28 receptor, but it is unlikely that these molecules are essentially required for the costimulatory pathway involving IL-2 secretion. Furthermore, most studies on CD28 signaling have been performed in T lymphoma cell lines. Transformed cells, however, may be poor models, because they have been selected to grow independently from costimulatory signals.

In the present study we have analyzed the early events of CD28 activation in primary T lymphocytes. Since most effects after CD28 stimulation are only observed in conjunction with TCR activation, we looked for a unique signal that was predominantly triggered by CD28 alone. We found that CD28 stimulation resulted in the rapid intracellular formation of reactive oxygen intermediates (ROIs) which have recently been implicated as important second messenger molecules (reviewed by Baeuerle and Henkel, 1994; Schulze-Osthoff and Baeuerle, 1995). ROI formation by CD28 involved the activation of 5-lipoxygenase and inhibition of this enzyme was able to block activation of the CD28-responsive complex and IL-2 secretion. These results suggest that the lipoxygenase metabolite leukotriene B4 may be an important effector molecule of the CD28 signaling pathway.

Results

CD28 ligation induces IL-2 secretion and NF-κB activation

To investigate the biochemical events associated with CD28 signaling, primary T lymphocytes were treated with agonistic anti-CD28 antibodies, with and without anti-CD3 antibodies activating the T cell receptor (TCR) complex. As shown in Figure 1A, ligation of neither CD28 nor the TCR led to a significant secretion of IL-2 into the supernatant. Combined treatment with anti-CD28 and anti-CD3, however, strongly induced IL-2 production. The enhancement of IL-2 gene expression by the CD28 costimulus has been found to be mediated by the CD28-responsive element (CD28RE) localized at position –162 to –152 within the IL-2 promoter (Fraser *et al.*, 1991; Verweij *et al.*, 1991). After costimulation, the CD28RE is bound by a CD28 response complex (CD28RC) which has been shown to contain members of the NF-κB family (Costello *et al.*, 1993; Ghosh *et al.*, 1993). Electrophoretic mobility shift assays (EMSAs) demonstrated that stimulation of T lymphocytes with anti-CD28 plus anti-CD3 induced a CD28RE-binding complex that was not detect-

able after stimulation with either agent alone (Figure 1B). To analyze the specificity of the newly formed complex, competition analyses with unlabeled oligonucleotides were performed. Formation of the slowly migrating CD28RC was competed by a 100-fold excess of unlabeled CD28RE oligonucleotide (Figure 1C, lane 2) but not by an unrelated oligonucleotide (lane 4) or by an oligonucleotide mutated in the CD28RE (lane 5). DNA binding was also competed by an oligonucleotide with a classical κB-binding site (Figure 1C, lane 3), indicating that CD28RC consisted of members of the NF-κB family.

NF-κB complexes may constitute a variety of different homo- and heterodimers of the NF-κB-Rel family of transcription factors. To analyze the subunit composition of the CD28-induced complex, supershift assays were employed. Antibodies against the NF-κB subunits p50, p65 and c-Rel were added to an extract of cells stimulated with anti-CD28 and anti-CD3. The CD28-induced DNA binding complex was totally retarded by anti-p50 (Figure 1D, lane 2) and also partially by anti-p65 (lane 3). Anti-c-Rel caused a reduction of DNA binding (Figure 1D, lane 4), whereas an unrelated (anti-TNF) IgG antibody had no effect (lane 5). These data indicate the presence of p50, p65 and c-Rel in the CD28-inducible DNA complex.

CD28 ligation induces a pro-oxidant condition in T lymphocytes

Biological effects of CD28, such as IL-2 production or NF-κB activation, can be observed only in conjunction with TCR activation. In order to investigate CD28-specific signaling events we looked for rapid effects that could be monitored solely by ligation of the CD28 receptor. It has previously been reported that CD28-mediated activation of NF-κB/CD28RC is prevented by antioxidants, suggesting that ROIs are involved in the CD28 signaling pathway (Costello *et al.*, 1993; Los *et al.*, 1994). Measurement of intracellular H_2O_2 levels by FACS analysis with the dye dichlorofluorescine demonstrated that anti-CD28 alone induced a strong increase of intracellular H_2O_2 levels (Figure 2A and B). This oxidative shift was detectable within 5 min after cell stimulation and was maximal after 30 min (data not shown). An isotype-matched control antibody, however, had no effects (Figure 2A). Production of H_2O_2 was significantly weaker after stimulation with anti-CD3 whereas the combined stimuli resulted in additive effects. In parallel, we analyzed the levels of intracellular glutathione (GSH), the major cellular antioxidant, using FACS analysis of monochlorobimane-stained cells (Rice *et al.*, 1986). Corresponding to the increase of H_2O_2, anti-CD28 alone induced a rapid decrease of GSH (Figure 2A and B). This decrease was visible 10 min after CD28 ligation, maximal after 30 min and was then gradually restored within 12 h (data not shown). These results demonstrate that CD28 signaling is associated with a rapid and transient shift towards pro-oxidant conditions. Measurement of H_2O_2 production and GSH depletion, therefore, should allow us to delineate the sequence of biochemical effects triggered upon CD28 ligation.

The activation of receptor-associated tyrosine kinases has been implicated as a primary step of CD28 activation and other signaling pathways. Treatment of T lymphocytes with the Src-like kinase inhibitor herbimycin A has been

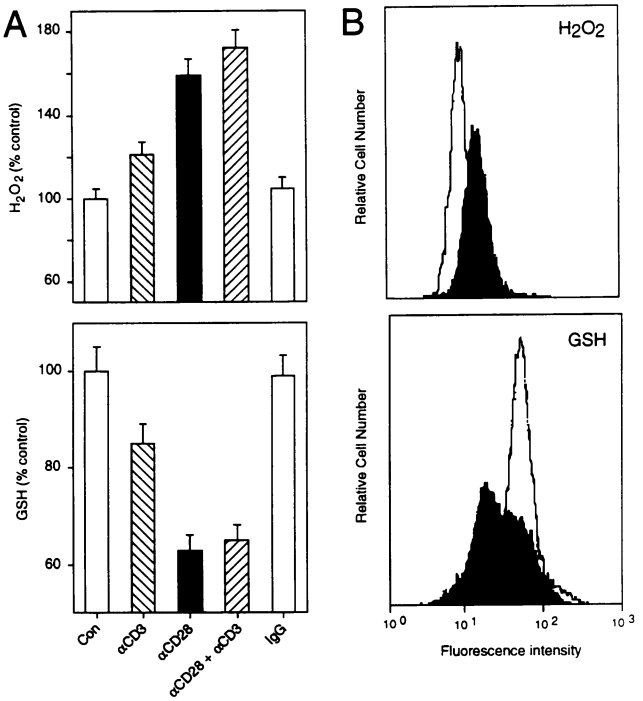

Fig. 2. Oxidative events in response to CD28 activation. (**A**) Effects of anti-CD28, anti-CD3, anti-CD28 + anti-CD3 and of an isotype-matched control antibody (IgG1) on intracellular H_2O_2 (upper panel) and glutathione levels (GSH, lower panel). Cells were stimulated for 30 min in the presence of the indicated reagents or left untreated (Con). H_2O_2 was measured by FACS analysis of dichlorofluorescine-stained cells, GSH was determined using monochlorobimane. Results are given as percentage ± SD of untreated cells. (**B**) Comparative FACS profiles of unstimulated (white area) and anti-CD28-stimulated cells (black area). A representative measurement of H_2O_2 levels (upper panel) and GSH levels (lower panel) is shown.

reported to inhibit CD28-mediated tyrosine phosphorylation and IL-2 expression (Lu *et al.*, 1992; Vandenberghe *et al.*, 1992). In accordance, anti-CD28-induced H_2O_2 production and GSH depletion (Figure 3A) as well as activation of NF-κB/CD28RC (Figure 3B) were completely inhibited by herbimycin A. These results illustrate that oxidative events can be linked to early steps of CD28 signaling, such as the activation of tyrosine kinases. Furthermore, certain enzymes producing ROIs upon CD28 ligation are likely to act downstream of tyrosine kinases.

Involvement of lipoxygenase in CD28 signaling

Several enzymes and intracellular electron transfer reactions are known to produce ROIs which may be involved in intracellular signaling events (Halliwell and Gutteridge, 1990). These include enzymes involved in lipid peroxidation, such as cyclooxygenase and lipoxygenase, the membrane-bound NADPH oxidase, xanthine oxidase and the mitochondrial respiratory chain. To identify the source of ROIs produced in response to CD28 ligation we investigated a number of inhibitors of these enzymes for an effect on CD28-mediated H_2O_2 production and GSH decrease. Initial experiments performed in primary and Jurkat T lymphocytes revealed that neither diphenylene iodonium, an inhibitor of NADPH oxidase, nor rotenone, a respiratory chain inhibitor, nor allopurinol, an inhibitor of xanthine oxidase, exerted any profound effects (data not shown). However, we observed that in particular redox inhibitors

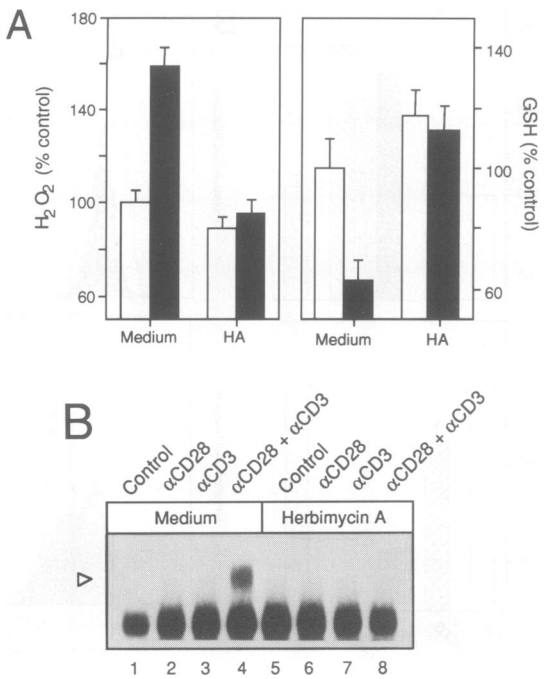

Fig. 3. Effects of the tyrosine kinase inhibitor herbimycin A on CD28 signaling. (**A**) Effects of herbimycin A on CD28-mediated increase of intracellular H_2O_2 formation (left panel) and decrease of GSH levels (right panel). Cells were pretreated for 8 h in the presence or absence of herbimycin A (HA, 0.5 μg/ml) and were then either left untreated (open bars) or stimulated for 30 min with anti-CD28 (black bars). (**B**) Effects of herbimycin A on the activation of CD28RC. Cells were pretreated as described in (A) and then stimulated for 2 h with anti-CD28, anti-CD3 or anti-CD28 + anti-CD3 in the absence (lanes 1–4) or presence of herbimycin A (HA) (lanes 5–8). Lysates were analyzed by EMSA using a ^{32}P-labeled CD28RE oligonucleotide. A section of the fluorogram is shown. The position of the CD28RC–DNA complex is indicated by an arrowhead.

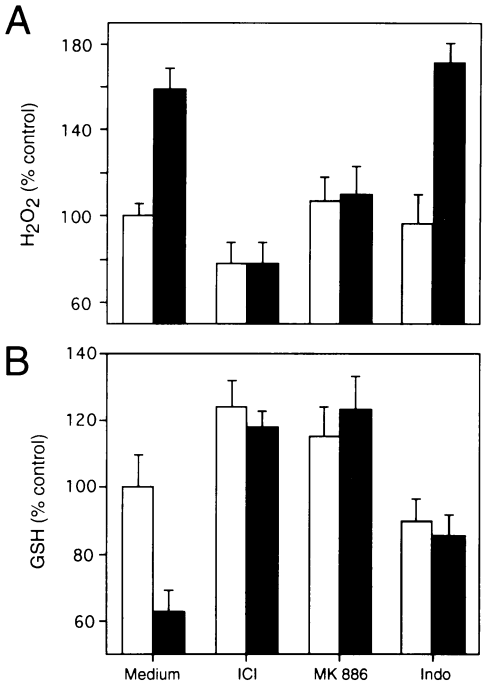

Fig. 4. Effects of lipoxygenase and cyclooxygenase inhibitors on CD28-mediated oxidative events. Cells were pretreated for 1 h with ICI 230487 (ICI, 5 μM), MK 886 (1 μM) or indomethacin (Indo, 10 μM) and then either left unstimulated (open bars) or stimulated with anti-CD28 (black bars) for 30 min. (**A**) Effects on CD28-mediated H_2O_2 formation. (**B**) Effect on GSH levels.

of lipid peroxidation, such as butylated hydroxyanisole (BHA) or nordihydroguaiaretic acid (NDGA), were able to inhibit CD28-mediated oxidative events as well as NF-κB activation and IL-2 production, indicating that enzymes of lipid peroxidation may be involved in CD28 signaling. Figure 4 shows that the methoxytetrahydropyran compound ICI 230487, a potent non-redox inhibitor of lipoxygenase (Crawley *et al.*, 1993), strongly inhibited CD28-induced H_2O_2 production and GSH decrease. Another compound, MK 886, which inhibits leukotriene biosynthesis by preventing the translocation of the 5-lipoxygenase-activating protein (FLAP) to the membrane (Rouzer *et al.*, 1990), had similar effects, indicating that 5-lipoxygenase or a closely related lipoxygenase may be involved in CD28 signaling events (Figure 4). In contrast, cyclooxygenases which have been also reported to initiate oxidative stress, are unlikely to be involved in the CD28 signaling pathway. Indomethacin (Figure 4) and aspirin (data not shown), two unrelated inhibitors of cyclooxygenase, did not inhibit, and even slightly augmented H_2O_2 production. This effect might be explained by the fact that inhibition of cyclooxygenase leads to the accumulation of arachidonic acid, thereby providing increased amounts of substrate for the lipoxygenase reaction.

CD28 ligation triggers arachidonic acid and leukotriene B4 release

The lipoxygenase pathway is coupled to phospholipase A_2 (PLA_2) which provides arachidonic acid for leukotriene biosynthesis. Inhibition of PLA_2 by dexamethasone significantly inhibited CD28-induced production of H_2O_2 and depletion of GSH (Figure 5A). The involvement of PLA_2 in the CD28 signaling cascade was further demonstrated by measurement of arachidonic acid release. Stimulation of T lymphocytes with anti-CD28 resulted in a strong release of arachidonic acid whereas anti-CD3 or a control antibody had no or only weak effects (Figure 5B). Consistent with oxidative events, CD28-triggered arachidonic acid release was inhibited by dexamethasone and the tyrosine kinase inhibitor herbimycin A (Figure 5B), whereas inhibitors of lipoxygenase had no effect (data not shown).

To confirm the involvement of lipoxygenase we measured the production of leukotrienes after crosslinking of CD28, with and without activation of the TCR. Figure 5C demonstrates that anti-CD28 induced a strong increase in leukotriene B4 (LTB4) production which was not observed after stimulation with anti-CD3 or a control antibody. Costimulation by both agents did not result in a significant difference, suggesting that lipoxygenase was selectively activated by a CD28-mediated signal. In addition, time course experiments revealed that the production of LTB4 was rapidly induced reaching a maximum 1 h after CD28 stimulation (Figure 5D). Liberation of other lipoxygenase products, such as the peptidyl leukotrienes LTC4, LTD4 and LTE4, however, was not significantly increased by anti-CD28 (data not shown). Furthermore,

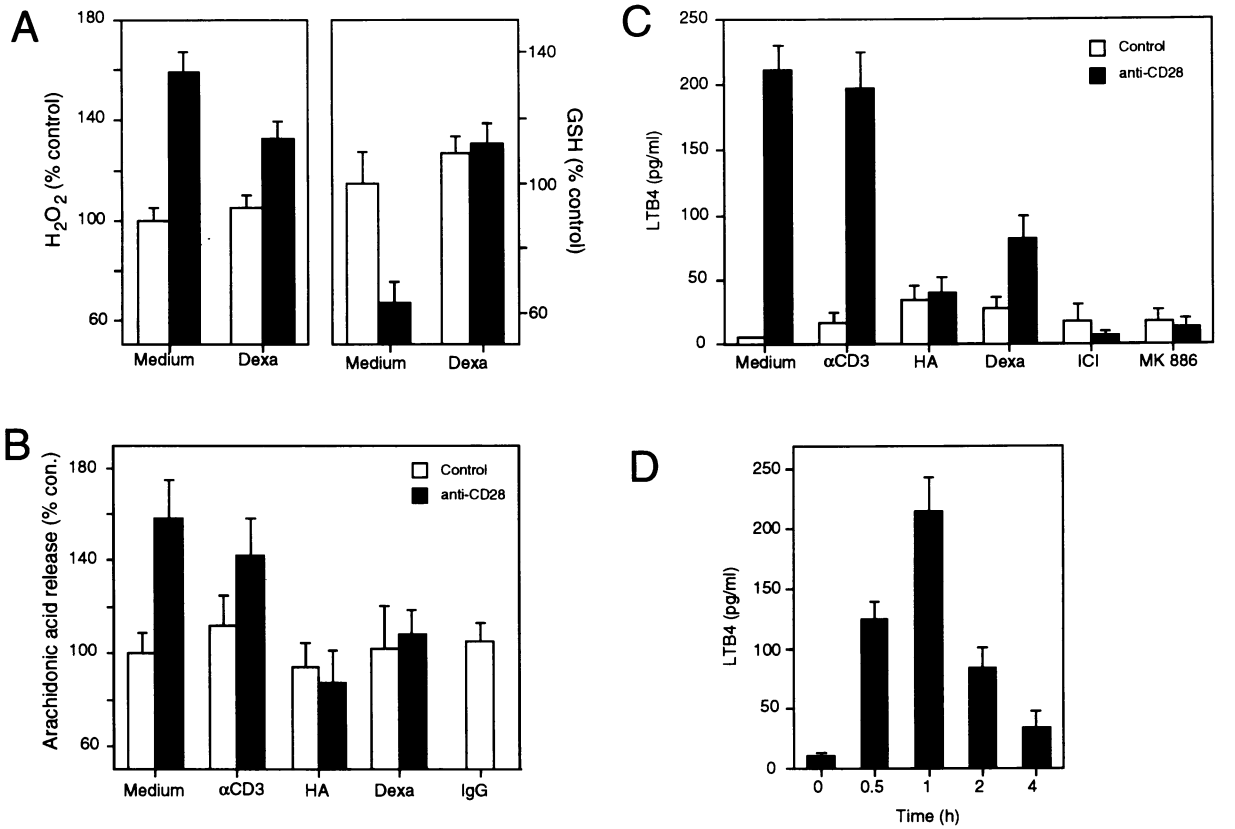

Fig. 5. Involvement of phospholipase A_2 and lipoxygenase in CD28 signal transduction. (**A**) Effect of the phospholipase A_2 inhibitor dexamethasone on CD28-mediated formation of H_2O_2 (left panel) and decrease of GSH (right panel). Cells were left untreated or treated with dexamethasone (Dexa, 5 μM) for 8 h. Unstimulated (open bars) or anti-CD28-treated (black bars) cells were analyzed 30 min after stimulation. (**B**) Arachidonic acid release assay. Cells were labeled with [^{3}H]arachidonic acid and either left untreated or treated with herbimycin A (HA, 0.5 μg/ml) or dexamethasone (Dexa, 5 μM). 8 h later, cells were treated with anti-CD28 (black bars) or medium (open bars), with or without anti-CD3. For control, an isotype-matched control antibody (IgG1) was used. Arachidonic acid release was measured following 2 h of stimulation in cell-free supernatants. 100% arachidonic acid release corresponded to 26 500 c.p.m. (**C**) Production of leukotriene B4 (LTB4) after stimulation with anti-CD28, anti-CD3 and anti-CD28 + anti-CD3 and effects of various inhibitors of CD28 signaling on leukotriene release. Cells were pretreated with herbimycin A (HA, 0.5 μg/ml), dexamethasone (Dexa, 5 μM), ICI 230487 (ICI, 5 μM), MK 886 (1 μM) or indomethacin (Indo, 10 μM) and stimulated with (black bars) or without (open bars) anti-CD28. Release of leukotrienes in cell-free supernatants was measured by an ELISA system 1 h following cell stimulation. (**D**) Time course of LTB4 release after stimulation with anti-CD28. Cells were stimulated with 1 μg/ml anti-CD28 and supernatants were harvested after the indicated time points.

pretreatment of T cells with herbimycin A or dexamethasone strongly inhibited anti-CD28-induced LTB4 release (Figure 5C). Corresponding to the effects observed on H_2O_2 production, LTB4 release was nearly completely prevented using the lipoxygenase inhibitors ICI 230487 and MK 886 (Figure 5C).

Lipoxygenase inhibition specifically affects NF-κB/CD28RC activation

The experiments suggested the involvement of tyrosine kinases, PLA_2 and 5-lipoxygenase in CD28 signaling. To prove the functional importance of these molecules, we investigated the effect of inhibition of these pathways on CD28-mediated activation of CD28RC and IL-2 production. Since T cells respond to anti-CD28 by ROI formation and since the lipoxygenases are known to give rise to elevated ROI formation (Gagnon *et al.*, 1989; Halliwell and Gutteridge, 1990), we also analyzed the effects of the antioxidant pyrrolidine dithiocarbamate (PDTC). In addition to the glucocorticoid receptor-dependent PLA_2 inhibitor dexamethasone, p-bromophenacyl-bromide (BPB) was included as an unrelated irreversible inhibitor of PLA_2 (Lister *et al.*, 1989). To this end, T lymphocytes were pretreated with herbimycin A, dexamethasone, BPB, ICI 230487, MK 886 or PDTC and then stimulated with anti-CD28 and anti-CD3. Non-toxic concentrations of all substances effectively inhibited DNA binding activity of CD28RC (Figure 6A) as well as anti-CD28-induced IL-2 production (Figure 6B). This indicated that activation of a tyrosine kinase, followed by activation of PLA_2 and lipoxygenase and the formation of ROIs are all functionally involved in CD28-triggered biological effects.

T cell activation and IL-2 expression is subject to a complex regulation by a number of transcription factors. Notably, the ubiquitous factors AP-1 and NF-IL2A (which is indistinguishable from the octamer binding factor Oct-1) as well as the T cell-specific transcription factor NF-AT have all been shown to be critical for IL-2 expression in response to TCR activation (Crabtree and Clipstone, 1994; Rao, 1994). To examine the specificity of the proposed pathway for CD28 responses, we monitored the effects of the inhibitors on TCR-specific transcription factors. NF-IL2A activity was constitutively expressed and not significantly affected by TCR and CD28 crosslinking nor by the inhibitors (Figure 6C). DNA binding of AP-1 and NF-AT was induced by anti-CD3 but not further enhanced by anti-CD28 (Figure 6C, lanes 2–4). Herbimycin A strongly prevented DNA binding of both transcription factors which

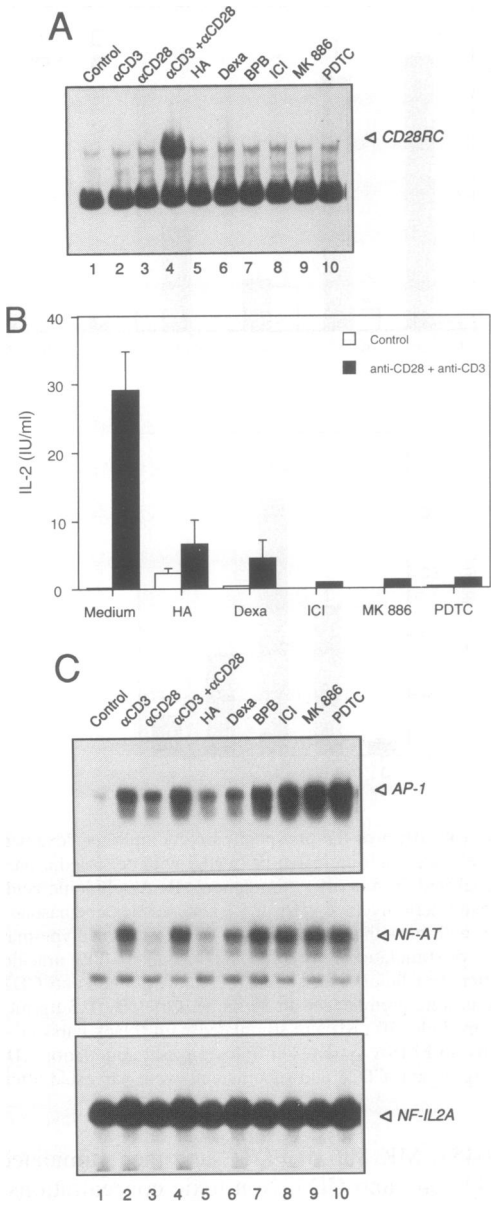

Fig. 6. Effects of inhibitors of tyrosine kinases, phospholipase A_2, lipoxygenase and antioxidants on biological activities of the CD28 costimulus. (**A**) Effects on anti-CD28-induced formation of CD28RC. Cells were pretreated with herbimycin A (HA, 0.5 μg/ml), dexamethasone (Dexa, 5 μM), p-bromophenacyl-bromide (BPB, 100 μM), ICI 230487 (ICI, 5 μM), MK 886 (1 μM) or PDTC (5 μM) and then stimulated for 2 h with a combination of anti-CD3 and anti-CD28 as described in Figure 1B. Extracts were analyzed by EMSA with a ^{32}P-labeled CD28RE oligonucleotide. A section of the fluorogram is shown. (**B**) Effects of inhibitors on anti-CD3 + anti-CD28-induced IL-2 secretion. Cells were pretreated with the inhibitors and then stimulated with saturating concentrations of anti-CD3 and anti-CD28 or left unstimulated (control). IL-2 was measured in the supernatants by an ELISA 48 h after stimulation. HA: 0.5 μg/ml herbimycin A; Dexa: 5 μM dexamethasone; ICI: 5 μM ICI 230487; MK 886: 5 μM; PDTC: 5 μM. (**C**) Effects of CD28 inhibitors on induction of AP-1, NF-AT and NF-IL-2A DNA binding activity. Cells were incubated with the indicated inhibitors as described in (A). Identical extracts as in (A) were used for EMSAs. Sections of the fluorograms are shown.

confirms that Src tyrosine protein kinases, such as p56^{lck} and p59^{fyn}, are important for TCR signal transduction (Rincón and Flavell, 1994; Weiss and Littman, 1994).

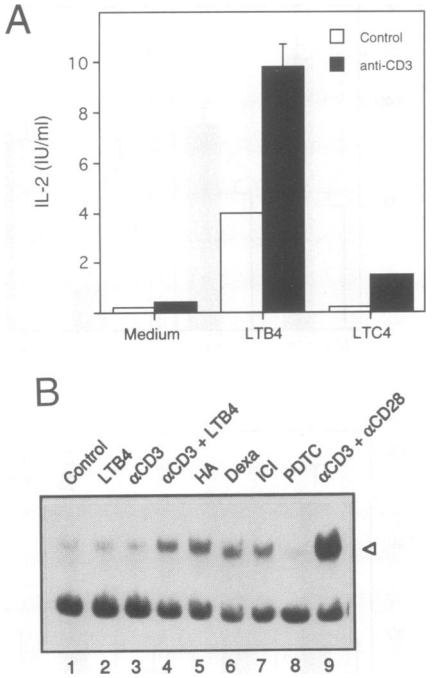

Fig. 7. Effects of exogenous leukotrienes on IL-2 secretion and CD28RC activation. (**A**) Effects on IL-2 secretion. T lymphocytes were treated with 50 nM LTB4 or LTC4. Supernatants were assessed for IL-2 48 h after cell stimulation. (**B**) Effects on CD28RC activation. Cells were stimulated with the indicated reagents and 50 nM LTB4. In lanes 5–8, cells were incubated with the indicated inhibitors as described in Figure 6A and then treated with anti-CD3 and LTB4 for 2 h. A section of the flurogram is shown.

Similar to CD28RC, both factors were also inhibited by dexamethasone (Figure 6C, lane 5), whereas BPB inhibited CD28RC but had no effect on AP-1 and NF-AT (lane 6). This suggested that dexamethasone inhibited both transcription factors independently of PLA_2 involvement. Indeed, previous reports have demonstrated that dexamethasone downregulates DNA binding of AP-1 through the direct interaction of the glucocorticoid receptor with AP-1 (Jonat *et al.*, 1990; Vacca *et al.*, 1992). NF-AT which consists of the calcium/calcineurin-responsive subunit NF-ATp and newly synthesized AP-1 components (Rao, 1994) has been reported to be also inhibited by glucocorticoids (Paliogianni *et al.*, 1993). However, unlike CD28RC, AP-1 and NF-AT were not inhibited by the two lipoxygenase inhibitors (Figure 6C, lanes 7–8), and also the antioxidant PDTC exerted no significant effects (lane 1). Thus these data indicate that signaling events downstream of protein tyrosine phosphorylation are different for CD28RC and NF-AT and AP-1, respectively.

LTB4 mimics anti-CD28 effects

The results indicated that leukotrienes, such as LTB4, may be important messenger molecules during CD28 signal transduction. We therefore investigated whether exogenous leukotrienes could substitute for anti-CD28 effects. As shown in Figure 7A, LTB4 slightly induced IL-2 secretion, whereas LTC4 had virtually no effects. Similar to the CD28 costimulus, however, LTB4-induced IL-2 production was potentiated by TCR stimulation. In addition, analysis of CD28RC–DNA binding demonstrated that LTB4, when added in combination with anti-CD3, could mimic anti-

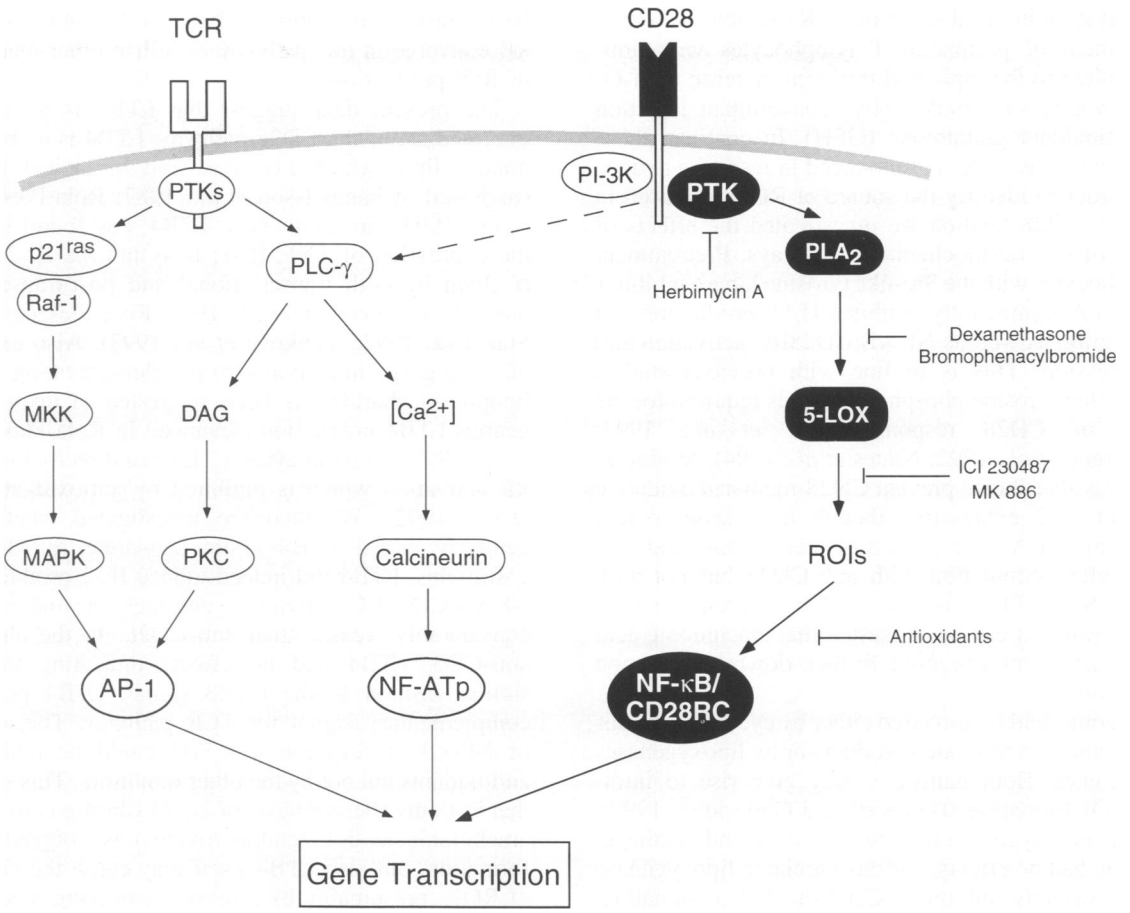

Fig. 8. Proposed model for signal transduction by CD28. CD28 ligation activates a Src-related protein tyrosine kinase (PTK) and PI-3 kinase (PI-3K) as initial events leading to the stimulation of phospholipase A_2 (PLA_2). Formed arachidonic acid is a substrate for 5-lipoxygenase (5-LOX) to generate hydroxyeicosatetranoic acids and leukotrienes. These products give rise to the formation of ROIs which then activate the transcription factor NF-κB/CD28RC. Also the TCR pathway requires the initial activation of PTKs, such as $p56^{lck}$, $p59^{fyn}$ and ZAP-70. These deliver signals that are coupled to at least three pathways including $p21^{ras}$/MAP kinase (MAPK) activation, stimulation of protein kinase C (PKC) and a calcium/calcineurin-dependent signal. These pathways result in the activation of the TCR-inducible transcription factors AP-1 and NF-AT. Although the signaling cascade differs between CD28 and the TCR, extensive crosstalk may allow complementation and synergy resulting in full IL-2 expression: CD28 may cooperate with the TCR in activation of $p59^{fyn}$ PTK. In some situations, CD28 ligation triggers a calcium-dependent signal (dashed line). Diacylglycerol (DAG) formed by phospholipase Cγl (PLCγl) may be converted by DAG lipase to arachidonic acid and redirected into the LOX pathway. A rise in cytosolic free calcium may activate calcium-dependent PLA_2 and LOX. Furthermore, CD28-mediated signals may be enhanced by ROI formation through the TCR which may be induced by a rise in calcium or PLCγ activation.

CD28 effects (Figure 7B, lane 4), whereas the sole addition of LTB4 to T lymphocytes had no effects (lane 3). Activation of CD28RC by LTB4 was considerably weaker than in response to anti-CD28 (Figure 7B, compare lanes 4 and 9). In contrast to anti-CD28 responses, induction of DNA binding was not prevented by the inhibitors of tyrosine kinase, PLA_2 and lipoxygenase activity (Figure 7B, lanes 5–7). The antioxidant PDTC, however, completely prevented CD28RC activation in response to LTB4 (Figure 7B, lane 8). Thus signals elicited after CD28 ligation can obviously be at least partially mimicked by exogenous LTB4.

Discussion

CD28 has been identified as a costimulatory receptor that provides the major second signal necessary for T cell function. Despite its fundamental importance for T cell development and the immune response, the biochemical basis of CD28 accessory signals is poorly understood. Most studies have been performed in transformed T cell lines which may be unsuitable since they may bypass costimulatory signals required for IL-2 expression and cell proliferation. Identification of CD28-specific second messenger molecules is further hampered by the fact that biological effects of CD28 signaling are observed only in conjunction with TCR activation. Thus a central question is whether the CD28 pathway is simply enhancing or sustaining the signals provided by the TCR or whether two independent pathways exist. Triggering of the TCR–CD3 complex initiates a signal cascade that leads to a rise in cytosolic free calcium, formation of diacylglycerol and the activation of protein kinase C and other kinases (Mueller *et al.*, 1989; Crabtree and Clipstone, 1994; cf. Figure 8). This calcium-dependent pathway is blocked by the immunosuppressive calcineurin inhibitors cyclosporin A and FK506. A characteristic feature of the CD28 pathway is its refractoriness to cyclosporin A, suggesting that CD28 triggers an independent pathway that is distinct from the TCR (June *et al.*, 1994).

In an attempt to investigate early events of the CD28 pathway we looked for signals that were triggered by

CD28 activation in the absence of TCR ligation. We found that treatment of peripheral T lymphocytes with anti-CD28 resulted in the rapid and transient increase of H_2O_2 formation which was paralleled by a concomitant depletion of the antioxidant glutathione (GSH). In contrast, these oxidative events were less pronounced in response to anti-CD3. In order to identify the source of ROIs produced in response to CD28 ligation we investigated the effects of inhibitors of several biochemical pathways. Pretreatment of T lymphocytes with the Src-like tyrosine kinase inhibitor herbimycin A significantly inhibited H_2O_2 production and GSH depletion as well as NF-κB/CD28RC activation and IL-2 expression. This is in line with previous studies indicating that tyrosine phosphorylation is required for the initiation of CD28 responses (Lu *et al.*, 1992; Vandenberghe *et al.*, 1992; Nunes *et al.*, 1994). Inhibition of PLA_2 was also able to prevent CD28-mediated oxidative events and IL-2 expression, though to a lesser extent than herbimycin A. The release of arachidonic acid was observed after stimulation with anti-CD28 but not with anti-CD3. Since PLA_2 is activated by protein tyrosine phosphorylation, it can be suggested that arachidonic acid release is an event triggered further downstream upon CD28 ligation.

Arachidonic acid is converted either by cyclooxygenases to prostaglandins and related products or by lipoxygenases to leukotrienes. Both pathways may give rise to intracellular ROI formation (Halliwell and Gutteridge, 1990). Whereas cyclooxygenase inhibitors, such as indomethacin and aspirin, had no effects, various unrelated lipoxygenase inhibitors strongly inhibited CD28-mediated signaling. These included the traditionally used redox-dependent inhibitors NDGA and BHA, and, in particular, the recently developed compounds MK 886 and ICI 230487, which selectively inhibit 5-lipoxygenase (Rouzer *et al.*, 1990; Crawley *et al.*, 1993). CD28 ligation resulted in the rapid release of the 5-lipoxygenase metabolite LTB4 which was not observed in response to TCR stimulation. Of note, the time course of LTB4 release coincided with the activation of NF-κB/CD28RC.

We further observed that CD28-induced NF-κB activation and IL-2 release were completely abolished by antioxidants, which concurs with our initial observation that formation of a pro-oxidant condition is selective for the CD28 response. Since leukotriene synthesis involves ROI production, for instance in the metabolism of hydroxyeicosatetranoic acids (Gagnon *et al.*, 1989), ROI formation may be the ultimate step leading to NF-κB/CD28RC activation. Several studies have shown that activation of NF-κB in response to other stimuli, such as cytokines or lipopolysaccaride, is abolished by antioxidants (Staal *et al.*, 1990; Mihm *et al.*, 1991; Schreck *et al.*, 1991; Meyer *et al.*, 1993; Schulze-Osthoff *et al.*, 1993; Schenk *et al.*, 1994), suggesting that ROIs may represent widely used second messenger molecules. However, the mechanisms by which ROIs are produced in response to these various NF-κB-activating stimuli have not carefully been investigated so far. In the case of TNF, it has been shown that ROIs produced during mitochondrial respiration are involved in gene-inducing effects of the cytokine (Schulze-Osthoff *et al.*, 1993). In the present study, we show that ROI formation in response to anti-CD28 involves 5-lipoxygenase or a closely related lipoxygenase, and it will be interesting to examine whether other inducers of NF-κB converge in this pathway or utilize other mechanisms of ROI production.

The present data suggest that LTB4 is an important intermediate of the CD28 pathway. LTB4 is a pro-inflammatory lipid which exerts several biological functions (reviewed in Samuelsson *et al.*, 1987; Rola-Pleszczynski *et al.*, 1993). In monocytes, LTB4 was found to induce the expression of TNF, IL-1, IL-6 and the IL-2 receptor α chain by both transcriptional and post-transcriptional mechanisms (Gagnon *et al.*, 1989; Rola-Pleszczynski and Stankova, 1992, Stankova *et al.*, 1993). Also expression of these genes in response to platelet-activating factor or lipopolysaccharide has been suggested to involve endogenous LTB4 production (reviewed in Rola-Pleszczynksi *et al.*, 1993). In monocytes, LTB4 can directly induce NF-κB activation which is inhibited by antioxidants (Brach *et al.*, 1992). We therefore investigated whether exogenously added LTB4 could substitute for the CD28 costimulus. LTB4 did indeed induce IL-2 production and NF-κB/CD28RC activation, although the induction was considerably weaker than anti-CD28. In the absence of anti-CD3, LTB4 had no effects indicating that, in a similar fashion to the CD28 signal, LTB4 provided a complementary signal for TCR pathway. The activation of NF-κB in response to LTB4 could be inhibited by antioxidants but not by the other inhibitors. This suggested that not only the synthesis of LTB4 (during conversion of arachidonic acid to endoperoxides) is coupled to ROI formation, but also LTB4 itself may cause the generation of ROIs, presumably by a reverse oxidizing reaction.

The model in Figure 8 proposes a CD28 signaling cascade which is initiated by the activation of a still unknown Src-like tyrosine kinase, followed by the activation of PLA_2 and 5-lipoxygenase which finally leads to pro-oxidant-induced activation of NF-κB/CD28RC and functional IL-2 expression. The proposed pathway differs substantially from the TCR signaling mechanism. To analyze the specificity for CD28, we monitored the effect of the inhibitors on the transcription factors NF-AT and AP-1 which are activated by TCR ligation and not further induced by anti-CD28. Neither lipoxygenase inhibitors nor antioxidants prevented the activation of these TCR-inducible transcription factors, indicating that the proposed pathway is indeed selective for CD28. However, despite substantial differences between both pathways, extensive crosstalk and interaction may exist which may explain the synergistic effect of TCR and CD28 stimulation. TCR signaling initially involves $p56^{lck}$, $p59^{fyn}$ and ZAP-70 tyrosine kinases, followed by the activation of the $p21^{ras}$ G-protein, phospholipase Cγ-mediated phosphoinositol turnover, calcium release and activation of Ser/Thr kinases (Crabtree and Clipstone, 1994; Izquierdo Pastor *et al.*, 1995). It is conceivable that the release of calcium triggered by the TCR may co-activate PLA_2 and 5-lipoxygenase which are dependent on calcium ions (Samuelsson *et al.*, 1987; Ford-Hutchinson *et al.*, 1994). DAG formed by TCR-activated PLCγ may be converted to arachidonic acid by DAG lipase and thereby redirected to the lipoxygenase pathway. In addition, it has been found recently that the protein phosphatase calcineurin may be involved in NF-κB activation in T cells by increasing IκB degradation (Frantz *et al.*, 1994). This might explain the observation

that activation of NF-κB/CD28RC requires two simultaneous signals and that neither anti-CD28 nor anti-CD3 alone are sufficient to induce activation. In summary, our present study provides important new details about the mechanism of the CD28 costimulus. Since CD28 plays an essential role in numerous immune and inflammatory processes, such as allograft rejection and cytotoxic T cell antitumor response, selective inhibition or potentiation of the biochemical events described may be useful for therapeutic targeting of the CD28 pathway.

Materials and methods

Reagents

The anti-CD28 monoclonal antibody LCB28 (mouse IgG1) was kindly provided by Dr R.van Lier (Amsterdam, NL). Anti-CD3 (OKT-3) was obtained from the American Tissue Culture Collection. These and isotype-matched control antibodies were used after purification by protein G affinity chromatography. The lipoxygenase inhibitors ICI 230487 and MK 886 were provided by Dr D.Steinhilber (Tübingen, Germany). Dexamethasone, p-bromophenacyl-bromide (BPB), nordihydroguaiaretic acid (NDGA), indomethacin, pyrrolidine dithiocarbamate (PDTC) and leukotrienes were from Sigma (Deisenhofen, Germany), herbimycin A was obtained from Biomol (Hamburg, Germany). Stock solutions of the reagents were routinely prepared in DMSO/ethanol (1:1) and used in dilutions of 1:500 or less, so that the final concentration of each solvent never exceeded 0.1%.

Preparation and treatment of primary T lymphocytes

Venous blood was collected from healthy volunteers, drawn in heparin-treated syringes and diluted in an equal volume of Hank's balanced salt solution. The suspension was layered over a Ficoll–Paque cushion (Pharmacia, Freiburg, Germany) and centrifuged at 700 *g* for 20 min. Mononuclear cells were collected at the interface, washed twice in PBS and resuspended in RPMI-1640 supplemented with 10% FCS and antibiotics. The cells were incubated on nylon wool for 45 min at 37°C to remove monocytes. B cells were depleted by incubation with anti-CD20-coated magnetic beads (Dynal, Hamburg, Germany). This procedure led to preparations of 93–98% purified T lymphocytes as judged by flow cytometry with anti-CD2, anti-CD14 and anti-CD20 antibodies. Experiments were routinely performed 12 h after T cell preparation in 24-well or 48-well plates, in a volume of 1 ml or 0.5 ml and a density of 2.5×10^6 cells/ml. Stimulation with anti-CD3 was performed after precoating the plates with 1 μg/ml of the antibody. Anti-CD28 was directly added to the cell cultures and used at a final concentration of 1 μg/ml.

Fluorescent measurement of intracellular hydrogen peroxide

Formation of hydrogen peroxide was measured using dichlorofluorescin diacetate (DFCH) according to Royall and Ischiropoulus (1993). A 10 mM stock solution of DFCH (Molecular probes, Eugene, OR) was prepared in DMSO under nitrogen and stored at –20°C. Cells were preloaded with 5 μM DFCH in culture medium for 0.5 h at 37°C. Measurements were performed in duplicates using a FACScan (Becton Dickinson) flow cytometer. Dead cells were excluded by forward/side scatter gating and staining with propidium iodide.

Measurement of intracellular glutathione (GSH)

Intracellular GSH levels were determined essentially as described (Rice *et al.*, 1986). Cells were stained with 20 μM monochlorobimane (Molecular Probes) for 10 min at 37°C. The staining reaction was stopped by the addition of ice-cold PBS. Unreacted dye was removed by centrifugation of the cells through a cushion of cold FCS and two washing steps in cold PBS. Samples were then analyzed in duplicates on a FACS Vantage (Becton Dickinson) flow cytometry system using an emission wavelength of 455 nm (Omega 450DF-65) and a 351–364 nm bandpass filter for excitation. Dead cells were excluded by forward/side scatter gating and propidium iodide staining.

Arachidonic acid release assay

Cells were incubated for 16 h in 2 μCi/ml [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (190 mCi/mmol, Amersham, Braunschweig, Germany) and pretreated with the inhibitors as indicated. After four washing steps in culture medium cells were stimulated with anti-CD28 or anti-CD3 in the presence of the inhibitors. Two hours later, supernatants were collected, centrifuged at 700 *g* for 10 min and at 10 000 *g* for 15 min. Release of arachidonic acid in the supernatants was measured by liquid scintillation counting.

Measurement of leukotrienes and IL-2

Cell-free supernatants of T lymphocytes, stimulated for 48 h, were assessed for IL-2 using a commercially available ELISA system (Dianova, Hamburg, Germany). The detection limit was 5 pg IL-2/ml. Results were calculated as IU/ml using an international standard preparation. 1 IU IL-2/ml corresponded to 78 pg/ml. Leukotriene release was measured 1 h after cell stimulation using a competitive ELISA system for LTB4 and LTC4, LTD4 and LTE4 (BIOTRAK, Amersham). The detection limit was 6.2 pg /ml for LTB4 and 10 pg/ml for LTC4, LTD4 and LTE4.

Electrophoretic mobility shift assay

For detection of DNA binding activities cells were seeded in a 2 ml volume at 2.5×10^6 cells/ml and pretreated with the inhibitors for 1 h and in the case of dexamethasone and herbimycin A (Uehara and Fukazawa, 1991) for 8 h. Then the cells were stimulated with anti-CD28 or anti-CD3. After a further 2 h of incubation cells were washed with ice-cold PBS and total and nuclear cell extracts were prepared essentially as described (Schulze-Osthoff *et al.*, 1993). Equal amounts of the extracts (~10 μg crude protein) were incubated with the ^{32}P-endlabeled oligonucleotides. Binding reactions were performed in a 20 μl volume containing 2–4 μl of extract, 4 μl 5× binding buffer (20 mM HEPES pH 7.5, 50 mM KCl, 1 mM DTT, 2.5 mM $MgCl_2$, 20% Ficoll), 2 μg poly(dI-dC) as non-specific competitor DNA, 2 μg BSA and 10 000–15 000 c.p.m. Cerenkov of the labeled oligonucleotide. Binding reactions for NF-AT were performed with 200 ng poly(dI-dC) per reaction. After 30 min incubation at room temperature, samples were loaded on a 4% non-denaturing polyacrylamide gel and run in 0.5× TBE buffer pH 8.3. For supershift assays, 2 μl of antibody was added to the reaction mixture simultaneously with the protein and the mixture was incubated as described above. Anti-p50, anti-p65 and anti-c-Rel were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

The sense sequences of the oligonucleotides were as follows:

(a) CD28RE (CD28-responsive element of the IL-2 promoter)
5′-GATCTGGTTTAAAGAAATTCCAAAGAG-3′

(b) CD28REmut (mutated CD28RE)
5′-GATCTGGTTTAAAGAAGCCTCAAAGAG-3′

(c) κB (NF-κB binding site of the mouse Ig κ light chain enhancer)
5′-AGCTTCAGAGGGGATTTCCGAGAGG-3′

(d) SP1
5′-ATTCGATCGGGGCGGGGCGAGC-3′

(e) AP-1 (TRE-responsive element of the collagenase promoter)
5′-AGCTTGATGAGTCAGGCCGGATC-3′

(f) NF-AT (distal NF-AT binding site of the IL-2 promoter)
5′-GGAGGAAAAACTGTTTCATACAGAAGGCGT-3′

(g) NF-IL2A (proximal octamer binding site of the IL-2 promoter)
5′-TAATATGTAAAAACATT-3′

Acknowledgements

The authors wish to thank R.van Lier and K.Khazaie for providing anti-CD28 and anti-CD3 antibodies, D.Steinhilber for lipoxygenase inhibitors, M.Li-Weber for oligonucleotides, M.Pach for antibody purification, and A.Böhme and H.Walczak for critical reading the manuscript.

References

Aruffo,A. and Seed,B. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 8573–8577.
Baeuerle,P.A. and Henkel,T. (1994) *Annu. Rev. Immunol.*, **12**, 141–179.
Bjorndahl,J.M., Sung,S.-S.J., Hansen,J.A. and Fu,S.M. (1989) *Eur. J. Immunol.*, **19**, 881–887.
Brach,M.A., de Vos,S., Arnold,C., Gruβ,H.-J., Mertelsmann,R. and Herrmann,F. (1992) *Eur. J. Immunol.*, **22**, 2705–2711.
Cerdan,C., Martin,Y., Brailly,H., Courcoul,M., Flavetta,S., Costello,R., Mawas,C., Birg,F. and Olive,D. (1991) *J. Immunol.*, **146**, 560–564.
Cerdan,C., Martin,Y., Courcoul,M., Mawas,C., Birg,F. and Olive,D. (1992) *J. Immunol.*, **149**, 2255–2261.

Costello,R., Lipcey,C., Algarté,M., Cerdan,C., Baeuerle,P.A., Olive,D. and Imbert,J. (1993) *Cell Growth Differ.*, **4**, 329–339.
Crabtree,G.R. and Clipstone,N.A. (1994) *Annu. Rev. Biochem.*, **63**, 1045–1083.
Crawley,G.C. *et al.* (1993) *J. Lipid Mediat.*, **6**, 249–257.
Ford-Hutchinson,A.W., Gresser,M. and Young,R.N. (1994) *Annu. Rev. Biochem.*, **63**, 383–417.
Frantz,B., Nordby,E.C., Bren,G., Steffan,N., Paya,C.V., Kincaid,R.L., Tocci,M.J., O'Keefe,S.J. and O'Neill,E.A. (1994) *EMBO J.*, **13**, 861–870.
Fraser,J.D., Irving,B.A., Crabtree,G.R. and Weiss,A. (1991) *Science*, **251**, 313–316.
Gagnon,L., Filion,L.G., Dubois,C. and Rola-Pleszczynski,M. (1989) *Agents Actions*, **26**, 141–147.
Ghosh,P., Tan,T.-H., Rice,N.R., Sica,A., and Young,H.A. (1993) *Proc. Natl Acad. Sci. USA.*, **90**, 1696–1700.
Guba,S.C., Stella,G., Turka,L.A., June,C.H., Thompson,C.B. and Emerson,S.G. (1989) *J. Clin. Invest.*, **84**, 1701–1706.
Halliwell,B. and Gutteridge,J.M.C. (1990) *Methods Enzymol.*, **186**, 1–85.
Harding,F.A., Mc Arthur,J.G., Gross,J.A., Raulet,D.H. and Allison,J.P. (1992) *Nature*, **356**, 607–609.
Hutchcroft,JE. and Bierer,B.E. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 3260–3264.
Izquierdo Pastor,M., Reif,K. and Cantrell,D. (1995) *Immunol. Today*, **16**, 159–164.
Jenkins,M.K. and Johnson,J.G. (1993) *Curr. Opinion Immunol.*, **5**, 361–367.
Jonat,C., Rahmsdorf,H.J., Park,K.K., Cato,A.C.B., Gebel,S., Ponta,H. and Herrlich,P. (1990) *Cell*, **62**, 1205–1215.
June,C.H., Ledbetter,J.A., Gillespie,M.M., Lindsten,T. and Thompson, C.B. (1987) *Mol. Cell. Biol.*, **7**, 4472–4481.
June,C.H., Ledbetter,J.A., Linsley,P.S. and Thompson,C.B. (1990) *Immunol. Today*, **11**, 211–216.
June,C.H., Bluestone,J.A., Nadler,L.M. and Thompson,C.B. (1994) *Immunol. Today*, **15**, 321–331.
Ledbetter,J.A., Parsons,M., Martin,P.J., Hansen,J.A., Rabinovitch,P.S. and June,C.H. (1986) *J. Immunol.*, **137**, 3299–3305.
Ledbetter,J.A., Imboden,J.B., Schieven,G.L., Grosmaire,L.S., Rabinovitch,P.S., Lindsten,T., Thompson,C.B. and June,C.H. (1990) *Blood*, **75**, 1531–1539.
Lister,M.D., Glaser,K.B., Ulevitch,R.J. and Dennis,E.A. (1989) *J. Biol. Chem.*, **264**, 8520–8528.
van Lier,R.A.W., Brouwer,M., De Groot,E., Kramer,I., Aarden,L.A. and Verhoeven,A.J. (1991) *Eur. J. Immunol.*, **21**, 1775–1778.
Lindsten,T., June,C.H., Ledbetter,J.A., Stella,G. and Thompson,C.B. (1989) *Science*, **244**, 339–343.
Linsley,P.S. and Ledbetter,J.A. (1993) *Annu. Rev. Immunol.*, **11**, 191–212.
Linsley,P.S., Brady,W., Urnes,M., Grosmaire,L.S., Damle,N.K. and Ledbetter,J.A. (1991) *J. Exp. Med.*, **174**, 561–569.
Los,M., Dröge,W. and Schulze-Osthoff,K. (1994) *Biochem. J.*, **302**, 119–123.
Lu,Y., Granelli-Piperno,A., Bjorndahl,J.M., Phillips,C.A. and Trevillyan, J.M. (1992) *J. Immunol.*, **149**, 24–29.
Lu,Y., Phillips,C. and Trevillyan,J.M. (1995) *Eur. J. Immunol.*, **25**, 533–537.
Martin,P.J., Ledbetter,J.A., Morishita,Y., June,C.H., Beatty,I.G. and Hansen,J.A. (1986) *J. Immunol.*, **136**, 3282–3287.
Meyer,M., Schreck,R. and Baeuerle,P.A. (1993) *EMBO J.*, **12**, 2005–2015.
Mihm,S., Ennen,J., Pessara,U., Kurth,R. and Dröge,W. (1991) *AIDS*, **5**, 497–503.
Mueller,D.L., Jenkins,M.K. and Schwartz,R.H. (1989) *Annu. Rev. Immunol.*, **7**, 445–480.
Nunès,J., Klasen,S., Franco,M.-D., Lipcey,C., Mawas,C., Bagnasco,M. and Olive,D. (1993) *Biochem. J.*, **293**, 835–842.
Nunès,J.A., Colette,Y., Truneh,A., Olive,D. and Cantrell,D.A. (1994) *J. Exp. Med.*, **180**, 1067–1076.
Pagès,F., Ragueneau,M., Rottapel,R., Truneh,A., Nunes,J., Imbert,J. and Olive,D. (1994) *Nature*, **369**, 327–329.
Paliogianni,F., Raptis,A., Ahuja,S.S., Najjar,S.M., Boumpas,D.T. (1993) *J. Clin. Invest.*, **91**, 1481–1489.
Prasad,K.V.S., Cai,Y.-C., Raab,M., Duckworth,B., Cantley,L., Shoelson, S.E. and Rudd,C. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 2834–2838.
Punt,J.A., Osborne,B.A., Takahama,J., Sharrow,S.O. and Singer,A. (1994) *J. Exp. Med.*, **179**, 709–713.
Rao,A. (1994) *Immunol. Today*, **15**, 274–281.
Rice,G.C., Bump,E.A., Shrieve,D.C., Lee,W. and Kovacs,M. (1986) *Cancer Res.*, **46**, 6105–6510.
Rincón,M. and Flavell,R.A. (1994) *EMBO J.*, **13**, 4370–4381.
Rola-Pleszczynski,M. and Stankova,M. (1992) *Blood*, **80**, 1004–1011.
Rola-Pleszczynski,M., Thivierge,M., Gagnon,N., Lacasse,C. and Stankova,J. (1993) *J. Lipid Mediat.*, **6**, 175–181.
Rouzer,C.A., Ford-Hutchinson,A.W., Morton,H.E. and Gillard,J.W. (1990) *J. Biol. Chem.*, **265**, 1436–1442.
Royall,J.A. and Ischiropoulus,H. (1993) *Arch. Biochem. Biophys.*, **302**, 348–355.
Samuelsson,B., Dahlén,S.-E., Lindgren,J.A., Rouzer,C.A. and Sherhan, C.A. (1987) *Science*, **237**, 1171–1176.
Schenk,H., Klein,M., Erdbrügger,W., Dröge,W. and Schulze-Osthoff,K. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 1672–1676.
Schreck,R., Rieber,P. and Baeuerle,P.A. (1991) *EMBO J.*, **10**, 2247–2258.
Schulze-Osthoff,K. and Baeuerle,P.A. (1995) *Adv. Mol. Cell Biol.*, in press.
Schulze-Osthoff,K., Beyaert,R., Vandevoorde,V., Haegeman,G. and Fiers,W. (1993) *EMBO J.*, **12**, 3095–3104.
Staal,F.J.T., Roederer,M., Herzenberg,L.A. and Herzenberg,L.A. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 9943–9947.
Stankova,J., Dupuis,G., Gagnon,N., Thivierge,M., Turcotte,S. and Rola-Pleszczynski,M. (1993) *J. Immunol.*, **150**, 4041–4051.
Su,B., Jacinto,E., Hibi,M., Kallunki,T., Karin,M. and Ben-Neriah,Y. (1994) *Cell*, **77**, 727–736.
Thompson,C.B., Lindsten,T., LedbetterJ.A., Kunkel,S.L., Young,H.A., Emerson,S.G., Leiden,J.M. and June,C.H. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 1333–1337.
Truitt,C.E., Hicks,C.M. and Imboden,J.B. (1994) *J. Exp. Med.*, **179**, 1071–1076.
Uehara,Y. and Fukazawa,H. (1991) *Methods Enzymol.*, **201**, 370–378.
Vacca,A. *et al.* (1992) *J. Exp. Med.*, **175**, 637–646.
Vandenberghe,P., Freeman,G.J., Nadler,L.M., Fletcher,M.C., Kamoun,M., Turka,L.A., Ledbetter,J.A., Thompson,C.B. and June,C.H. (1992) *J. Exp. Med.*, **175**, 951–960.
Verweij,C.L., Geerts,M. and Aarden,L.A. (1991) *J. Biol. Chem.*, **266**, 14179–14182.
Weiss,A. and Littman,D.R. (1994) *Cell*, **76**, 263–274.

Received on December 2, 1994; revised on April 10, 1995