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Pimecrolimus inhibits up-regulation of OX40 and synthesis of inflammatory cytokines upon secondary T cell activation by allogeneic dendritic cells

F. S. KALTHOFF, J. CHUNG & A. STUETZ

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SUMMARY

Pimecrolimus is a new non-steroidal inhibitor of T cell and mast cell activation. In the present study, we compared the potency of pimecrolimus and cyclosporin A (CyA) to inhibit cytokine synthesis of alloantigen-primed T cells and the expression of CD134 (OX40), an inducible co-receptor molecule thought to be critical for the survival and expansion of inflammation-mediating T cells. To mimic the physiological situation of recurrent antigenic stimulation, we have used dendritic cells (DC) as stimulators of purified $CD4^+$ T cells in the primary and secondary allogeneic mixed lymphocyte culture (allo-MLC). Pimecrolimus inhibited surface expression of OX40 and prevented the up-regulation of CD25 and CD54 with a 10-fold higher potency compared to CyA. Similarly, 50% inhibition of allo-DC-mediated T cell proliferation by pimecrolimus was obtained at 0·55 nM, compared to about 12 nM for CyA. Furthermore, pimecrolimus blocked the increase of OX40 on primed T cells restimulated on day 10 in secondary allo-MLC. Allo-DC-primed T cells showed a restricted cytokine profile characterized by the production of TNF-α, IFN-γ and IL-2 but low to undetectable levels of IL-4 and IL-10. The synthesis of TNF-α and IFN-γ and the up-regulation of OX40 on T cells after secondary allogeneic stimulation were almost entirely blocked by 10 nM pimecrolimus. Taken together, pimecrolimus inhibits T cell proliferation and Th1 cytokine synthesis and also prevents the up-regulation of the OX40 co-receptor on primed T cells indicating its potential in the therapy of chronic inflammation and autoimmunity.

Keywords allogeneic MLC cytokines dendritic cells OX 40 T cells

INTRODUCTION

Successful activation of naïve T cells requires recognition of antigenic peptide in association with major histocompatibility complex proteins and the concomitant stimulation by co-receptor molecules. The most extensively studied co-receptor on T cells is the CD28 molecule, which is expressed constitutively on all T cells [1,2]. CD28 can bind either CD80 or CD86, two receptors belonging to the B7 family of co-stimulatory ligands that are expressed on antigen-presenting cells (APC). Simultaneous activation by antigen and co-receptors triggers cytokine secretion and primary proliferation of naïve T cells [3]. In addition, antigenic activation in alliance with co-stimulation induces a variety of other surface molecules that are critical for sustained T cell proliferation and the differentiation into specialized effector T cells. An example is the co-receptor CD134, also known as OX40 molecule, which belongs to the TNF-receptor superfamily [4]. CD134 is expressed exclusively on Ag-stimulated T cells and peaks around 4–5 days after primary activation [5]. The specific ligand, OX40-L, has been reported as an inducible surface molecule on various types of APC that have encountered CD40-ligand on recently activated T cells [6]. In turn, the engagement of CD134 on preactivated T cells by OX40-L expressed on APC was shown to enhance T cell survival by inducing anti-apoptotic molecules of the Bcl-2 family [7] and appears to be of paramount importance for the generation of $CD4^+$ T memory cells [8,9]. Many recent studies have shown the importance of the CD134/OX40-L molecular pair as a co-stimulatory pathway that is operative in a variety of inflammatory disorders and autoimmune diseases. Previously, it was demonstrated that the blockade of CD134 could ameliorate ongoing inflammatory bowel disease [10] and decrease disease severity in experimental allergic encephalomyelitis, a model of human multiple sclerosis [11]. Furthermore, CD134 expression was increased on synovial fluid T cells but not peripheral blood T cells of patients suffering from rheumatoid arthritis [12]. OX40-L knock-out mice showed impaired sensitization for contact hypersensitivity reactions to oxazolone due to a failure of efficient T cell priming by APC [13]. In another study, OX40-L-deficient mice revealed a suppression of recall responses of T cells that were

Correspondence: Frank Kalthoff, Novartis Research Institute, Department AID, Brunnerstrasse 59, A 1235 Vienna, Austria.
E-mail: frank.kalthoff@pharma.novartis.com

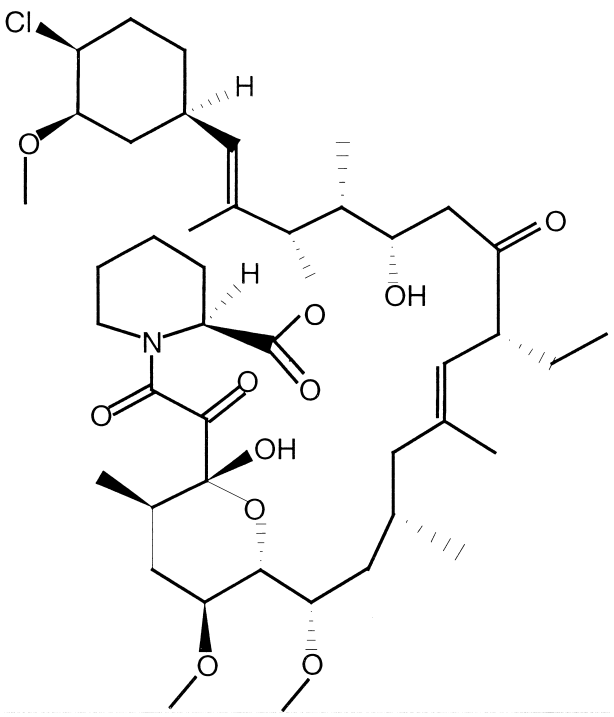

Fig. 1. Chemical structure of pimecrolimus (SDZ ASM 981, Elidel®). Reproduced from Meingassner *et al.* Br J Dermatol 1997; **137**:568–76, with kind permission of the publisher.

primed to protein antigens or alloantigens [14]. Taken together, these data imply that the inhibition of CD134 expression on activated T cells or the prevention of proliferation of CD134$^+$ T cells should prove beneficial in the treatment of chronic inflammatory diseases and autoimmunity.

In the present study, we have shown that pimecrolimus, a new ascomycin derivative referred to previously as SDZ ASM 981 (Fig. 1), potently and dose-dependently inhibited the up-regulation of CD134 on CD4$^+$ human T cells activated by allogeneic dendritic cells (DC) in the mixed lymphocyte culture (MLC). More importantly, pimecrolimus also prevented the up-regulation of CD134 on antigen-primed T cells and inhibited their production of inflammatory cytokines upon secondary stimulation. In animal models, pimecrolimus combines high anti-inflammatory activity in the skin with a low potential to impair systemic immune responses [15]. This profile could be corroborated in clinical studies, where pimecrolimus has proven to be well tolerated, safe and effective in the short- and long-term topical treatment of patients with atopic dermatitis [16,17]. The new findings reported in this study suggest a broader therapeutic potential of pimecrolimus and indicate its usefulness in the treatment of autoimmune and chronic inflammatory disorders.

MATERIALS AND METHODS

Cell preparation and culture

Mononuclear cells (MNC) were isolated from leukapheresis samples of healthy human volunteers by density gradient centrifugation over Lymphoprep® (Nycomed Pharma, Oslo, Norway). The cells were frozen and stored in liquid nitrogen until required for the purification of T cells or monocytes.

Primary MLC

As responders in one-way allogeneic MLC, CD4$^+$ T cells were purified from MNC aliquots by negative immunomagnetic selection (CD4$^+$ T cell isolation kit, Miltenyi, Bergisch-Gladbach, Germany). For strong activation of primary CD4$^+$ T cells, DC derived from monocytes of an unrelated donor were used as stimulatory cells in the MLC. For this purpose, monocytes were isloated from aliquots of MNC by immunomagnetic depletion of nonmonocytic cells (monocyte isolation kit, Miltenyi) and their differentiation into DC was induced by culture in RPMI-1640 medium containing 10% fetal calf serum (FCS) and supplemented with human recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) (300 U/ml) and IL-4 (200 U/ml), both from Novartis Biomolecular Productions (Novartis Pharma, Basel Switzerland). At the end of the 6-day culture period, the cells were analysed by flow cytometry to confirm their differentiation into DC (CD1a^{++}, CD14$^{low/-}$, CD40$^+$, CD54$^+$, CD80$^+$, CD86^{weak+}, HLA class II Ag$^{strong+}$). Aliquots of 2×10^6 DC were frozen in liquid nitrogen for subsequent use as stimulator cells. T cell stimulation in primary MLC was started by adding 1×10^5 purified CD4$^+$ T cells to $2{\cdot}5 \times 10^3$ allogeneic DC in 100 μl medium per well of 96-well round-bottom microtitre plates (Costar®, Corning Inc., NY, USA). Irradiation of the DC was not required as they lacked proliferative capacity. Subsequently, 100 μl of culture medium containing appropriate dilutions of pimecrolimus or cyclosporin A (CyA) were added to each well to determine inhibition of T cell stimulation. All samples were assayed in quadruplicate. T cell proliferation was measured by adding 1 μCi/well of tritiated thymidine (specific activity 3000 mCi/mMol, Amersham, UK) during the last 16 h of a 5-day culture period. The results are expressed as the mean cpm ± s.d. of quadruplicate wells. CD4$^+$ T cells and DC were always seeded separately in order to control for background proliferation.

Secondary MLC

To study secondary responses of allo-MHC-primed T cells, bulk primary MLC were performed by coculture of 2×10^6 CD4$^+$ T cells together with 5×10^4 DC in 24-well culture plates (Costar®). T cells were harvested from primary MLC on day 10, washed and viable cell counts were determined microscopically on the basis of trypan blue dye exclusion. CD4$^+$ T cells with a viability of greater than 90% were restimulated in secondary MLC using the same allogeneic DC batch as used in primary MLC. The actual ratio of DC and CD4$^+$ T cells depended on the aim of subsequent analysis. A DC/T cell ratio of 1/20 was chosen for the assay of secondary surface marker up-regulation. For this purpose, 2×10^5 CD4$^+$ T cells were stimulated by 1×10^4 DC in round-bottom 96-well plates and cultured in the presence or absence of appropriate concentrations of test compounds. After 4 days, T cells were harvested and processed for surface staining. For stimulation of intracellular cytokine synthesis in CD4$^+$ T cells, the DC : T cell ratio was increased to 1 : 6 allowing a more frequent and intense cellular interaction during secondary MLC. In this case, cells were harvested and processed for intracellular staining of cytokines after 22 h, as described in detail below.

Analysis of T cell surface antigen expression by flow cytometry

Cells were stained on ice with fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated murine monoclonal antibodies for 30 min in phosphate-buffered saline supplemented with 2% FCS and 0·1% sodium azide. After extensive washing, 7-amino-

actinomycin D (7-AAD, Sigma, St Louis, MO, USA) was added to each sample at a final concentration of 4 μg/ml prior to FACScan™ measurements, thus enabling the exclusion of dead cells during analysis of specific fluorescence with the CellQuest® software (Becton Dickinson, Mountain View, CA, USA). Appropriate evaluation of specific cell staining was achieved by compensation of nonspecific fluorescence in each channel. The following monoclonal antibodies were used for surface marker analysis of T cells or differentiated DC: anti-CD25-FITC or -PE (M-A251); anti-CD40-FITC (5C3); anti-CD83-PE (HB15e); anti-CD86-FITC or -PE (FUN-1); anti-CD134-PE (ACT35), all purchased from Pharmingen, San Diego, CA, USA; anti-CD3-FITC (UCHT1); anti-CD4-FITC (MT310); anti-HLA class II (CR3/43) were obtained from Dako, Glostrup, Denmark; anti-CD54-FITC (84H10) was obtained from Serotec, UK, and the anti-CD14-FITC or anti-CD1a-PE were purchased from Chemicon, Temecula, CA, USA or Coulter Corp., Miami, FL, USA, respectively.

Detection of intracellular cytokines by flow cytometry

To induce intracellular cytokine synthesis specifically after allo-MHC antigen-priming, aliquots of 3×10^6 T cells derived from bulk primary MLC were restimulated by 5×10^5 allogeneic DC in 48-well Costar plates each containing 1 ml culture medium supplemented with different concentrations of test compound as appropriate. After 10 h, exocytosis of cytokines was prevented by adding 10 μl/well of Golgi Plug® (PharMingen) containing Brefeldin A. Secondary MLC was continued for another 12 h and T cells were washed and stained with anti-CD40-FITC conjugated monoclonal antibody (MoAb) to allow clear distinction between DC and T cells in FACS analysis. Subsequently, aliquots of 3×10^5 T cells were fixed and saponin-permeabilized (Perm/fix solution, BD PharMingen) and incubated with one 0·4 μg/sample of the following PE-conjugated MoAbs: anti-IL2, anti-IL4, anti-IFN-γ, anti-TNF-α or isotypic control MoAb, all purchased from BD PharMingen. Specific detection of intracellular cytokines by the fluorochrome-conjugated MoAb was confirmed in each experiment by incubating one activated cell sample with an excess of nonlabelled, cytokine-specific blocking MoAb purchased from BD Pharmingen as additional control. In some experiments, 3×10^5 T cells were activated by phorbol myristate acetate (PMA) and ionomycin in the presence of Brefeldin A (activation cocktail supplied by BD PharMingen) for 6 h to achieve a rapid and maximal intracellular cytokine accumulation. Cell fixation, permeabilization and staining with PE-conjugated cytokine-specific MoAbs were performed in 96-well round-bottom plates according to the protocol of the manufacturer.

RESULTS

Pimecrolimus inhibits up-regulation of CD134 and T cell proliferation in primary MLC

Pimecrolimus is a novel drug that binds to macrophilin-12 and inhibits the phosphatase activity of calcineurin, thereby preventing antigen-mediated activation of T cells and mast cells [18]. In contrast to T cell cytokines or activation markers such as CD25 and CD54, the transcriptional regulation of co-receptors of the TNF-R superfamily such as CD134 is less well studied [19]. In the first series of experiments, pimecrolimus was compared to the calcineurin inhibitor CyA with respect to its potency to inhibit the induction of CD134 after primary T cell activation. To mimic the physiological situation, purified CD4$^+$ T cells were stimulated by allogeneic DC because they are known as powerful T cell activators. The allo-MLC was performed with a 40-fold excess of CD4$^+$ T cells over DC in the presence and absence of pimecrolimus and CyA. Figure 2a shows that the up-regulation of CD134, CD25 and CD54 expression was inhibited dose-dependently by pimecrolimus. The concentration range tested for pimecrolimus was 10 nM, 1 nM and 0·1 nM, and CyA was tested at concentrations of 100 nM, 10 nM and 1 nM. Figure 2b shows the parallel shift of dose-dependency for inhibition of each surface marker, indicating that pimecrolimus was at least 10-fold more potent than CyA. Notably, greater than 80% inhibition of CD134 increase was observed at a concentration of 10 nM pimecrolimus or 100 nM of CyA. In addition to the analysis of surface marker regulation, pimecrolimus and CyA were also compared for their potency to inhibit primary T cell proliferation stimulated by allogeneic DC. The analysis of proliferation data revealed IC_{50} values of 0·55 nM and 11·9 nM for pimecrolimus and CyA, respectively (Fig. 3). These values are in line with those published previously for the inhibition of antigen-specific T cell proliferation [18].

Secondary increase of CD134 expression on allo-activated T cells is prevented by pimecrolimus and CyA

Co-stimulation via CD134 on T cells upon secondary encounter with OX40-L expressing APC is believed to expand the antigen-specific T cell pool [8,9]. Thus, interference with the up-regulation of CD134 may be one possibility to stop the cycle of recurrent T cell restimulation in chronic inflammation and autoimmunity. This led us to analyse whether pimecrolimus would effectively inhibit the increase of CD134 co-receptor expression as a result of secondary T cell activation. For this purpose, CD4$^+$ T cells were stimulated by allogeneic DC and maintained in the priming culture for 10 days. Subsequently, the T cells were restimulated by the priming DC and CD134 expression levels were analysed in comparison to CD25 on day 14. Figure 4 shows that both CD134 and CD25 were expressed on 28% or 62% of the T cells, respectively, on day 10 prior to secondary allogeneic stimulation. Expression of both activation molecules declined unless T cells were subjected to restimulation by allogeneic DC. The secondary rise of CD134 and CD25 expression as determined by FACS on day 14 was inhibited by 10 nM of pimecrolimus or 100 nM of CyA. Relative to the difference between of the levels of both antigens obtained on day 14 for stimulated *versus* not restimulated T cells, CD134 and CD25 expression were inhibited to about 60% and 73%, respectively.

Inhibition of cytokine synthesis in restimulated CD134$^+$ T cells by pimecrolimus and CyA.

Chronic antigenic stimulation of T cells as occurring in autoimmune diseases is known to be associated with the local production of TNF-α and IFN-γ in the affected tissues [20]. In this study, we used secondary stimulation of allo-MHC-primed T cells as a reasonable approach to mimic antigenic restimulation *in vivo* and to evaluate the potency of drugs to inhibit cytokine synthesis. In these experiments, the DC : T cell ratio was increased to 1 : 6 to achieve a more potent and timely coordinated stimulation of the T cells. Production of cytokines was evaluated by intracellular staining with PE-labelled MoAb and analysis by flow cytometry. DC could be excluded from analysis based of their distinct side and forward scatter characteristics and by staining with the DC

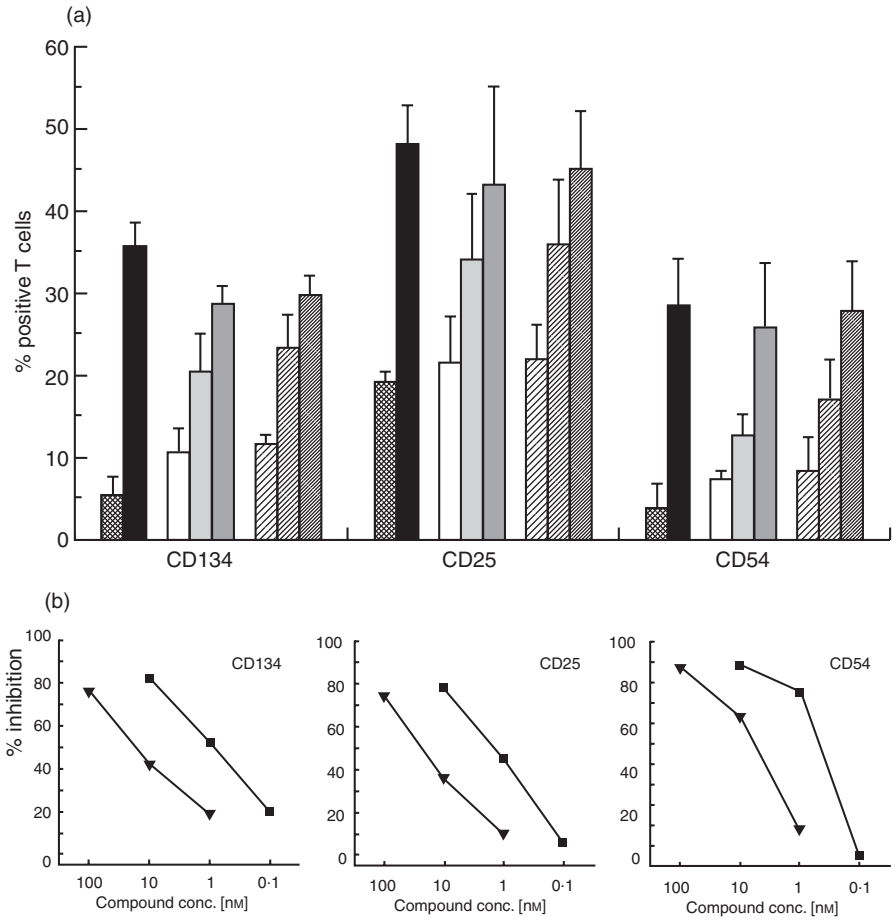

Fig. 2. Inhibition of surface antigen expression on CD4+ T cells activated in primary MLC. (a) Purified CD4+ T cells were stained with antibodies specific for OX40 (CD134), CD25 and CD54 before stimulation (cross-hatched) and after stimulation for 5 days by allogeneic DC at a DC/T cell ratio of 1/40 in the absence of compounds (black bars) and in the presence of either pimecrolimus used at 100 nM (white), 10 nM (grey) or 1 nM (dark grey) or cyclosporin A used at 1000 nM, 100 nM or 10 nM (left-hatchted bars with increasing density). The percentage of positive T cells is shown as the mean (± s.d.) obtained in three independent experiments. (b) Mean percentage inhibition of surface marker induction by pimecrolimus or cyclosporin A was calculated according to the formula given in the legend to Table 1. The data displayed were obtained in one out of three similar and independently performed experiments.

selective MoAb CD40-FITC prior to cell permeabilization (Fig. 5a). About 8–9% of all T cells were activated to produce IFN-γ and TNF-α that accumulated intracellularly due to the presence of Brefeldin A for the last 12 h of the 22-h stimulation period (Fig. 5b, upper panel). IL-2 accumulation was detected in about 2% of the T cells (range 2–4% in four independent experiments), whereas virtually no synthesis of IL-4 was seen. IL-10 was also undetectable in T cells stimulated in the secondary allo-MLC (data not shown). This pattern of cytokine production is characteristic for Th1 cells regarded as the major effector cell type in autoimmunity. As shown in Fig. 5b, the presence of 10 nM pimecrolimus during secondary MLC completely blocked the induction of IL-2 and TNF-α and inhibited synthesis of IFN-γ to about 70%. One nM pimecrolimus, a concentration corresponding to the IC_{50} determined in primary allo-MLC, reduced IL-2, TNF-α and IFN-γ synthesis to 87%, 65% and 36%, respectively, as shown in Table 1, summarizing the results obtained in four similar and independently conducted experiments. In line with the data shown for the inhibition of surface antigen up-regulation, the potency of CyA to inhibit intracellular cytokine synthesis was approximately 10-fold lower as compared to pimecrolimus. We also analysed the pattern of cytokine production after maximal T cell stimulation using the leucocyte activation cocktail provided by BD PharMingen containing PMA, ionomycin and Brefeldin A. As shown in Fig. 6, TNF-α and IL-2 were strongly induced in two-thirds of all T cells, whereas IFN-γ was detected in about one-third of the T cells. The lower percentage of IFN-γ-producing T cells can be explained by the short stimulation period (6 h), as IFN-γ production is known to have delayed kinetics. IL-4 synthesis was very low (5% after PMA/ionomycin stimulation) and IL-10 was not detectably produced. Also under conditions of maximal stimulation, 10 nM pimecrolimus blocked the synthesis of IFN-γ, TNF-α, IL-2 by 69%, 95% and 97%, respectively. Taken together, these results show that CD4+ T cells which were primed by allogeneic DC show a profound bias towards Th1 responses after restimulation. Moreover, in comparison to CyA, pimecrolimus showed a superior efficacy to inhibit the production of inflammatory cytokines.

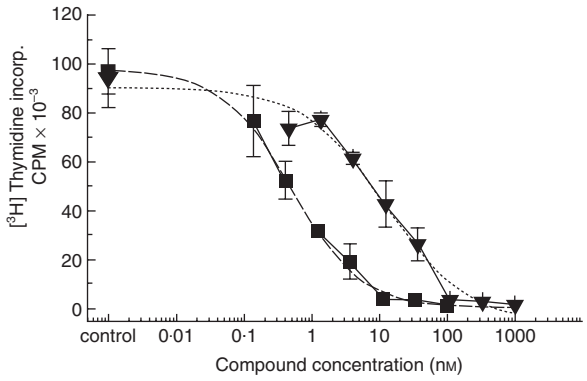

Fig. 3. Compound-mediated inhibition of proliferation of $CD4^+$ T cells in primary allogeneic MLC with DC as stimulator cells was determined by addition of [^{3}H]-thymidine during the last 16 h of the 5-day MLR. Results are shown as mean cpm ± s.d. of quadruplicate culture wells and are representative of three independently performed MLC assays. Sigmoidal curve fitting of plots obtained for CyA (dotted line) and pimecrolimus (dashed line) allowed determination of IC_{50} values using the Origin® software (version 6·1). ▼, CyA; $IC_{50} = 11{\cdot}8$ nM; ■, pimecrolimus; $IC_{50} = 0{\cdot}55$ nM.

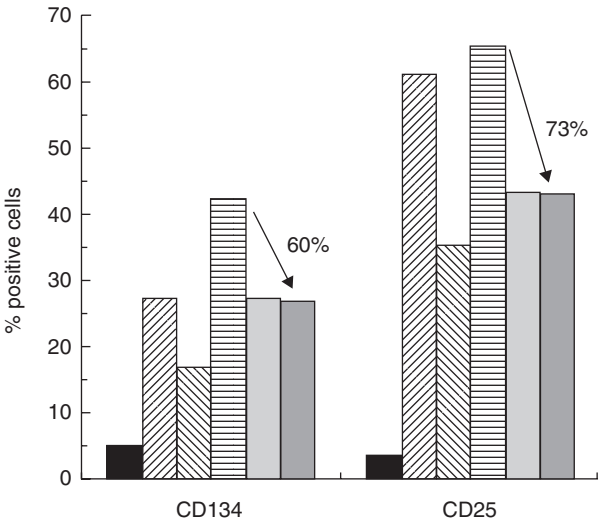

Fig. 4. Pimecrolimus inhibits the increase of CD134 expression on primed T cells. Only few resting $CD4^+$ T cells express CD134 or CD25 (filled black). DC-mediated T cell activation in MLC led to up-regulation of CD134 and CD25 as determined on day 10 (right-hatched). Thereafter, expression of both activation molecules dropped unless T cells were subjected to restimulation (RS) by DC on day 10. Relative to the level of surface expression on non-stimulated T cells (no RS control, set as 100% inhibition), CD134 and CD25 were inhibited to about 60% and 73% in the presence of 10 nM pimecrolimus (PiC) or 100 nM cyclosporin A (CyA) as indicated by downward arrows. Data are representative of three similar and independently conducted experiments. ■, Day 1: start of culture; ▨, day 10: time of RS; ▧, day 14: no RS control; ▤, day 14: RS control; ▩, day 14: RS + CyA (100 nM); ▩, day 14: RS + PiC (10 nM).

DISCUSSION

Since its discovery in the early 1990s [5,21,22], evidence has accumulated for a function of OX40/CD134 as co-stimulatory receptor which delivers positive signals for the differentiation of previously activated but not resting T cells. As a member of the TNF-receptor superfamily, CD134 has been shown to counteract apoptosis of activated T cells and to synergize with other signals in fostering memory T cell development. Several recent studies have indicated a critical role of OX40L in the co-stimulation of $CD134^+$ T cells in pathological conditions, such as inflammatory skin disease [23], graft-*versus*-host disease [24] and autoimmune disease [25,26]. For instance, in the experimental autoimmune encephalomyelitis (EAE) model in SJL mice, an antagonistic OX40L-specific MoAb inhibited the accumulation and the activation of pathogenic OX40-expressing T cells in the central nervous system (CNS) [27]. Similar to the situation in EAE, T cells isolated from the synovial fluid of rheumatoid arthritis patients strongly expressed CD134 [28], and OX40L was detected on the sublining cells of the synovial tissue [12]. Given the relevance of CD134/OX40L interaction in several diseases with an chronic inflammatory component, we investigated the relative potency of a new drug, pimecrolimus, to inhibit the up-regulation of the co-stimulatory receptor CD134. Pimecrolimus complexes with its intracellular protein ligand macrophilin-12 and inhibits the activity of the threonine/serine phosphatase calcineurin [18]. Consequently, T cell stimulation is blocked at the level of gene activation through the transcription factor NFAT, which depends on calcineurin activity for dephosphorylation-mediated translocation from the cytoplasm to the cell nucleus. In this respect, the mode of action of pimecrolimus is comparable to the known drug, cyclosporin A (CyA). As shown in primary allogeneic MLC, pimecrolimus was highly effective to prevent the up-regulation of surface expression of CD134. This activity was shared by CyA which, however, required a 10-fold higher concentration to block expression to a similar degree. The inhibition data for CD134 are in good agreement with the results obtained for CD25 and CD54, two activation molecules known to be sensitive to calcineurin inhibition. Together with the results for T cell growth inhibition in allo-MLC, these data confirm the one log difference in potency between pimecrolimus and CyA in primary assays. A major focus of this study was the up-regulation of CD134 and cytokine synthesis in T cells after secondary challenge by allogeneic DC. In this respect, pimecrolimus used at a concentration of 10 nM prevented CD134 up-regulation on about two-thirds of all T cells. Moreover, the same concentration of pimecrolimus almost completely inhibited the production of T cell cytokines. Additional time-course studies have demonstrated the first 24 h of secondary MLC as the crucial period for drug-mediated inhibition of CD134 up-regulation. Withdrawal of pimecrolims after this time point did not revert sustained inhibition of CD134 on allo-MLC restimulated T cells for a period of 6 days (data not shown).

In line with the T cell disbalance observed in many chronic inflammatory disorders, the allo-MHC-primed T cells showed a strong bias to synthesize Th1 cytokines, such as TNF-α and IFN-γ, in addition to the growth factor IL-2. The intracellular production of Th2 cytokines such as IL-4 and IL-10 was low to undetectable. The Th1 pattern of cytokine production also emerged after maximal activation by phorbol ester and ionomycin. This argues against the possibility that the production of anti-inflammatory cytokines such as IL-4 and IL-10 was missed in the analysis due to the low number of responding T cells. In any case, we feel that, in contrast to non-physiological activation by phorbol ester and Ca^{2+} ionophore, secondary MLC should mimic the antigenic restimu-

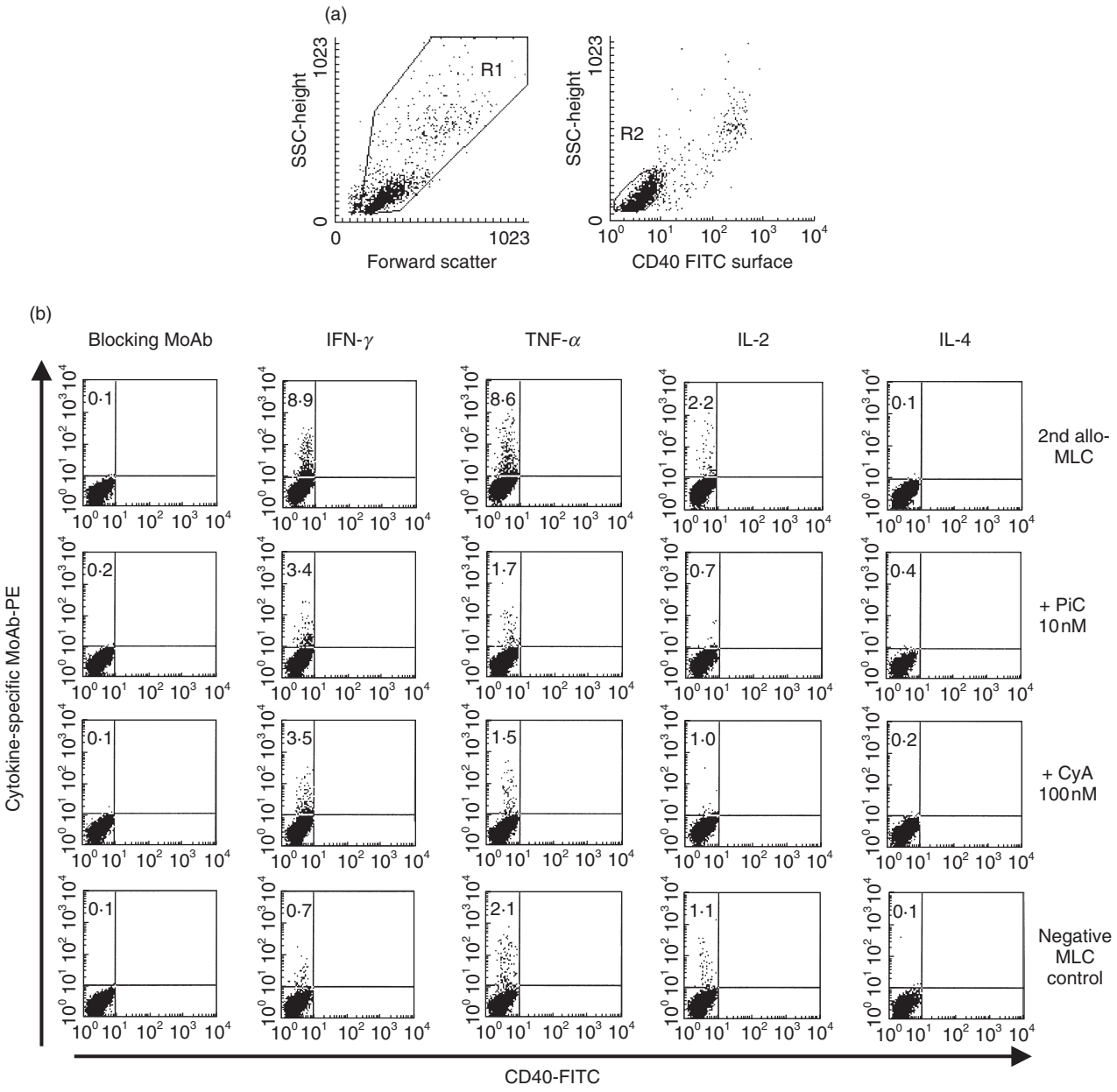

Fig. 5. Intracellular analysis of cytokine synthesis induced in MLC. Following priming in bulk MLC for 10 days, $CD4^+$ T cells were restimulated by the addition of the same batch of allogeneic DC in the presence and absence of PiC or CyA and incubated for 22 h, the final 12 h thereof in the presence of Brefeldin A. Subsequently, appropriate aliquots of the secondary MLC were processed for intracellular cytokine staining as described in Materials and Methods. (a) Gates for analysis were set to exclude cellular debris based on forward and side scatter (R1) and CD40-FITC staining (R2) to exclude DC from evaluation. (b) Specific intracellular staining of T cell cytokines as determined by competition with unlabelled anti-cytokine MoAb (blocking control) in each staining reaction. Inserted numbers indicate percentage of cytokine-expressing T cells. The data shown were obtained in one of four independent experiments with similar results (see also Table 1).

lation in inflammatory diseases more closely because only those T cells bearing antigen receptors with sufficient affinity become activated upon allogeneic restimulation and respond more rapidly as characteristic for primed T cells.

A strong bias for Th1 cell cytokine production was evident in two different rodent models for inflammatory bowel disease [10] and for myelin basic protein (MBP)-specific, encephalitogenic T cells isolated from the spinal cord of rats after adoptive transfer of EAE [29]. Moreover, in both models the disease-mediating T cells were found to be highly enriched for $OX40/CD134^+$ T cells. This observation prompted the investigators to use toxin-conjugated CD134-specific MoAbs as a therapeutic compound with which they showed selective eradication of pathogenic MBP-specific T cells *in vivo* and a drastic amelioration of disease symptoms [30]. On the other hand, enhancing the co-stimulation via OX40 by using an agonistic MoAb was shown to break established T cell

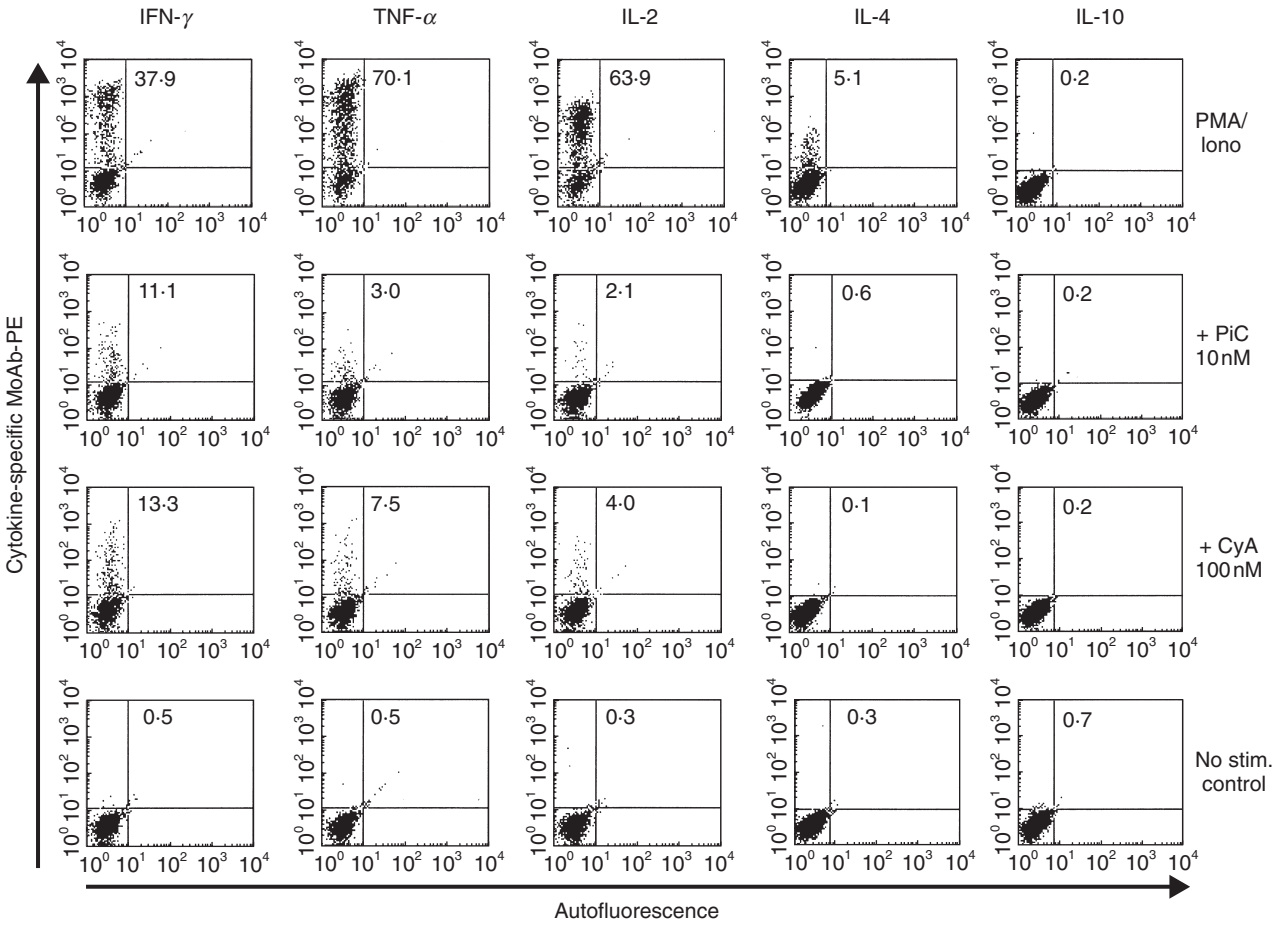

Fig. 6. Induction of intracellular cytokine synthesis by phorbol ester and Ca^{2+} ionophore. Allo-DC primed $CD4^+$ T cells were restimulated on day 10 of MLC by addition of PMA and ionomycin for 6 h in the presence of Brefeldin A. T cell samples were fixed, permeabilized, stained with cytokine-specific or isotype-control MoAb and analysed after addition of 7-AAD to exclude dead cells from evaluation. Inserted numbers indicate percentage of cytokine-expressing T cells. The data shown were obtained in one of two independent experiments with similar results.

Table 1. Percentage inhibition (mean ± s.d.) of cytokine synthesis in T cells as determined after secondary stimulation by allogeneic DC

	Pimecrolimus		CyA	
Cytokine	10 nM	1 nM	100 nM	10 nM
IL-2	94% (±10) (1)	87% (±11)	94% (±5)	84% (±9)
IFN-γ	76% (±17)	36% (±19)	73% (±19)	44% (±9)
TNF-α	100% (0)	65% (±23)	99% (±3)	69% (±11)

Data were derived from evaluation of intracellular cytokine staining in four independent experiments. Inhibition values were calculated by using the following formula:

$$\frac{\%\text{pos.cells stimulated} - \%\text{pos.cells stimulated with compound}}{\%\text{pos.cells stimulated} - \%\text{pos.cells unstimulated}} \times 100\%$$

tolerance *in vivo* or anergy *in vitro*, suggesting the therapeutic usefulness of such antibodies in tumour therapy [31].

Ideally, therapeutic intervention in chronic inflammation and autoimmune diseases should target the memory pool of lymphocytes and also inhibit the recurrent activation of naïve T cells to antigen. In this sense, pimecrolimus appears as an attractive new drug with high potency to interfere with CD134 expression, T cell proliferation and inflammatory cytokine synthesis.

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