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Platelet Factor 4 Inhibits Proliferation and Cytokine Release of Activated Human T Cells¹

Jens Fleischer,* Evelin Grage-Griebenow,[†] Brigitte Kasper,* Holger Heine,* Martin Ernst,* Ernst Brandt,* Hans-Dieter Flad,* and Frank Petersen^{2*}

Platelet factor 4 (PF-4), a platelet-derived CXC chemokine, has been shown to induce the differentiation of monocytes into a subset of macrophages that lack the expression of HLA-DR Ag. This suggests a potential role for PF-4 in the modulation of monocyte-dependent T cell activation. Using an Ag-specific stimulation model in which T cells were cocultured with monocytes in the presence of recall Ags, we could show that under these conditions PF-4-treatment caused a strong decrease of T cell proliferation as well as of IFN- γ release. However, inhibition of T cell functions such as proliferation, IL-2 release, and IL-2 mRNA production did also occur when isolated T cells were activated in the absence of monocytes with immobilized Abs directed against CD3 in combination with cross-linked anti-CD28 Abs. The effect could be reversed when low concentrations of exogenous IL-2 instead of anti-CD28 were used as a costimulus in combination with anti-CD3 Abs. Further evidence for direct modulation of T cell function by PF-4 was obtained by the detection of specific binding sites for the chemokine on the surface of these cells. Taken together, our results show that specific binding of PF-4, resulting in the down-regulation of the IL-2-release correlates with the inhibition of functions in activated T cells. *The Journal of Immunology*, 2002, 169: 770–777.

T cell activation is a tightly controlled process which does not only involve the TCR, but also a variety of costimulatory signals. Besides well-known cytokines like IL-2 or IL-4, which have been shown to participate in this regulatory process (1), more recent reports describe that also members of the chemokine family are able to modulate the activation state of T cells. Various CC chemokines including RANTES, the monocyte chemotactic proteins (MCP)³ -1, -2, -3, and -4, and the monocyte inflammatory peptides (MIP)-1 α and -1 β are able to induce chemotaxis and adherence, as well as transendothelial migration of T cells or specific T cell subsets (reviewed in Ref. 2). Furthermore, three members of the CXC subfamily of chemokines, the IFN- γ -inducible protein 10 (IP10), the human Mig, and the most recently discovered IFN-inducible T cell α chemoattractant have been shown to attract activated T cells through binding to their common receptor CXCR3 (3, 4). Besides these typical chemokine functions, little is known about a potential role of these molecules in the control of TCR-mediated responses. In a pioneering work, Taub and coworkers (5) demonstrated that CC chemokines RANTES, MCP-1, and MIP-1 α are capable of directly costimulating human T cell proliferation as well as IL-2 production in the presence of anti-CD3 Abs. In addition, these chemokines enhanced Ag-specific proliferation and increased the level of CD25 surface expression on T cells.

Platelet factor 4 (PF-4), a member of the CXC subgroup of the chemokine family, is released in high concentrations from activated platelets (6, 7). The functional role of PF-4 appears to be quite exceptional. In a recent report we could show that highly purified PF-4 lacks chemotactic activity for polymorphonuclear neutrophil (PMN), but in the presence of TNF- α stimulates these cells to exhibit functions like exocytosis of secondary granule markers or tight adhesion to different surfaces (8). Investigating PF-4 binding sites, we could demonstrate that PF-4-induced functions were not elicited through binding to IL-8Rs or another seven-transmembrane-domain molecule, but through interaction with an integral chondroitin sulfate proteoglycan expressed on the surface of human PMN (9, 10). Apart from short-term responses, PF-4 activity was shown to be also involved in long-term differentiation and regulatory processes such as the control of endothelial cell and fibroblast proliferation (11–13) and the support of the survival of hemopoietic stem cells as well as of progenitor cells (14). In a most recent report, we could show that PF-4 prevents human monocytes from spontaneous apoptosis and induces differentiation of monocytes into a certain subtype of macrophages (15). In contrast to GM-CSF-exposed or serum-derived macrophages, PF-4-treated cells were characterized by a total lack of surface-expressed HLA-DR Ag, while the costimulatory molecule B7-2 was found to be significantly up-regulated on the cell membrane.

Because a loss of HLA-DR could affect the capacity of macrophages to present Ag to T cells, we studied potential direct or indirect effects of PF-4 on human T cell activation in vitro. Our results demonstrate that PF-4 can act as a potent regulator of T cell functions by down-modulating cell proliferation and cytokine release. These results may indicate a potential role for PF-4 as a mediator of long-term effects in the regulation of inflammatory processes in vivo.

*Department of Immunology and Cell Biology, Research Center Borstel, Borstel, Germany; and [†]Department of Experimental Pneumology, University Hospital Bergmannsheil, Bochum, Germany

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² Address correspondence and reprint requests to Dr. Frank Petersen, Department of Immunology and Cell Biology, Research Center Borstel, Parkallee 22 D-23845, Borstel, Germany. E-mail address: fpetersen@fz-borstel.de

³ Abbreviations used in this paper: MCP, monocyte chemotactic protein; IP10, IFN- γ -inducible protein 10; MFI, median fluorescence intensity; MIP, monocyte inflammatory peptide; PF-4, platelet factor 4; PPD, purified protein derivative of tuberculin; TFA, trifluoroacetic acid; TTX, tetanus toxoid; PMN, polymorphonuclear neutrophil.

Materials and Methods

Preparation of PF-4

Human natural PF-4 was purified to homogeneity from release supernatants of thrombin-stimulated platelets in a three-step procedure as previously described (8). The preparations contained <0.125 ng LPS/mg PF-4

(i.e., below 4 pg/ml at 4 μ M PF-4) as determined by the *Limulus* amoebocyte lysate assay, ruling out potential side effects caused by contaminating LPS. PF-4 was lyophilized, stored at -80°C , and reconstituted to stock solutions of 1 or 2 mg/ml in 0.1% trifluoroacetic acid (TFA) before use.

Abs and fluorescence labeling

For stimulation of T cells, murine mAbs against CD3 (clone X35; Dianova, Hamburg, Germany) and CD28 (clone 28.1; Serotec, Wiesbaden, Germany) in combination with a rat-anti-mouse Ig antiserum (Dianova) were used. All Abs were without sodium azide and tested for low endotoxin levels.

Immunophenotyping of the cells was performed with the following mAbs: anti-CD3 (UCHT-1, IgG1; DAKO, Hamburg, Germany), anti-CD14 (M5E2, IgG2A; BD Biosciences, Heidelberg, Germany), anti-CD16 (DJ130c, IgG1; DAKO), anti-CD19 (HD37, IgG1; DAKO), anti-HLA-DR (L243, IgG2A; BD Biosciences), and the appropriate IgG1 and IgG2A isotype control Abs (DAKO), all directly labeled with PE. Briefly, 1×10^6 cells were stained at 4°C with amounts of Ab according to the recommendations of the manufacturer. Thereafter, the cells were washed, resuspended in PBS, and fixed with 3% paraformaldehyde solution for storage until analysis in the flow cytometer (FACSCalibur; BD Biosciences). The Abs directed against CD16, CD19, and HLA-DR were also used for T cell isolation (see next section). A murine mAb against PF-4 (clone PF63.1) was generated in our laboratory (15).

Cell preparation and culture

PBMC were prepared from venous blood of healthy single donors using density gradient centrifugation (16). The obtained PBMC were further separated into lymphocyte and monocyte fractions using counterflow centrifugation as previously described (17). Purity of the monocyte fractions exceeded 95% as determined by flow cytometry analysis of CD14 expression and α -naphthyl-esterase staining (17). Peripheral blood T cells were isolated from the lymphocyte fractions by MACS as previously described (18). Briefly, 50×10^6 lymphocytes were suspended in 5 ml PBS and labeled with Abs directed against CD16, CD19, and HLA-DR (50 μ l each), and incubated for 20 min on ice. After a washing-step, cells were incubated with a biotin-conjugated goat-anti-mouse Ig antiserum (diluted 1/100) and finally combined with streptavidin-coupled magnetic microbeads (diluted 1/10; Miltenyi Biotec, Bergisch Gladbach, Germany). The labeled cells were passed through a magnetic separation column, the effluent cells were collected and analyzed by flow cytometry. Purity of T cells was determined by flow cytometry analysis of CD3 expression and passed 97% without detectable platelet contamination in all experiments.

All cell cultures (T cells, T cells with monocytes, and PBMC) were performed in RPMI 1640 medium containing 10% v/v heat-inactivated FCS (Biochrom, Berlin, Germany), 100 U/ml penicillin G (Biochrom), 100 μ g/ml streptomycin (Biochrom), and 2 mM L-glutamine (Biochrom) in 96-well flat-bottom microtiter plates (200,000 cells/well; Nunc, Roskilde, Denmark). Viability of cells was routinely tested before and after stimulation by trypan blue exclusion and exceeded always 95%.

Binding of PF-4 to T cells

Iodination of PF-4 and binding experiments with iodinated PF-4 to T cells were performed as described for PMN in detail elsewhere (9). Briefly, cells were suspended at 2×10^7 cells/ml in D-PBS supplemented with 20 mg/ml BSA (binding buffer), and duplicate samples of 2×10^6 cells were incubated on ice for 2 h with 5-nM ^{125}I -labeled PF-4 in the presence or absence of increasing concentrations of unlabeled PF-4.

Alternatively, the association of PF-4 with T cell surfaces was determined by using indirect immunofluorescence labeling: 1×10^6 T cells were incubated in 100- μ l volumes with increasing concentrations of PF-4 for 1 h on ice or left untreated. After repeated washing steps, cells reacted for 30 min on ice with saturating concentrations of mAb PF63.1 (10 μ g/ml) to allow direct correlation between the Ab signal obtained and the relative amount of cell-associated PF-4. Following another washing step, the cells were incubated with a FITC-conjugated goat-anti-mouse Ig Ab (Dianova) for 30 min on ice and finally analyzed by flow cytometry. In some experiments, PF-4 (4 μ M) was preincubated with heparin (20 μ g/ml; Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C before addition to the cells. In assays where chondroitinase ABC-digested T lymphocytes were used, 5×10^6 cells were treated with 1 U/ml of the enzyme (protease-free; Sigma-Aldrich) for 30 min at 37°C as described previously for neutrophils (9) and subsequently tested for their capacity to bind PF-4.

Activation of T cells

For polyclonal stimulation, anti-CD3 Abs (clone X35) were immobilized to the surface of 96-well flat-bottom microtiter plates (Nunc) using a modification of the method described by Geppert and Lipsky (19). Briefly, volumes of 30 μ l of Ab solution (1 μ g/ml in PBS) per well were incubated for 1 h at 37°C . Following removal of unbound Ab by washing with HBSS, cells were distributed into the wells and, when indicated, anti-CD28 Abs were added (final concentration 1 μ g/ml). After 30 min at 37°C , cross-linking was performed by addition of rat-anti-mouse Ig (0.5 μ g/ml). PF-4 was added directly to the cell suspension before seeding into the culture plates (final concentration 4 μ M).

In some experiments, native human IL-2 (Blutspendedienst Niedersachsen, Springe, Germany) at a concentration of 10 U/ml was used instead of anti-CD28 Abs.

For antigenic stimulation, PBMC or T cells reconstituted with 10% autologous monocytes were seeded into culture plates (in the presence and absence of PF-4 at concentrations indicated in the text) and stimulated with purified protein derivative of tuberculin (PPD; Statens Serum Institut, Copenhagen, Denmark) at a concentration of 10 μ g/ml or with tetanus toxoid (TTX; Behring Werke, Marburg, Germany) at a concentration of 5 LF/ml.

Determination of lymphoproliferation and cytokine release

Cell proliferation was determined after 80 h of culture by pulsing the cells for 16 h with 17.4 kBq [^3H]methylthymidine (Amersham-Buchler, Braunschweig, Germany) per well. Thereafter, the cells were harvested onto glass fiber filtermats and the radioactivity was measured by beta scintillation counting. The release of cytokines into the supernatant was determined by sandwich ELISA. Cell culture supernatants were harvested after 24 and 96 h and analyzed for their content of IL-2 and IFN- γ , respectively. ELISA for IL-2 (BD PharMingen, Hamburg, Germany) was performed as recommended by the manufacturer while ELISA for IFN- γ (a kind gift from Dr. H. Gallati, Intex, Mutans, Switzerland) was done as described elsewhere (20).

Quantitative RT-PCR

Cells (10^6 /well) were cultured for 24 h in a 24-well culture plate using various stimuli, centrifuged, and the resulting cell pellets were frozen at -80°C . T cell mRNA was isolated using mRNA Direct MicroKit (Dyna, Hamburg, Germany) and subsequently reverse transcribed into cDNA (Superscript II; Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocols. Quantitative PCR was performed in a LightCycler instrument (Roche, Mannheim, Germany) using 2 μ l cDNA and 8 μ l PCR master reaction (FastStart DNA Master SYBR Green I; Roche) for the following primer combinations and PCR parameters: GAPDH, 5' primer: accacagtcacatgccatcac, 3' primer: tccaccacctgtgtctgtctgta, annealing temperature 58°C ; quantification at 88°C after 19 s elongation time at 72°C ; IL-2, 5' primer: cacagctacaactggagcattta, 3' primer: agaaattctacaatgtgtctgtc, annealing temperature starting at 68°C and touching down to 58°C with a decrease of $0.5^{\circ}\text{C}/\text{cycle}$, quantification after 16 s elongation time at 72°C . For quantification, several dilutions of stimulated human mononuclear cell cDNA was used as internal standard and calculation was performed using LightCycler software version 3.5.3 (Roche) and the second derivative maximum algorithm. Data were expressed as ratio between amounts of mRNA of IL-2 and GAPDH.

Determination of intracellular free calcium and chemotaxis

Intracellular Ca^{2+} transients were measured using the fura 2 method as described previously for human neutrophils (8). T cell chemotaxis was measured using a 48-well Boyden's chamber (NeuroProbe, Cabin John, MD) according to the method of Ludwig et al. (21). Briefly, agonists were serially diluted in RPMI 1640 (without phenol red) containing 0.1% BSA and added to the bottom wells of the chamber. These were covered with a polycarbonate membrane (pore size 5 μ m; Costar Nucleopore, Tübingen, Germany), and the top wells received 1×10^5 purified T cells suspended in 50 μ l RPMI 1640 supplemented with 0.1% BSA. After incubation for 2.5 h at 37°C in an atmosphere containing 5% CO_2 cells in the bottom, wells were lysed and the number of migrated cells was determined by measurement of endogenous β -glucuronidase enzymatic activity and calculation from a standard of lysed cells run in parallel.

Statistics

All experiments were performed four to eight times. Due to the quantitative differences measured for single donors, the data from proliferation and cytokine responses were not pooled. The statistical significance of these

results was analyzed by using Wilcoxon's signed-ranks test for paired samples. This nonparametric test does not require a normal gaussian distribution of the samples.

Results

PF-4 reduces lymphoproliferation of mononuclear cells

In a first approach, a potential impact of PF-4 on the proliferation of human T lymphocytes was investigated. Mononuclear cells were isolated as described and subsequently stimulated with the recall Ag tuberculin (PPD, from *Mycobacterium tuberculosis*) in the presence or absence of increasing concentrations of PF-4 (0.1–8 μ M). After 96 h of culture, the rate of lymphoproliferation was determined by incorporation of [3 H]methylthymidine. As depicted in Fig. 1, the proliferation rate of cells treated with PF-4 concentrations up to 0.1 μ M did not differ from that receiving no PF-4 (~5600 cpm). However, PF-4 dosages of 0.5 μ M or higher provoked a dose-dependent decrease in cell proliferation, with maximal inhibition by ~60% observed at 4 μ M PF-4. No effect was seen in cultures run in parallel receiving only medium supplemented with an equivalent proportion of the PF-4 solvent TFA (data not shown). In the absence of PPD, no cell proliferation was seen, irrespective of whether PF-4 was present or not (data not shown). Because a dosage of 4 μ M PF-4 appeared to be optimal, this dosage was chosen for all additional experiments. These data suggested a potent immunomodulatory function of PF-4. However, because mononuclear cells represent a mixed fraction of T cells, B cells, NK cells, and monocytes, it remained unclear whether the observed effect was caused by changes in the interaction between monocytes and T cells or whether other cell types could also participate in this process.

PF-4 reduces Ag-specific proliferation and IFN- γ release in cocultures of monocytes and T cells

In a next series of experiments, we analyzed the effects of PF-4 on Ag-specific T cell activation by the use of highly purified leukocyte subsets. T cells were cultured with 10% autologous monocytes as APCs and stimulated with PPD or TTX (from *Clostridium tetani*). Additional to the rate of lymphoproliferation, the release of the typical Th1 cytokine IFN- γ was determined as an important activation marker for the recall Ags tuberculin and TTX. Stimulation of Ag-specific T cells with the appropriate Ag, either tuberculin or TTX, led to cell proliferation (~16,200 and ~28,700 cpm, respectively; Fig. 2A) and secretion of IFN- γ (~9,200 and ~6,600 pg/ml, respectively; Fig. 2B). Neither proliferation nor IFN- γ re-

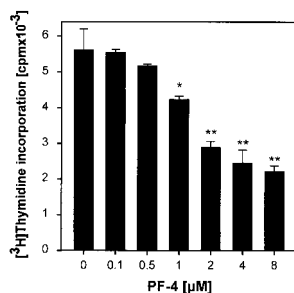


FIGURE 1. Effect of PF-4 on the proliferation of mononuclear cells. Isolated PBMC were stimulated with tuberculin (10 μ g/ml) in the absence or presence of increasing concentrations of PF-4 for 96 h. Proliferation of PBMC was determined by incorporation of [3 H]methylthymidine as described in *Materials and Methods*. Data represent mean \pm SD of one representative experiment of four, each performed in triplicate. Statistically significant differences between PF-4-treated and untreated samples are indicated (*, $p < 0.04$ and **, $p < 0.01$, respectively).

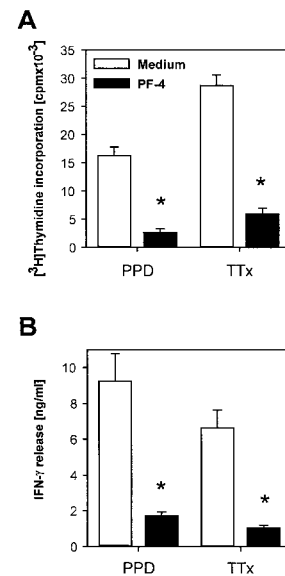


FIGURE 2. Effect of PF-4 on the proliferation and IFN- γ release of Ag-activated T cells. Purified T cells were combined with 10% autologous monocytes and stimulated with PPD (10 μ g/ml) or TTX (5 LF/ml) in the absence (\square) or presence (\blacksquare) of a constant concentration of PF-4 (4 μ M). **A**, Proliferation of T cells was determined after 96 h as described in the legend to Fig. 1. **B**, At the same time point, supernatants from parallel cultures of cells from the same donor were taken and monitored for the presence of IFN- γ by ELISA. Data represent mean \pm SD of one representative experiment performed in triplicate of 10. *, Significant differences ($p < 0.001$) between PF-4-treated and untreated samples based on the data from 10 experiments.

lease were observed in control cultures run in parallel and receiving no Ag (data not shown). The presence of PF-4 caused a substantial and significant ($p < 0.001$, calculated on the basis of 10 experiments) inhibition of both cell proliferation (84 and 79% of inhibition) and IFN- γ release (81 and 84% of inhibition) in PPD and TTX-stimulated cells, respectively. Again, treatment of cells with the solvent TFA was without effect on the responses measured. These data provide evidence that either monocytes or T cells are the principal effector cells responsible for the inhibitory effects of PF-4 on T cell proliferation and cytokine release, while the presence of other cell types from the mononuclear cell fraction is not required.

PF-4 reduces proliferation and IL-2 release of purified T cells

To investigate whether there existed a direct effect of PF-4 on T cells, experiments with highly purified T cells in the absence of monocytes were performed. In a polyclonal stimulation model, PF-4 or a corresponding amount of medium was added to purified T cells, which were activated with immobilized anti-CD3 and cross-linked anti-CD28 as described in *Materials and Methods*. Control cultures were performed in parallel in the absence of any Ab or with anti-CD3 alone. Because T cells do not produce IFN- γ in such a stimulation model, the secretion of IL-2 and the rate of lymphoproliferation were determined as activation markers after 24 and 96 h, respectively. Stimulation with immobilized anti-CD3 alone did not induce a relevant cell proliferation (~780 cpm in [3 H]thymidine incorporation, Fig. 3A), indicating that no accessory cells were left in the T cell preparation. Furthermore, treatment with PF-4 alone or in combination with CD3 Abs was without effect on this cell function. However, the combination of anti-CD3 and anti-CD28 induced a strong proliferative response (~34,500 cpm), which became significantly ($p < 0.005$) reduced by >64%

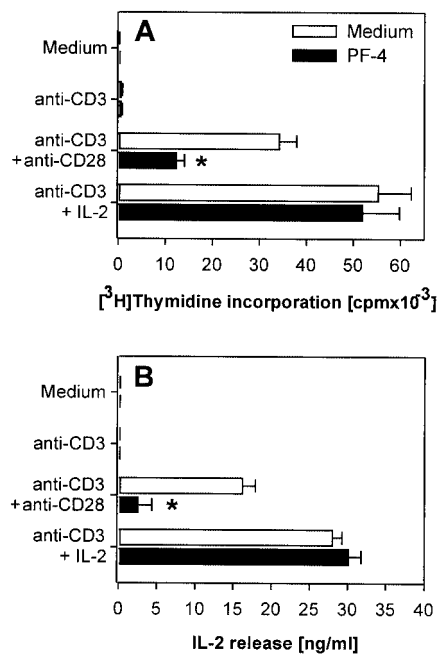


FIGURE 3. Effect of PF-4 on the proliferation and IL-2 release of purified T cells. Isolated cells were stimulated with Abs directed against CD3 alone or in combination with either anti-CD28 Abs or exogenous IL-2 (10 U/ml) in the absence (□) or presence (■) of a constant concentration of PF-4 (4 μ M). *A*, Proliferation of T cells was determined after 96 h as described in the legend to Fig. 1. *B*, After 24 h, supernatants were monitored for the presence of IL-2 by ELISA. In costimulation experiments where exogenous IL-2 was added as a costimulus, the amount of endogenous IL-2 was determined by subtracting the amount of exogenous IL-2 (10 U/ml equivalent to 4 ng/ml) from the total IL-2 measured. Data represent mean \pm SD of one representative experiment of six, each performed in triplicates. Statistically significant differences between PF-4-treated and untreated samples are indicated (*, $p < 0.005$).

in the presence of PF-4. Interestingly, this inhibitory effect of PF-4 did not occur when cell stimulation was performed with anti-CD3 in combination with exogenous IL-2 (10 U/ml) instead of Abs against CD28 (Fig. 3A). Cultures of T cells with IL-2 alone without anti-CD3 stimulation did not show a significant proliferation response (data not shown). These data provide first evidence that PF-4 is directly active on T cells; and moreover, suggest a critical role for IL-2 in the regulation of this process.

Consequently, in a next step we examined whether there was an effect of PF-4 on the release of endogenous IL-2 by stimulated T cells. In these experiments, results very similar to those seen with lymphoproliferation were obtained. As shown in Fig. 3B, neither medium nor immobilized anti-CD3 alone were able to induce measurable amounts of IL-2 (<78 pg/ml), while as expected, costimulation of cells with anti-CD3 and anti-CD28 provoked the release of high amounts of the cytokine (~16,000 pg/ml). However, the presence of PF-4 caused a strong and significant ($p < 0.005$) inhibition of IL-2 release by >84%. The addition of exogenous IL-2 (10 U/ml, equivalent to 4,000 pg/ml) instead of anti-CD28 Abs led to the induction of additional endogenous IL-2 (a total of 28,000 pg/ml as calculated by subtraction of the exogenous IL-2 added to the cultures). In contrast to its inhibitory effect in anti-CD3/anti-CD28-stimulated cultures, PF-4 did not affect IL-2 induction in the latter cultures.

FACS analysis of anti-CD3/anti-CD28-stimulated T cells revealed that treatment of these cells with PF-4 did neither affect the surface expression of CD25 (IL-2R α -chain) nor that of CD28

itself, indicating that the inhibitory effect of PF-4 was not mediated through down-regulation of these receptors (data not shown). Taken together, our data indicate that PF-4 directly inhibits T cell proliferation and that the observed antiproliferative effects are most likely due to inhibition of the autocrine IL-2 release.

PF-4 inhibits the induction of IL-2 mRNA

As PF-4 strongly reduced the release of IL-2 from activated T cells, the question arose whether this could be due to a reduced expression of IL-2 mRNA in these cells. Therefore, purified T cells were stimulated with immobilized anti-CD3 alone or in combination with anti-CD28 in the presence and absence of PF-4 as described above. After 24 h of culture, total mRNA was extracted and the presence of IL-2 mRNA was quantitatively analyzed by real-time fluorescence RT-PCR with the LightCycler system and expressed as ratio to the amount of the housekeeping gene GAPDH. The amplified product was further identified by DNA-sequencing of the product. As expected, unstimulated cells as well as cells stimulated with immobilized anti-CD3 alone express only very low amounts of IL-2 mRNA (1- to 2-fold over GAPDH-mRNA, Fig. 4). Costimulation of cells with anti-CD3 and anti-CD28 led to a clear up-regulation of IL-2 mRNA expression (6,600-fold over GAPDH-mRNA). Addition of PF-4 to the stimulated cells resulted in clear and significant ($p < 0.04$) reduction of the expression of IL-2 mRNA (1,760-fold over GAPDH-mRNA) as compared with CD3/CD28 costimulated cells. Although IL-2 mRNA expression in anti-CD3/CD28-stimulated cells showed a strong variation between the individual experiments (from 1,600-fold to 185,000-fold over GAPDH), PF-4-mediated inhibition appeared to be rather constant (between 74 and 88.9%, data not shown). These data clearly show that the observed decrease in IL-2 release correlates with a reduced IL-2 mRNA expression and indicate that PF-4 exerts its regulatory effect upstream of the transcription event.

Binding of PF-4 to human T cells

Our unexpected finding that PF-4 has the capacity to directly modulate T cell functions as described above raised the question as to

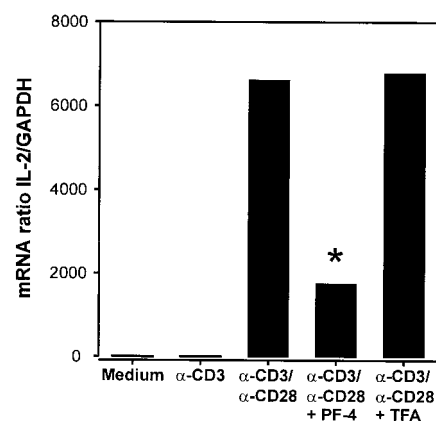


FIGURE 4. Effect of PF-4 on the expression of IL-2 mRNA in purified T cells. Cells were left untreated or stimulated with immobilized anti-CD3 Abs alone, in combination with anti-CD28 Abs, or in combination with anti-CD28 in the presence of 4 μ M PF-4 or a respective amount of the solvent TFA instead of PF-4. After 24 h of culture, total mRNA was extracted from the cells and IL-2 mRNA was quantitatively analyzed by real-time fluorescence RT-PCR with the LightCycler system. Data of one representative experiment of three are given and expressed as ratio of amounts of IL-2 mRNA vs amounts of the mRNA of the housekeeping gene GAPDH. Statistically significant differences between PF-4-treated and untreated stimulated samples are indicated (*, $p < 0.04$).

whether these effects were mediated through binding of PF-4 to specific receptors on these cells. Although the rather high PF-4 concentrations required for T cell activation suggested that the putative receptors were of relatively low affinity, we first performed binding analyses to examine T cells for the presence of potential high-affinity binding sites. Therefore, cells were incubated with a low concentration of iodinated PF-4 (5 nM) in the presence of increasing dosages of unlabeled PF-4. With concentrations up to 50 nM of cold PF-4, binding of the labeled ligand remained unchanged, indicating the absence of PF-4 high-affinity binding sites (Fig. 5). However, at dosages of cold PF-4 higher than 50 nM, ^{125}I -labeled PF-4 binding dramatically increased over background levels, reaching a maximum with 1 μM of cold PF-4. Further enhancement of cold ligand concentration led to a dose-dependent decrease in the amount of bound ^{125}I -labeled PF-4 down to background levels. This kind of "bell-shaped" competition kinetics has been previously described for the interaction of PF-4 with the chondroitin sulfate side chain of a proteoglycan which represents the PF-4 receptor on human PMN (9) and was found to be due to the selective interaction PF-4 receptors with the tetrameric form of the chemokine. This aspect will be discussed in more detail later on.

Because of the rather low affinity of PF-4 receptors on T cells, equilibrium binding experiments appeared impracticable, as these would have required extraordinarily high concentrations of the radioactively labeled ligand (up to 8 μM) and even higher concentrations of unlabeled PF-4 to achieve full competition for receptor binding (at least 160 μM ; 9).

To circumvent these problems, we chose an alternative approach to characterize the kinetics of PF-4 binding to T cells. To assess the binding kinetics of PF-4 to T cells, we made use of a newly developed mAb (PF63.1) which binds PF-4 in its soluble as well as in its cell-associated form. Different to another anti-PF-4 Ab previously established in our laboratory (mAb PF1; Ref. 22), binding of PF-4 to mAb 63.1 could not be inhibited by soluble chondroitin sulfate or other glycosaminoglycans (data not shown). Furthermore, PF-4 in complex with the latter Ab was still able to bind to GAG-chains associated with the PF-4 receptor on neutrophils (data not shown), all indicating the binding sites for GAGs and Ab on

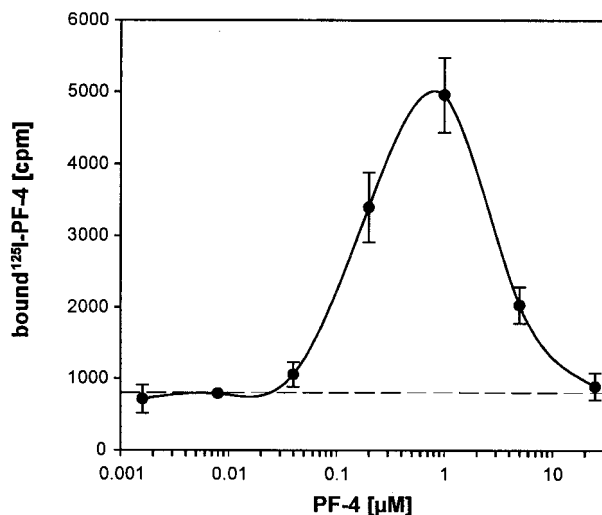


FIGURE 5. Competition of unlabeled PF-4 for binding of ^{125}I -labeled PF-4 to T cells. T cells were incubated with 5 nM ^{125}I -labeled PF-4 alone (dashed line) or in the presence of increasing concentrations of unlabeled PF-4 (●). Data represent total bound radioactivity and are given as mean \pm SD of three independent experiments.

the PF-4 molecule are different. The applicability of the Ab for the analysis of PF-4 binding kinetics to PMN was verified by FACS Analysis, where binding of saturating amounts of the Ab to cells receiving increasing concentrations of all cell-associated PF-4 followed saturation kinetics identical with those observed with the binding of radiolabeled PF-4 (data not shown). In accordance with experiments performed with iodinated PF-4 in parallel, neither binding of PF-4 nor of mAb PF63.1 was observed with PMN pretreated with chondroitinase ABC or under conditions where PF-4-binding was blocked by the addition of heparin (data not shown). These results demonstrate that mAb PF63.1 detects PF-4 bound to its receptor on cell surfaces.

In a first approach, isolated T cells were incubated with increasing concentrations of PF-4, ranging from 0.001–8 μM , and cell-associated PF-4 was subsequently detected with saturating concentrations of the Ab. As depicted in Fig. 6A, a first signal exceeding background levels (median fluorescence intensity (MFI) of 5) was observed at 0.1 μM PF-4 (MFI of 45). Fluorescence intensity increased dose dependently, reaching a maximum at 4 μM PF-4 (MFI of 912). Cells not preincubated with PF-4 did not bind the Ab. The observed binding kinetics correlated with the kinetics of PF-4-induced T cells functions (refer to Fig. 1). However, with regard to affinity and selectivity, PF-4 binding sites appear to be rather untypical for chemokine receptors which are activated already at nanomolar concentrations of their ligands. This

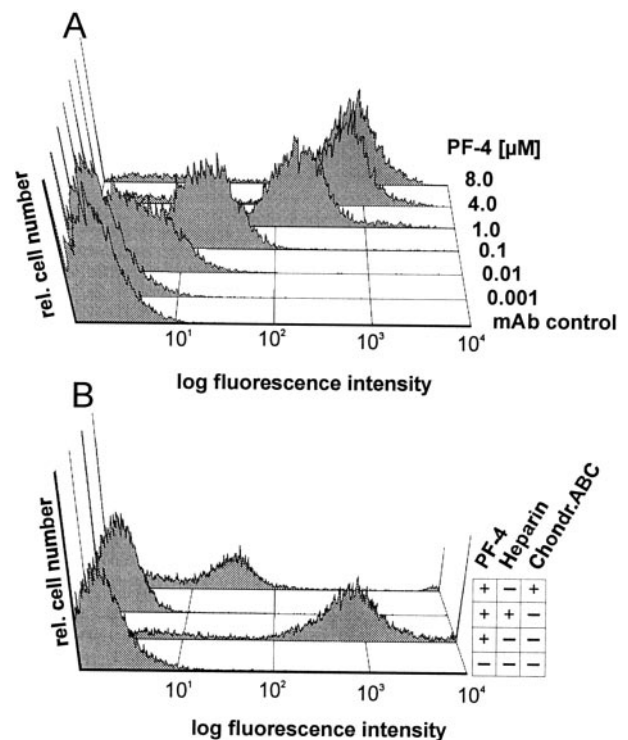


FIGURE 6. Binding of PF-4 to isolated T cells. *A*, Purified cells were incubated with increasing concentrations of PF-4 as indicated or were left untreated (Ab control), and cell-bound PF-4 was detected by flow cytometry by indirect fluorescence staining using saturating concentrations of mAb PF63.1 as the detecting Ab. *B*, Effect of heparin or treatment of cells with chondroitinase ABC on the binding of PF-4. Cells were incubated in the absence (*first row*) or presence of a constant concentration of PF-4 (4 μM) alone (*second row*) or in the additional presence of heparin (10 $\mu\text{g}/\text{ml}$, *third row*). In a parallel set, cells were preincubated for 30 min with 1 U/ml chondroitinase ABC before PF-4 binding (*fourth row*). Cell-associated PF-4 was determined as described above. The data from one representative experiment of four are given.

impression was strengthened by our observations that PF-4 does not induce characteristic chemokine functions in T cells such as changes in the intracellular free calcium concentration at dosages up to 4 μM (data not shown). Furthermore, we were unable to detect any chemotactic activity of the chemokine at concentrations varying between 0.125 and 4 μM (data not shown). Because we have shown previously that PF-4 binding to neutrophils does not involve typical seven-transmembrane domain receptors, but is mediated by chondroitin sulfate proteoglycans (9, 10), we wondered whether glycosaminoglycans could also be responsible for its interaction with T cells. To investigate this, PF-4 binding to T cells was conducted either in the presence of heparin or, in a parallel set of experiments, with cells digested with chondroitinase ABC before incubation with PF-4. Although cells treated with PF-4 showed a strong positive signal upon detection with mAb 63.1 (MFI of 995, Fig. 6B), the presence of heparin completely abrogated PF-4 binding to the cells (MFI of 5). Furthermore, binding of PF-4 was reduced by >94% with cells pretreated with chondroitinase ABC (MFI of 64), indicating that indeed glycosaminoglycans of the chondroitin sulfate type are involved in this process. Therefore, our data provide first evidence that PF-4 binding to cell surface glycosaminoglycans may not be restricted to neutrophils and endothelial cells, but could represent a more general mechanism in the interaction of PF-4 with cellular receptors.

Discussion

In the present study, we report on the discovery of novel biological activities of the platelet-derived CXC chemokine PF-4 for human T cells. Although rapidly inducible T cell functions like chemotaxis, adherence, or transmigration can be mediated by several chemokines, including members of the CXC group like stromal cell-derived factor-1, Mig, and IP10 (reviewed in Refs. 2 and 23), the role of chemokines in the regulation of long-term biological functions in these cells has remained largely unknown and is only beginning to emerge.

Our study was initiated by the observation that treatment of monocytes with PF-4 leads to a complete loss of HLA-DR Ag expression on their surface. By looking for a function of this specific subtype of macrophages, we hypothesized that PF-4 could potentially act as an indirect suppressor of T cell activation by reducing the capacity of monocytes to present Ag to these cells. However, in this report we show for the first time that not only monocytes are affected by the chemokine, but that PF-4 directly elicits long-term biological effects in T cells and can act as a potent suppressor of T cell function in terms of reducing lymphoproliferation and IL-2 mRNA expression, as well as inhibiting the release of IL-2 and IFN- γ . Thus, PF-4 acts in a manner quite opposite to several other chemokines, which were reported to promote T cell functions. In a first report, Bacon et al. (24) showed that a high concentration of RANTES (1 μM) can directly induce T cell proliferation and IL-2 production in the absence of additional activating signals. In a more detailed study, Taub et al. (5) could demonstrate that at physiological concentrations the CC chemokines MIP-1 α and -1 β , RANTES, and MCP-1 are capable of directly costimulating purified human T cell proliferation and IL-2 production in the presence of anti-CD3 mAb and of augmenting lymphoproliferation in an Ag-specific stimulation model *in vitro*. Nevertheless, in the same study the authors found PF-4 to lack any capacity for the modulation of these T cell responses. Although these findings appear to contradict our present observations, it has to be taken in account that the experimental approaches used differed from ours by several important features. Taub et al. (5) used T cell preparations that responded vigorously to immobilized anti-CD3 alone, indicating a residual costimulatory potential within

their cell preparation which may have overcome the PF-4-mediated effects. Even more important may be the fact that these authors used concentrations of the different chemokines up to maximally 1 $\mu\text{g/ml}$ (~ 0.14 μM). However, we observed first biological effects with PF-4 at ~ 3 - to 4-fold higher concentrations. Although in general it may be sufficient to investigate chemokine-induced cellular functions within the nanomolar concentration range, conditions for PF-4 are likely to be somewhat different. PF-4 is not secreted from cells after a prolonged time of stimulation, but is released at high concentrations from activated platelets within minutes. Although no data exist in the literature concerning the PF-4 concentrations existing at sites of acute platelet activation *in vivo*, normal serum concentrations of PF-4 (1–2.5 μM ; Refs. 6 and 25 and our unpublished observations) would be sufficient to modulate T cell responses. Interestingly, according to our findings, PF-4-mediated modulation of T cell functions occurs in the same concentration range (0.5–4 μM) as that required for the activation of human neutrophils (22) and monocytes (15, 26).

The capacity of PF-4 to inhibit proliferation of various cell types has been reported earlier. However, the reported mechanisms of PF-4 action appear to be different from those we observed in T cells. Antiproliferative activity of the chemokine for endothelial cells and fibroblasts was shown by several authors (12, 13) to involve competition of PF-4 for coreceptor binding of growth factors, the formation of heterooligomers with growth factors as well as direct effects of PF-4 on these cells (11). However, we could show that PF-4 suppresses significantly the secretion of IL-2 by purified T cells. This will most likely lead to disturbance of the IL-2 autocrine loop required for the activation of these cells. There exists several possibilities of how PF-4 could affect T cell activation. One aspect could be the induction of immunosuppressive cytokines by PF-4, for example, TGF- β or IL-10. These cytokines are known to be secreted by T cells in response to polyclonal stimulation and could inhibit functions of these cells by an autocrine mechanism. However, in view of the reported principles by which these cytokines suppress T cell functions, their participation in PF-4-mediated processes appears unlikely. TGF- β has been shown to arrest the cell cycle progression of T cells induced by IL-2 (27). By contrast, we found that PF-4-mediated suppression of proliferation could be overcome by low concentrations of exogenous IL-2 (4 ng/ml), indicating that IL-2 secretion rather than the susceptibility of T cells to IL-2 becomes altered by the chemokine. Furthermore, Weller et al. (28) reported that TGF- β induces apoptosis in activated T cells, a phenomenon we did not see during culture with PF-4 (our unpublished observations). Finally, treatment of murine T cells with TGF- β resulted in an enhanced expression of IL-2 mRNA (29), while PF-4 stimulation mediated the opposite effect. IL-10, a further potential candidate for indirectly mediating PF-4-induced effects, was reported to suppress T cell proliferation by down-regulating the expression of the IL-2R, but not by modulating the IL-2 mRNA expression (30). According to our own observations, the amount of surface-expressed IL-2R α -chain (CD25) remained unchanged during stimulation with PF-4. These phenomena provide indirect evidence that neither IL-10 nor TGF- β are involved as indirect mediators of PF-4-induced effects on T cells.

A further possibility of PF-4 action could be an interference with the intracellular signaling of CD28. The latter molecule has been shown to be involved in the induction and stabilization of IL-2 mRNA expression (31, 32). In contrast, it should be mentioned that PF-4 stimulation did not alter the level of CD28-expression on the surface of activated T cells. An even more complicated situation is encountered with the interpretation of the effects of PF-4 in the more physiological Ag-specific system using

autologous monocytes. Comparable to its effect on isolated T cells, PF-4 provoked a significant decrease of lymphoproliferation in response to TTX as well as to tuberculin and inhibited the release of IFN- γ in these cells. The release of IFN- γ in the Ag-specific system depends on the presence of memory Th1 cells (33). IFN- γ is not only a typical marker for a Th1-type of response, but also a strong activator of monocyte functions (34). Therefore, the observed inhibition of IFN- γ release indicates not only a change in the T cell response but may also have an effect on the subsequent activation of monocytes.

With respect to this, one has to take into account our previous findings that apart from activity on T cells, PF-4 also affects the APCs. The fact that PF-4 provokes the down-modulation of HLA-DR on monocytes (15) may interfere with PF-4-induced effects on T cells in the Ag-specific stimulation model. Thus, the question whether PF-4-mediated suppression of T cell functions in this model is due to modulation of either monocytes or T cells or represents a combinatory effect involving both cell types must be finally left open. However, our data clearly show that the capacity of PF-4 to suppress T cell functions is not limited to isolated cells but takes also place within the physiological context of an antigenic activation.

Finally, it is possible that PF-4 directly induces an inhibitory signal without liberation of secondary mediators that would act in an auto or paracrine manner. Current investigations are on the way to clarify this by the analysis of early signal transduction events induced by the chemokine. However, irrespective of the mechanisms underlying PF-4-mediated effects, the direct modulation of T cell functions requires the presence of specific receptors expressed on the cell surface. Analysis of potential PF-4 receptors on T cells revealed a pattern showing remarkable similarities in the binding profile and the biochemical composition as compared with those previously found on human neutrophils (9). First, binding sites on both cell types preferentially interact with the tetrameric form of the chemokine. Although ^{125}I -labeled PF-4 alone did not bind at low concentrations (<50 nM), addition of increasing dosages of cold PF-4 (0.2–1 μM) led to a successive increase in cell-bound radioactivity, suggesting that mixed oligomers of iodinated and cold PF-4 had formed and interacted with the receptor. Displacement of the ligand at further increased dosages of cold PF-4 (5–25 μM) was most likely due to the formation of unlabeled oligomers that competed for binding. Second, PF-4 binding could be completely inhibited in the presence of heparin and was sensitive to digestion with chondroitinase ABC. Binding of PF-4 to T cells was specific and saturable and occurred within a range of concentrations where PF-4-mediated effects were observed, i.e., half maximal binding occurred at ~ 1 –2 μM PF-4 and half maximal inhibition of T cell proliferation was seen at ~ 0.7 μM PF-4. These data indicate that binding to the receptor on T cells involves interaction to a chondroitin sulfate proteoglycan; and therefore, shares characteristics with the PF-4 receptor we previously characterized on the surface of human neutrophils (9, 10). Interestingly, binding of PF-4 to membrane-associated proteoglycans of the heparan sulfate-type on bovine aortic endothelial cells (35) as well as to human umbilical cord vein-derived cells (36) has been reported earlier, and Luster et al. (11) found that PF-4 and IP10 share the same heparan sulfate proteoglycans for binding to human endothelial cell lines. However, because we found chondroitin sulfate glycosaminoglycan chains to be involved in PF-4 binding to T cells as well as to neutrophils, and preliminary data exist that this may also apply to binding to monocytes (our unpublished observations), this type of glycosaminoglycan rather than heparan sulfates appear to be responsible for PF-4 binding to cells of the hemopoietic lineage. However, our results do not exclude that PF-4 apart

from binding to chondroitin sulfate proteoglycans could interact with a second surface molecule on T cells. Although PF-4 binding was completely blocked in the presence of heparin, chondroitinase ABC treatment with optimal concentrations of the enzyme revealed that $\sim 5\%$ of the binding sites are not susceptible to the enzyme. However, the lacking capacity of PF-4 to induce characteristic chemokine functions in T cells such as chemotaxis or intracellular calcium transients argue against the participation of a typical seven-transmembrane domain receptor in this context. Our present investigations are directed to the identification of the receptors and to the mechanism that are involved in PF-4 receptor signaling.

In summary, our results provide unexpected new insight into the regulation of T cell functions by a rather atypical chemokine. Speculating on the biological effects of PF-4 in vivo at the site of inflammation one could envisage that PF-4 may play a role in maintaining peripheral tolerance and in suppressing responses of autoreactive T cells in a situation where high amounts of proinflammatory cytokines are present. Future investigations will focus on more detailed analysis of PF-4 effects on T cells and monocytes during coculture, a situation where one could expect synergistic effects between monokines and PF-4 on T cell activation and between T cell cytokines and PF-4 on monocyte functions, respectively. In conclusion, we were able to show previously unknown properties of PF-4 regarding T cell activation. These findings may give new insights into possible mechanisms of immunoregulation at the site of inflammation.

Acknowledgments

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