The role of proteinase 3 (PR3) and the protease-activated receptor-2 (PAR-2) pathway in dendritic cell (DC) maturation of human-DC-like monocytes and murine DC

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ABSTRACT

Objective. *The aim of the study was to assess PAR-2 expression on dendritic cell (DC) subsets and other immune cells of Wegener's granulomatosis (WG) patients and healthy controls (HC) and to investigate whether Proteinase 3 (PR3, a serine protease which can activate PAR2) induces maturation of human DC-like monocytes and murine Flt-3 ligand- and GM-CSF-generated DC.*

Methods. *Human peripheral blood cells including DC subsets and Flt-3l- and GM-CSF-generated mouse DC were analysed for expression of PAR-2 and DC maturation markers by flow cytometry before and after stimulation with PR3, trypsin, PAR-2 agonist or LPS for 24 h.*

Results. *There was no difference of PAR-2 expression on PMNs, monocytes, lymphocytes and DC between all WG samples and HC. However, in inactive WG, expression of PAR-2 was downregulated on the cell surface of PMNs, monocytes, lymphocytes, and CD11c+DC compared to active WG and HC. PR3 and PAR2-agonists did not induce upregulation of PAR-2 or maturation markers of human DC-like monocytes in WG and HC. Likewise, murine PR3 did not induce upregulation of PAR-2 or maturation markers in murine DC.*

Conclusion. *PAR-2 expression is downregulated on human peripheral blood cells including CD11c+ DC in inactive WG compared to active WG and HC, possibly reflecting a non-activated status of these cells in inactive disease. PR3 and PAR-2- agonists did not induce maturation of human ex vivo DC-like monocytes in WG and HC and of murine DC, suggesting this pathway is not singularly involved in the maturation of these cell subsets.*

Introduction

In previous studies, Wegener's autoantigen Proteinase 3 (PR3) has been shown to induce maturation of human monocyte-derived DC via the PAR-2 pathway and to license PR3-matured DC for Th1 priming (1-3). PAR-2 is also implicated in the activation of neutrophils (4, 5) and neutrophil-endothelial interactions (6) that may be relevant for the induction of small vessel vasculitis in WG. Furthermore, PAR-2 is important in T-cell and monocyte activation (7, 8), cell subsets that are important players in the pathogenesis of WG as well. The expression of PAR-2 on immune cells such as PMNs, monocytes, lymphocytes and DC in WG has not been assessed so far. Furthermore, DC maturation via PR3 and PAR-2 has only been tested *in vitro* using monocyte-derived DC (1), but not in circulating DC subsets of WG patients. In mice, soy bean trypsin inhibitor has been shown to prevent mouse dendritic cell maturation *in vitro*, suggesting that serine proteases may be important for mouse dendritic cell maturation (9). DC-like monocytes may also be relevant in the initiation of an adaptive immune response, as these monocyte-subsets have recently been shown to correlate with disease activity in inflammatory systemic diseases and seem to represent a tool for monitoring immunosuppressive therapy (10-12).

Furthermore, there is evidence of a crosstalk of the PAR-2 pathway with TLR4. In the presence of TLR4, PAR-2-AP, a synthetic PAR2-agonistic peptide, augments the PAR-2 mediated NF-κB-driven pathway (13). Furthermore, in PAR-2 -/- macrophages, the expression of LPS (TLR4 agonist)-induced genes is dysregulated and TLR4 -/- macrophages respond poorly to PAR-2-AP (13). Additionally, PAR-2-AP and LPS

both amplify IL-6 production (14). This crosstalk may be relevant for WG, as nasal carriage with S. aureus been shown to trigger relapses in WG (15), implicating bacterial infections could enhance the PAR-2 mediated adaptive immune response against PR3 in WG.
The aim of the study was to assess PAR-2 and TLR4 expression on dendritic cell (DC) subsets, DC-like monocytes and other immune cells of Wegener's granulomatosis patients and healthy controls (HC) and to investigate whether PR3 induces monocyte/DC maturation and if DC-maturation was induced if it occurred via the PAR-2 pathway, either alone or in synergy with other receptor systems. In parallel, the same analyses were performed on murine DC populations generated from mouse bone marrow cells in order to establish a genetically tractable model for putative co-stimulatory factors.

Methods

Patients

PAR-2 expression was assessed on peripheral blood cells from 31 WG patients (7 patients in remission/inactive stage as defined by the Birmingham Vasculitis Activity Score, version 3 (BVAS3) (16) corresponding to a BVAS 3 of 0, 24 active patients (BVAS 2003 >0, mean BVAS 2003=8.727 ±1.535, median BVAS 2003=6) and 28 age- and gender-matched HC. For *in vitro* stimulation experiments with PR3, PBMC from 7 WG patients and 7 HC were used.

In vitro *generation of murine DC*

Murine bone marrow cells were isolated from C57BL/6 mice obtained from Charles River (Sulzfeld, Germany) and DC-generation was performed according to the protocol by Brasel *et al*. (17). Briefly, bone marrow-derived cells were cultured in 6-well tissue culture plates either at a concentration of 4 x 10^5 cells/ml and in the presence of 20 ng/ml murine GM-CSF (R&D Systems, Wiesbaden, Germany) or at a concentration of 1 x 10^6 cells/ml in the presence of 200 ng/ml murine Flt-3 ligand (R&D Systems, Wiesbaden, Germany) for 8 days (18). At day 3 and 6, 2/3 of the supernant of the GM-CSF cultures was replaced by fresh medium containing 20 ng/ml GM-CSF. All Flt-3 ligand treated cells and the non-adherent fraction of GM-CSF treated DC were harvested and washed with PBS (PAA) before using for stimulation. In several experiments murine DC were also generated with a combination of GM-CSF and IL-4, but results are similar to that obtained with GM-CSF DC.

Reagents for flow cytometry and stimulation experiments

The following mouse monoclonal antibodies (mAbs) were used for flow cytometry analysis of human cells: FITC-conjugated mAbs against CD80 and isotype control IgG2a and IgG1, PE-conjugated mAbs against CD64, CD14, BDCA1-3, CD19, CD11b, CD86, HLA-DR and isotype control IgG2a and IgG1 and APC-conjugated mAbs against CD11c, CD16, CD3, CD56, CD83 and isotype control IgG1 (all BD Pharmigen Company). Anti-human PAR2 mouse (SAM11, sc13504) was obtained from Santa Cruz Biotechnology. For PBMC stimulation experiments, various stimulators were used: LPS (Sigma), human PR3 (Athens Research&Technology, USA), Trypsin (Calbiochem, Germany), PAR2 agonist peptide (PAR-2-AP, SLIGKV-NH2, H5042) and agonist control peptide (AP-control, VKGILS-NH2, H5882, Bachem, Germany). RPMI 1640 containing 5% human serum (HS, heat inactivated at 56 C for 30 min, PAA lab, GmbH, Germany), 2mM L-glutamine (PAA), 1% penicillin an 1% streptomycin (PAA) was used as culture medium. The following antibodies and reagents were used for flow cytometry analysis and culture of murine cells: Cells were cultured in RPMI 1640 (PAA, Cölbe, Germany) containing 10% heat-inactivated FCS (PAA) and antibiotics. Murine PR3 was kindly provided by U. Specks (Div. of Pulmonary and critical Care Medicine, Mayo Clinic Foundation, Rochester, USA) and D. Jenne (Dept. of Neuroimmunology, Max-Planck-Institute of Neurobiology, Martinsried, Germany). LPS from *S. friedenau* was kindly provided by Helmut Brade (Research Center Borstel, Germany). For flow cytometry the following monoclonal antibodies and secondary reagents were used (all from Becton Dickinson GmbH, Heidelberg, Germany): PerCP-conjugated streptavidin, and APC-conjugated hamster IgG1, PE-conjugated rat IgG2a, and PE-conjugated mouse IgG2a, FITC-conjugated rat IgG2a, -IgG2b, Biotin-conjugated rat IgG2b-, -IgG2a isotype control antibodies, and APC-conjugated hamster anti-mouse CD3e, -CD11c, FITC-conjugated rat anti-mouse CD11b, Biotin-conjugated rat anti-mouse CD45RA, -CD86, Gr-1, PE-conjugated rat anti-mouse CD45R/B220, Pe-conjugated mouse anti-mouse NK-1.1, -I-Ab specific antibodies and FITC-conjugated mouse anti-mouse, -rat, -human PAR2.

Flow cytometry analysis of human peripheral blood cells and murine DC

For analysis of human cells, 5 ml anti-coagulated venous blood was centrifuged (1200 rpm, 4 min.) and washed three times with PBS. Cells were then aliquoted at 100µl and incubated with mAbs against PAR-2, markers of DC (CD11c, BDCA1-3), of neutrophils/ myeloid cells (CD16, CD64, CD14, CD11b), of B-lymphocytes (CD19), of T-lymphocytes (CD3), of NK-cells (CD56) and isotype controls at 4°C for 30 min in the dark. Then lysing buffer (BD Biosciences) was added for 10 min at 4°C in the dark, cells were centrifuged at 1200 rpm for 10 min at 4°C and resuspended in PBS+ 0.5% BSA. For analysis of murine markers, cells were also washed with PBS, resuspended in PBS/0.5% BSA and then aliquoted in 50µl. Then they were incubated, as described for human blood above, with different cell-specific antibodies for T (CD3e)-, B (B220)-, NK (NK.1.1)-, granulocytes (Gr-1), myeloid (CD11b) and DC (CD11c, CD45RA) to define the purity and composition of the generate DC populations and with Ab for MHC-class-II (I-Ab), CD40 and CD86 to analyse cell maturation as well as with isotype control Ab and anti-PAR2. Labelling was usually performed as four-colour staining.
FACS Calibur (BD Biosciences, San Jose, CA) or LSRII were used for flow cytometry and CellQuest software (Becton-Dickinson) or WinMDI for data analysis.

Stimulation experiments

Isolated human PBMC 1x10^6/ml were stimulated with either human PR3 (10μg/ml), or trypsin (2μg/ml), PAR2-AP (0.1mM), AP-control (0.1 mM) or LPS (10 ng/ml) for 24 h (37°C, 5% CO_2) and then stained for DC maturation markers as outlined below. Murine Flt-3 ligand and GM-CSF generated DC were seeded in 96-well tissue culture plates at a concentration of 1 x 10^6/ml and incubated with either 10 μg/ml murine PR3 or 10 ng/ml of LPS *(S. friedenau)* and stained for cell type-specific or maturation markers.

Statistical analysis

Data are presented as mean values ± standard deviation (SD). Data analysis was done using two-tailed Mann Whitney and Wilcoxon *t*-test. A p-value <0.05 was considered statistically significant.

Results

Cell surface expression of PAR-2 on human blood cells

There was no significant difference of PAR-2 expression levels between all WG and HC on PMNs, lymphocytes and monocytes. However, in inactive WG the percentage of PAR-2 expression was significantly lower on PMNs, monocytes and lymphocytes compared to active WG (mean BVAS 2003 = 8.727±1.546) and HC (p<0.05) (Fig. 1A). PAR2- MFI ratio was significantly lower in PMNs of inactive WG compared to active WG and HC (p<0.05) (Fig. 1B).

PAR-2 expression on human blood DC subsets

PAR-2 expression was assessed in myeloid (BDCA1$^+$, (CD1c)$^+$, BDCA3high$^+$, all CD11c$^+$) and plasmacytoid (BDCA2$^+$, BDCA4$^+$) DC. Both in HC and WG, cells numbers of BDCA1+, BDCA2+ and BDCA4+ DC were too few for reliable PAR2-expression analysis. Among the whole CD11c$^+$ DC population, the percentage of PAR-2+ cells and antigen density (MFI) was also downregulated in inactive WG (mean percentage: 11.61±5.61, respectively) compared to active WG (16.26±7.82) and HC (13.16±4.46, p<0.05, Fig. 2 A and B). There was no significant difference in the MFI and percentage of PAR2 expression between BDCA3^{high} DC populations.

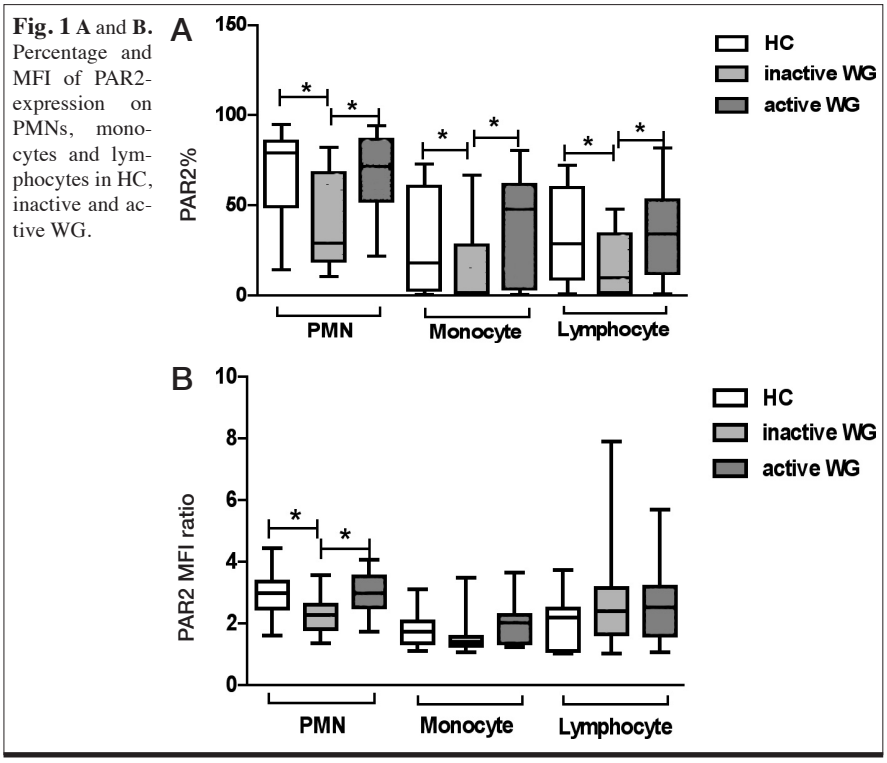

Fig. 1 A and **B.** Percentage and MFI of PAR2-expression on PMNs, monocytes and lymphocytes in HC, inactive and active WG.

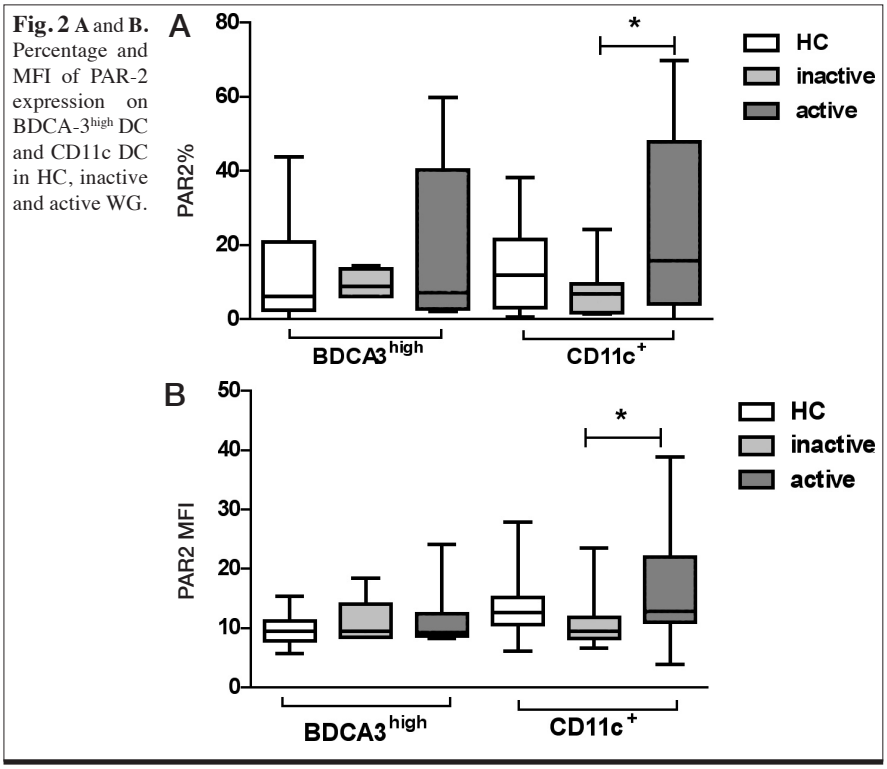

Fig. 2 A and **B.** Percentage and MFI of PAR-2 expression on BDCA-3^{high} DC and CD11c DC in HC, inactive and active WG.

PAR-2 expression on human peripheral blood DC-like monocytes

The percentage of PAR-2 expression on a subset of CD16$^+$ monocytes was not significantly different in HC, active and inactive WG, (data not shown). However, PAR2-expression (in percent) on CD64$^+$ monocytes was increased in active WG compared to inactive WG (p=0.028) and HC (p=0.010) (data not shown). The number of CD64$^+$CD16$^+$ monocytes, another subset described to

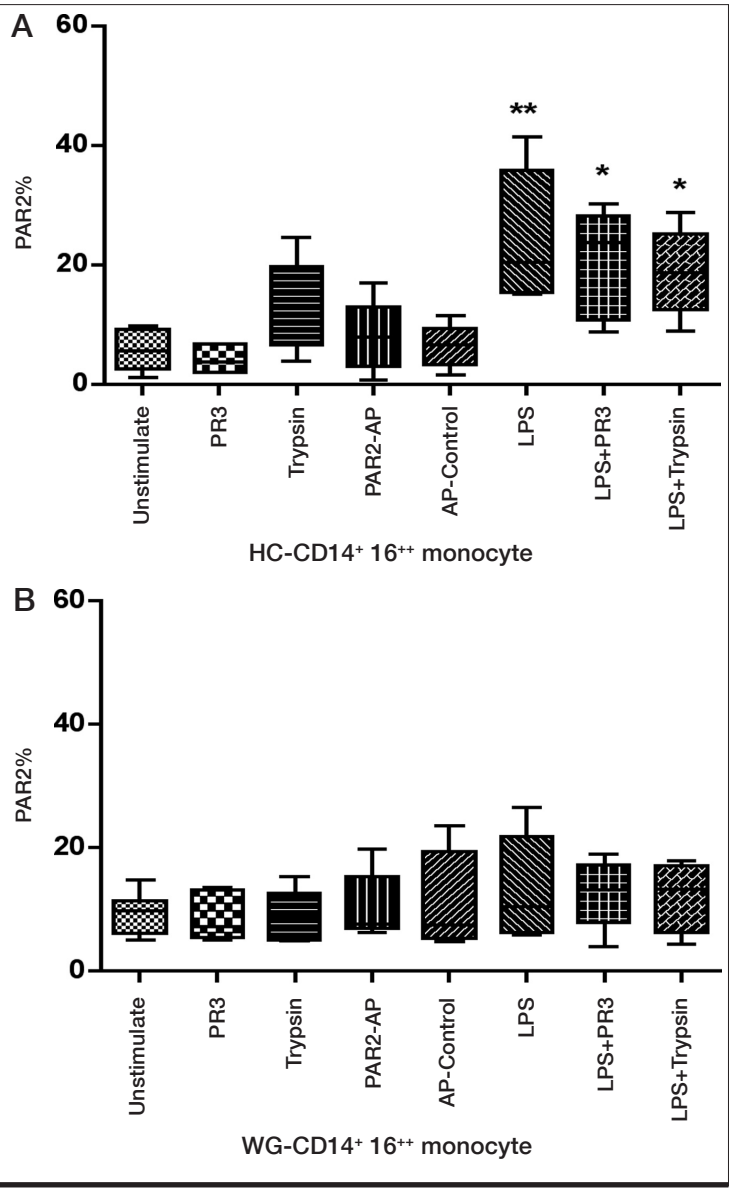

Fig. 3 A and **B.** Percentage of PAR2- expression on CD14+CD16+ monocytes of HC (3A) and WG (3B) without stimulation and after stimulation with PR3, Trypsin, PAR-2, PAR2-agonist peptide (PAR2-AP), PAR2-agonist control peptide (PAR-AP), LPS, LPS + PR3 and LPS + Trypsin for 24 h.

exhibit DC-like properties (11), was very low among MNC (mean 2.4% ± 2.59 in WG vs. 0.88% ± 0.61 in HC), but was significantly higher in WG compared to HC (p<0.05). Due to the low amount of cells, PAR-2 expression could not be assessed in the $CD64^+CD16^+$ monocyte subset. Therefore, PAR2- expression was analysed on another monocyte cell subset (CD14+CD16+ monocytes) that shares many features with the CD64+CD16+ monocyte cell subset (10-12). The CD14+CD16+ monocyte subset allowed analysis of PAR-2 expression and showed a low PAR-2 expression on unstimulated cells of active WG, inactive WG and HC; there was no significant difference between these groups (data not shown).

TLR4-expression on human peripheral blood cells

Percentage and MFI ratio of TLR4 expression on lymphocytes was higher in WG (percentage: 1.643±2.15, MFI ratio: 1.64±2.15) compared to HC (percentage: 0.61±0.42, MFI ratio: 0.61 ±0.42, p<0.05). There was no significant difference of TLR4 expression in PMNs, CD16+ monocytes, CD11c+ DC, $BDCA3^{high}$ DC between HC and WG (data not shown). Co-expression of PAR-2 and TLR4 on the cell surface of $BDCA3^{high}$ DC and CD11c+ DC was not different between HC and WG (data not shown).

PAR-2 expression on murine in vitro generated DC

The incubation of murine bone marrow cells with Flt3-ligend or GM-CSF resulted in the generation of two distinct DC populations which differ in typical DC- and maturation markers, as described elsewhere (16). Flt3-ligand generated DC consisted of a mixture of plasmacytoid (defined as B220+/CD45RA+/CD11c+/$CD11b^{low}$) and myeloid DC (B220-/CD45RA-/CD11c+/$CD11b^{high/low}$) with an immature phenotype characterised by low expression of MHC-class-II (data not shown) and CD86. In contrast, GM-CSF generated DC represented only myeloid DC ($CD11b^{high}$/CD11c+) highly expressing these maturation/activation markers (Fig. 4 B). On both murine DC populations PAR-2 expression was generally low and comparable. PAR-2 appeared downregulated in cultured compared to fresh DC (1.86±1.31 (n=7) *vs.* 16.25 ± 22.38 (n=4) for GM-CSF DC and 4.59±3.83 (n=10) *vs.* 14.25±12.38 (n=4) for Flt3L DC (Fig. 4 A).

Effect of PR3, PAR-2 agonists and LPS on PAR-2 expression and maturation of human DC-like monocytes ($CD14^+CD16^+$) and murine DC phenotype

As the number of $CD64^+CD16^+$ DC-like monocytes was too small to analyse in peripheral blood, $CD14^+CD16^+$ DC-like monocytes, a similar but somewhat larger subset was followed during stimulation experiments within whole blood. There was no significant difference in the expression of PAR-2 on the cell surface of CD14+CD16+ monocytes between WG and HC and between stimulated and unstimulated cells in WG and HC after stimulation with natural PAR2-activating serine proteases (PR3, trypsin) or synthetic PAR2-ligand PAR-2-AP and AP-control peptide. However, PAR-2 expression induced by LPS was significantly higher in LPS-stimulated HC compared to LPS-stimulated WG (mean 29.8%±2.55 *vs.* 9.55%±1.42, p<0.01) (Fig. 3).

When PAR2-expression of unstimulated and stimulated CD14+CD16+ DC-like monocytes was compared within the populations of HC and WG, LPS induced a significant up regulation of PAR-2 expression in stimulated cells of HC compared to unstimulated cells (p<0.01, Fig. 3A), but PR3, Trypsin and

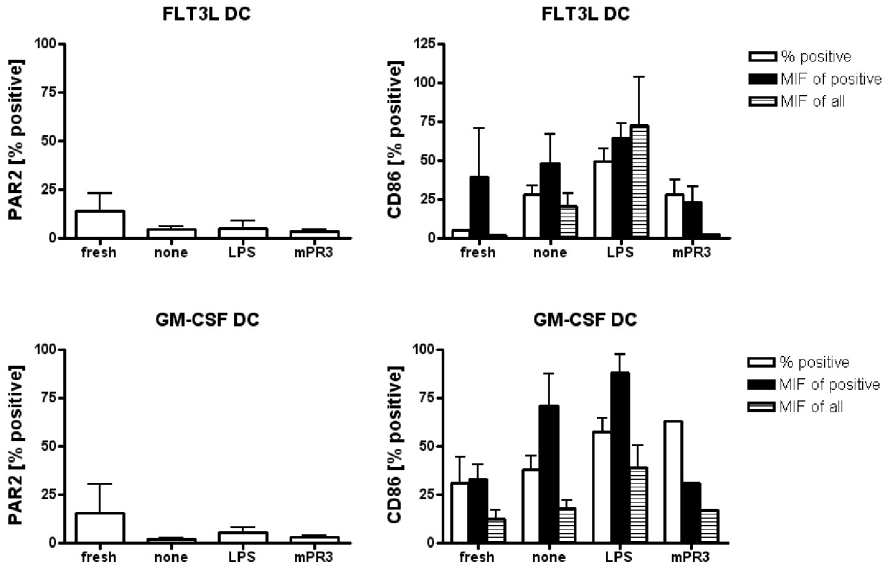

Fig 4. A and **B.** PAR-2 (A) and CD86 (B) expression on murine bone marrow derived DC. Cells were stained and flow cytometrically analysed immediately after generation and after overnight culture in the absence (none) or presence of LPS and murine PR3 (mPR3).

PAR2-AP did not. PAR-2 expression of WG CD14+CD16+ monocytes was not significantly different between unstimulated and stimulated cells of WG (Fig. 3B). However, there was a trend of a higher PAR-2 expression on LPS-stimulated cells compared to control.

An increase in expression of maturation markers CD83, CD80, CD86 and HLA-DR on CD14+CD16+ human DC-like monocytes of HC and WG was not induced by stimulation with PR3, trypsin and PAR-2-AP compared to unstimulated DC-like monocytes, but by LPS (data not shown).

Neither LPS nor murine PR3 induced upregulation of PAR2-expression (mean percent ± SD for GM-CSF DC: 5.33±4.89 (LPS), 3.17±1.83 (mPR3), both n=6 *vs*. 1.86±1.31 (none, n=7) and for FLT3L DC: 5.29±6.2 (LPS, n=7), 3.64±3.24 (mPR3, n=11) *vs*. 4.59±3.83 (none, n=10)) (Fig 4A), however, in GM-CSF stimulated DC, LPS induced a slight increase of PAR2-expression compared to unstimulated (cultured) cells, even if this was not significant ($p>0.05$). Stimulation with LPS induced upregulation of maturation markers CD86 (mean percent ±SD: 49.71±16.33 (n=7) *vs*. 28.11±14.79 (n=9) for Flt3L DC and 57.8±12.16 (n=5) *vs*. 38.33±12 (n=6) for GM-CSF DC (Fig. 4B), whereas murine PR3 did not (28.5±12, n=4 for Flt3L DC). Similar results were obtained with CD40 and MHC-class-II (not shown) and when DC where generated by GM-CSF together with IL-4 (data not shown).

Discussion

Recently, we showed that Wegener's autoantigen PR3 induces maturation of monocyte-derived DC and licenses them for Th1-priming of autologous CD4+ T-cells of Wegener's granulomatosis patients (1). The aim of this study was to assess PAR-2 expression on DC subsets and other immune cells in the blood of WG patients and to test whether PR3 induces maturation of DC-like monocytes in WG and of murine DC. Whereas there was no difference between all WG patients and healthy individuals in the expression of PAR-2 on PMNs, lymphocytes and monocytes, PAR-2 expression was found to be down regulated on PMNs, lymphocytes, monocytes and CD11c+ DC in inactive WG compared to active WG and HC. As PAR-2 plays an important role in the mediation of cell activation in PMNs (4-62-4), T-lymphocytes (75) and monocytes (86), the downregulation of PAR-2 expression may reflect a state of inactivity of these cells and may occur during efficient immunosuppressive therapy. PAR-2 expression on murine DC cell populations was low but well detectable on fresh DC and downregulated in cell culture. The reason for this remains unclear but indicates that PAR-2 expression may be influenced by multiple factors.

Neither PR3 nor other PAR-2 agonists (trypsin, PAR-2-AP) induced a significant upregulation in expression of PAR-2 and of maturation markers on human CD14+CD16+ DC-like monocytes in WG and HC compared to controls. Only LPS increased expression of PAR-2 and induced dendritic cell maturation markers in HC and murine DC, but hardly in WG. The significant increase in PAR-2 expression after LPS stimulation in HC compared to WG may be a consequence of immunosuppressive therapy in WG. The mechanism of LPS-induced PAR-2 upregulation remains speculative, but a cross-talk between LPS (TLR4 agonist) and PAR-2 seems likely (13). Furthermore, the finding that LPS is able to induce upregulation of PAR-2 in DC-like monocytes may be relevant with regard to the triggering of WG relapses by bacterial stimuli (15).

Serine proteases have been implicated in triggering murine DC development from bone marrow progenitor cells cultured with GM-CSF and IL-4 via PAR-2 (1). However, similar to the results obtained with human cells, we found no significant upregulation of expression of PAR-2 and of maturation markers in GM-CSF- or GM-CSF/IL-4- generated murine DC stimulated with murine PR3. Obviously, PR3 alone is not sufficient to trigger the development of bone marrow derived murine DC.

Interestingly, whereas LPS induced upregulation of PAR-2 expression on human CD14+CD16+ monocytes in HC, this was not observed in murine Flt-3l stimulated DC; in GM-CSF stimulated DC, there was a trend to upregulation of PAR-2 by LPS, but this was not significant.

In summary, there is evidence that PAR-2 expression on peripheral blood cells (PMNs, lymphocytes, monocytes and CD11c+ DC) is downregulated in inactive disease. However, contrary to previous results using *in vitro* generated monocyte-derived DC, no upregulation of PAR-2 and DC maturation could be induced by PR3 stimulation of circulating human DC-like monocytes

and murine GM-CSF and Flt-3l generated DC. For these cell populations, additional factors rather than PR3 alone may be required to induce DC maturation, such as bacterial stimuli (*e.g. S. aureus* (15)) or NETs (neutrophil extracellular traps) (19) containing PR3 and other proteases as well as DNA.

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