Transdifferentiation of polymorphonuclear neutrophils to dendritic-like cells at the site of inflammation in rheumatoid arthritis: evidence for activation by T cells

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Objective: To investigate infiltrated cells in the synovial fluid (SF) of inflamed joints of patients with rheumatoid arthritis (RA), with special reference to polymorphonuclear neutrophils (PMN) and their interaction with T cells.

Methods: Expression on PMN of activation associated receptors CD14, CD64, CD83, and major histocompatibility complex (MHC) class II was examined in the SF of 15 patients with RA, as were the infiltrated T cells. SF cytokines were determined by enzyme linked immunosorbent assay (ELISA). To mimic the in vivo situation, coculture experiments were carried out using PMN and T cells of healthy donors.

Results: The SF contained activated T lymphocytes and abundant PMN. SF PMN expression of CD14 and CD64 was enhanced compared with peripheral blood. Of special interest was the observation that only the SF PMN expressed MHC class II antigens and CD83. Exposure to SF, which contained considerable amounts of cytokines (for example, interferon-γ (IFN-γ)), tumour necrosis factor-α, and interleukin-2, induced a similar receptor pattern on blood derived PMN of healthy donors. Furthermore, PMN acquired MHC class II and CD83 within 24 to 48 hours, when co-cultured with autologous T cells or T cell lines. This effect was also achieved by T cell supernatants, dependent on protein synthesis, and could be inhibited by antibodies against IFN-γ.

Conclusions: SF PMN from patients with RA undergo major alterations, including transdifferentiation to cells with dendritic-like characteristics, probably induced by T cell derived cytokines. Because MHC class II positive PMN are known to activate T cells, the mutual activation of PMN and T cells might contribute to the perpetuation of the local inflammatory process, and eventually to the destructive process in RA.

The actiology and pathogenesis of rheumatoid arthritis (RA) are still elusive as are the mechanisms leading to joint destruction. Autoimmune phenomena have been suggested as the initiating event—for example, the activation of T and B lymphocytes and monocytes.1-5 Increasing interest has focused on the role of polymorphonuclear neutrophils (PMN), mainly because prominent PMN infiltrates are found in inflamed joints.6-7 Furthermore, in numerous studies evidence for activation of PMN in synovial fluid (SF) has been reported, particularly the modulation of activation associated surface receptors, including for example, CD66a-c, CD35, CD46, or CD11a/b.8-10 Because PMN have a reservoir of receptors and cytokines, and a propensity to generate and release cytotoxic and proteolytic entities, they may contribute to cartilage destruction. Numerous reports on the role of PMN in the development of tissue damage have been published, including, for example, lesions occurring in ischaemia reperfusion and lung injury,11,12 in the development of chronic wounds,13 or in chronic inflammatory diseases, including RA.14-16

Although the presence of activated PMN in the SF is well documented, it is unknown how the PMN are activated and how they persist at the site of inflammation. As protagonists of acute inflammation, PMN are short lived cells, rapidly undergoing apoptosis after having fulfilled their tasks—for example, phagocytosis of bacteria. Apoptosis is considered to be a prerequisite for the clearance of an inflamed site, and for limitation of the inflammatory response. A failure to undergo apoptosis is thought to result in an unrestrained release of cytotoxic entities by PMN, and thus in damage of the surrounding tissue.17

In this study we analysed the leucocytes in inflamed joints of patients with RA in order to examine whether PMN indeed have a prolonged lifespan, evident as transdifferentiation, and whether T lymphocytes might be involved in this process.

PATIENTS AND METHODS

Patient characteristics

The study was approved by the ethics committee of the University of Duesseldorf. Fifteen patients with RA (diagnosed according to the American College of Rheumatology criteria for the classification of RA18) gave their informed consent and were recruited to the study. Six patients were “naïve” with onset of symptoms <3 months previously and no treatment with disease modifying antirheumatic drugs (early RA). Nine patients had a longstanding disease with a mean (SD) duration of 10.4 (6.6) years. SF bacteria were not identified in any of the patients.

Abbreviations: ELISA, enzyme linked immunosorbent assay; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte macrophage-colony stimulating factor; IFN-γ, interferon-γ; MHC, major histocompatibility complex; NHD, normal healthy donors; PBS, phosphate buffered saline; PE, phycoerythrin; PMN, polymorphonuclear neutrophils; RA, rheumatoid arthritis; SF, synovial fluid; SN, supernatant(s); TNF-α, tumour necrosis factor-α.
Activation of PMN by T cells in RA

Cytofluorometry
Cells from SF or whole blood were double labelled using a fluorescein isothiocyanate (FITC) labelled antibody to CD66b (Immunotech, Marseilles, France, clone 80H3) as PMN marker and phycocerythrin (PE) labelled antibodies to CD14 (clone UCHM1), CD64 (10.1), major histocompatibility complex (MHC) class II (HLA DR-DP-DQ, clone WR18, all Serotec, Oxford, GB), or CD83 (Immunotech, HBI5A). For lymphocytes, antibodies to CD3 (SK7), CD4 (SK3), CD8 (SK1), CD69 (L78; all Becton Dickinson), CD19 (Immunotech, J4.119), and CD25 (Caltag, Burlingame, USA, CD25-3G10) were used. The final concentration of antibodies varied between 1 and 10 μg/test. Isotypic controls IgG1 FITC, IgG2a, and IgG2b PE were used in the same concentration. To reduce non-specific binding Fc receptors were saturated with 10 μg/ml human IgG (Venimmun, Cventon, Marburg, Germany) and with 1% bovine serum albumin before and during incubation. After incubation of SF cells (1×10⁶) or of whole blood (100 μl) with the respective antibodies for 15 minutes at 25˚C in the dark, cells were washed twice in FACS buffer, fixed in PBS/1% paraformaldehyde and stored at 4˚C until analysed by FACSort and CellQuest software (Becton Dickinson). Whole blood was lysed by FACS lysing solution (BD; 10 minutes in 2 ml; 1:10 in aqua dest.). Results are expressed as the percentage of positive cells in the respective gate or quadrant, or as the mean fluorescence intensity, respectively.

For comparison, receptor expression on peripheral PMN of 20 normal healthy donors (NHD) was measured. Expression of the newly acquired receptors (for example, CD83 and MHC class II) was considered to be positive when the mean plus 2 SD of all NHD was exceeded.

Isolation of PMN and in vitro activation
PMN were separated from 50 ml whole blood by centrifugation on PolymorphPrep (Nycomed, Oslo, Norway), which yielded a highly homogeneous preparation with a purity >98% (judged by cytofluorometry with an antibody to CD66b). PMN where cultivated in AIM (Gibco, Eggenstein, Germany) with 2.5% autologous heat inactivated serum. PMN were either stimulated with the indicated concentration of supernatants of phytohaemagglutinin activated T cells or 100 U/ml IFNγ for 24–48 hours. For co-culture 4×10⁶ PMN and 4×10⁶ T cells were put together for up to 48 hours in 4 ml AIM. In whole blood, PMN (100 μl) were stimulated with 100 μl SF of patients with RA for 30 minutes or 24 hours and used as described above. The T cell clone A37/2 was obtained from Dr Kabelitz/Heidelberg. To prolong the life of the cells they were restimulated with irradiated monocytes and B cells (as feeder cells) every 14–18 days with 1 ng/ml phytohaemagglutinin. Supernatants of T cells were obtained after 48 hours by centrifugation of cells and stored at −20˚C until used.

Multiplex enzyme linked immunosorbent assay (ELISA)
Cytokine levels in SF were determined using the Luminex multianalyte technology (BioPlex system by BioRad, Hercules, CA, USA) in combination with multiplex cytokine kits. In total, eight different cytokines (cytokines 8-plex-kit BioRad) were measured in 50 μl SF. The detection sensitivity of all cytokines was <10 pg/ml. Data from previous studies (unpublished observation; not shown) suggested that (PBS)/1% bovine serum albumin/0.1% sodium azide (FACS buffer) and subjected to cytofluorometry. Viability was assessed by trypan blue exclusion and microscopic evaluation. Peripheral blood was taken at the same time from all patients.

Recovery of SF cells
By puncture, between 5 and 50 ml of SF was collected in tubes containing sodium-heparin (Becton Dickinson, Heidelberg) and processed within 2 hours. After centrifugation, cells were washed once with phosphate buffered saline (PBS)/1% bovine serum albumin/0.1% sodium azide (FACS buffer) and subjected to cytofluorometry. Viability was assessed by trypan blue exclusion and microscopic evaluation. Peripheral blood was taken at the same time from all patients.

Figure 1  Expression of surface receptors on leucocytes from synovial fluid (left panels) or peripheral blood of the same patient (right panels). (A) By forward-side scatter three major cell populations could be identified in the synovial fluid: R2 are PMN; R3, monocytes; R4, lymphocytes. Distribution of cells in the blood was similar. (B) Left panels: By double labelling PMN were identified by anti-CD66b FITC as a marker for PMN (in R7; right quadrant), and either IgG PE (as negative control), or PE conjugated antibodies to CD14, CD64, CD83, and MHC class II. Monocytes express similar antigens as PMN (except CD66b); they appear in R6 (middle quadrant) as a distinct cell population, constitutively expressing MHC class II and CD14. The lymphocytes (in R5; left quadrant) are negative for CD14, CD64, CD66b, CD83, but express MHC class II antigens. In whole blood (right panels), PMN are identified by anti-CD66b and are located in the lower and upper right quadrants. Monocytes and lymphocytes are co-located in the lower and upper left quadrants, both negative for CD66b.
detected fluorescence intensity reaches a fixed value. The polymerase chain reaction cycle number at which the known input concentration of a plasmid against the standard curve, obtained by plotting a horizontal bar the median. Differences between the groups were summarised as the percentage of positive PMN or as mean fluorescence intensity as detected by flow cytometry with box plots. The box contains 50% of the values, the square indicates the mean values, and the horizontal bar is the median. Differences between the groups were calculated using analysis of variance; the mean values were considered different when p was < 0.05 (ND indicates no difference between the groups). There was no difference in the receptor expression pattern on SF PMN between patients with early and longstanding RA.

Transdifferentiation of locally activated PMN to dendritic-like cells

To analyse the SF PMN further, expression of activation associated surface receptors was studied and compared with that in peripheral blood PMN and in PMN of 20 NHD. CD14 was drastically up regulated on the SF infiltrated PMN, but not on peripheral blood PMN of the same patients or of NHD. Similarly, CD64 was up regulated only on the PMN in the SF. To study the transdifferentiation of SF PMN, we looked for the surface expression of MHC class II antigens and of CD83. Neither receptor is constitutively expressed by PMN and the expression after stimulation depends on de novo protein synthesis. On SF PMN, but not on blood PMN, CD83 expression was found in 11/15 patients (mean (SD) 37.9 (37.8)%). Moreover, MHC class II expression was seen on the SF derived PMN in 11/15 patients (mean (SD) 50.1 (44.6)%), whereas the peripheral blood PMN were negative for MHC class II. Of note, MHC class II and CD83 were not necessarily coexpressed: only the PMN of nine patients were double positive for MHC class II and CD83. The amount of both CD83 and MHC class II varied between the patients. Figure 1 shows an example and fig 2 summarises the data for all the patients.

There was, however, no correlation with the number of infiltrated total leucocytes, T cells, PMN, or monocytes, or with the systemic C reactive protein levels of the MHC class II and CD83 expression. Moreover, there was no difference between the patients with early or longstanding RA.

As not all patients were positive for either CD83 or MHC class II, we studied the kinetics of receptor expression of PMN derived from SF ex vivo. Cells with no CD83, but expressing MHC class II were cultivated further for 16 hours; then the viability, CD83 and MHC class II expression were measured. The PMN were still viable, the PMN acquired CD83, and MHC class II antigens increased further on a subpopulation of PMN. Of note is, that the SF contained also a subpopulation of PMN. Of note is, that the SF contained also a subpopulation of PMN. Of note is, that the SF contained also a subpopulation of PMN. Of note is, that the SF contained also a subpopulation of PMN. Of note is, that the SF contained also a subpopulation of PMN.
summarises the data for all patients. There was no correlation between the amount of any cytokine in the SF and the expression of MHC class II antigens or CD83 on PMN. In line with the presence of cytokines in the SF, the short term culture (30 minutes) of PMN derived from NHD with patients’ SF resulted in activation of PMN, seen as an up regulation of CD14 and CD64. Culture for 48 hours induced CD83 expression and, to a lesser extent, MHC class II. Figure 5 shows an example.

**Figure 3** Induction of CD83 and MHC class II expression on SF derived PMN derived cultured ex vivo. (A) Expression of CD83 and MHC class II was measured either directly after recovery of the PMN from the SF (line), or after culture for 16 hours at 37°C (filled peaks). (B) In parallel, activation associated antigens (CD69 and CD25) were determined on T cells after 16 hours (one of three experiments with cells of three patients is shown).

**Figure 4** Cytokines in the SF of all 15 patients: the cytokines were quantified by ELISA and expressed as pg/ml. Data of all patients are summarised as box and whiskers plots. The box contains 50% of the values, the square indicates the mean values, the horizontal bar the median (note, there is a logarithmic y axis). The dots at the bottom symbolise patients who were negative for the respective cytokines. Data from previous studies (unpublished observation; not shown) suggested that pretreatment of the SF with hyaluronidase and filtration of the samples to remove rheumatoid factors is not necessary.

**Activation of PMN by T cells in vitro: expression of MHC class II and CD83 on PMN**

Because the ex vivo analyses described above suggested a local activation of PMN by the infiltrated T cells or their cytokines, respectively, a series of in vitro experiments were carried out to test the interaction of highly purified T cells with PMN of healthy donors. Firstly, the expression of MHC class II on PMN was measured after incubation with supernatants (SN) of stimulated T cells. SN of the T cell clone A37/2 induced MHC class II on PMN in a concentration dependent manner (fig 6). By an ELISA, IFNγ was detected in the SN; correspondingly, the expression of MHC class II could be inhibited by a neutralising antibody to IFNγ (fig 6).

Also, after co-culture with T cells (clone A37/2), PMN expressed MHC class II after 24 hours. Again, the percentage of MHC class II positive PMN increased with the number of T cells. The expression of MHC class II on PMN was significantly decreased when either PMN or T cells were pretreated for 2 hours with a protein synthesis inhibitor (cycloheximide 0.1 μg/ml; the dose did not affect the viability of the PMN or T cells; fig 7), indicative of protein de novo synthesis of MHC class II by PMN, and of a mediator produced by T cells.

By real time polymerase chain reaction, IFNγ mRNA was detected in T cells that had been co-cultivated with autologous PMN for 2 hours; expression was further increased after 6 and 24 hours (table 1).

**DISCUSSION**

Leucocyte infiltration into infected tissues is essential for an efficient host defence; it is, however, also seen at sites of sterile or chronic inflammation—for example, in inflamed joints of patients with RA. In our study the majority of infiltrated leucocytes in the SF of patients with RA were PMN. Although activation of PMN at the inflamed site has been well described by others, the unexpected finding...
was that a portion of the PMN had acquired CD14, CD83, and MHC class II antigens. Neither CD83 nor MHC class II are stored in resting PMN and normally are only expressed by professional antigen presenting cells—for example, monocytes or dendritic cells. Their expression, however, can be induced by stimulating PMN—for example with IFN-γ, TNF-α, or GM-CSF, and requires de novo protein synthesis. This implies, that the lifespan of the PMN can be extended by appropriate conditions, which in turn leads to a further differentiation, especially obvious when the PMN were further cultured ex vivo. Although this presumption is in contrast with the traditional image of PMN as terminally differentiated cells, there is growing evidence that not only in vitro but also under certain pathological conditions—for example, chronic inflammatory diseases and severe trauma, PMN can escape from apoptosis and differentiate further, thereby acquiring new surface receptors such as MHC class II, CD86, CD80, or CD83.

Figure 5  Activation of PMN in whole blood of NHD by SF of patients with RA. The left panel shows that culture for 48 hours activated PMN as seen by the expression of CD83 (line: cells cultured with PBS alone; filled peak: cultured with 50% SF; PMN were identified by anti-CD66b and gated accordingly). The right panel summarises the results of three independent experiments carried out with the blood of three healthy donors (NHD 1–3) and the SF of two patients with RA (SF-P1 and SF-P2); the CD83 expression after 48 hours stimulation with SF compared with PBS alone is shown.

Figure 6  (A) Induction of MHC class II (HLA DP-DQ-DR) expression on PMN of a healthy donor after stimulation with 20% vol/vol supernatant (SN) of the T cell clone A37/2 for 48 hours (line: culture medium alone, filled peak: 20% SN added). (B) The acquisition of MHC class II was dependent on the SN concentration (10–50% vol/vol) (note: the experiment was performed with cells of a different donor). (C) The effect of the SN could be inhibited by antibodies to IFN-γ and to a lesser extent by anti-GM-CSF (concentration 2 μg/ml each; conditions: 30% SN of the clone A37/2 for 24 hours; mouse IgG as isotype control did not inhibit the induction of MHC class II).

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* SN of clone A37/2 (48 h stimulated with 1 ng/ml PHA; IFN-γ concentration > 1000 pg/ml by ELISA)
MHC class II antigen expression on PMN in SF and on PMN in the peripheral blood of patients with RA has also been described by Cross et al. In contrast with our data, their results showed that PMN were negative for MHC class II after isolation, but expression could be induced when PMN were further cultivated. This discrepancy cannot be fully explained. An explanation may be that in our study a more sensitive detection system (for example, different antibody, different fixation) was used, which was supported by the observation that under our culture conditions the surface expression of MHC class II and CD83 was also increased further. Moreover, MHC class II expression was not seen in all of our patients; one possible explanation might be the difference in culture conditions. In this situation, we presume that activated T cells, having infiltrated the inflamed joint, provide the required cytokines.

The presence of CD14, MHC class II, and CD83 on PMN in SF indicates not only that PMN have an extended lifespan in RA but also argues for a transdifferentiation of PMN to cells with characteristics of dendritic cells. As shown before by us and by others and reviewed by Ashitekar and Saha, MHC class II positive PMN can present superantigens or antigens to T cells in an MHC class II dependent or restricted manner, respectively. Although this function has only been demonstrated in vitro, it is quite feasible that similar reactions also occur in vivo, because antigen presentation to T cells is the only known biological role for MHC class II antigens. The function of CD83, known as a specific marker for dendritic cells, is still not well understood, but its association with antigen presenting cells is highly suggestive of its role in the specific immune response, especially of CD4+ T cells (reviewed by Lechmann et al). Regardless of the biological consequences, our data point to a "cross talk" between PMN and T cells at the site of inflammation, and further support the concept of a link between the innate (PMN) and the specific (T cells) immune response. Nevertheless, a statement about the defined localisation of this "cross talk" between PMN and T cells alone is misleading, because of the unrestrained release by PMN of their cytotoxic and proteolytic entities.

Another important implication of our data is that the locally activated PMN obviously have escaped from their constitutive apoptosis, because they could still synthesise protein de novo. On the one hand, apoptosis of PMN, and the subsequent phagocytosis by macrophages is considered to be a prerequisite for the resolution of an inflammatory process; on the other hand, escape from apoptosis is thought to contribute to tissue damage, because of the unrestrained release by PMN of their cytotoxic and proteolytic entities. These arguments suggest that the prolonged lifespan of activated PMN in patients with RA may contribute to the progression of the inflammatory process to chronicity; moreover, the PMN may inflict local tissue damage, and participate in the destruction of cartilage and bone.
REFERENCES


