The CD14⁺CD16⁺ monocytes in erysipelas are expanded and show reduced cytokine production

Alexia Horelt[1], Kai-Uwe Belge[1], Birgit Steppich[1], Jörg Prinz[2] and Löms Ziegler-Heitbrock[1,3,4]

[1] Institute for Immunology, University of Munich, Munich, Germany
[2] Department of Dermatology, University of Munich, Munich, Germany
[3] GSF-National Research Center, Institute for Inhalation Biology, Gauting, Germany
[4] Department of Microbiology and Immunology, University of Leicester, Leicester, GB

In human peripheral blood the classical CD14⁺⁺DR⁺ monocytes and the pro-inflammatory CD14⁺CD16⁺DR⁺⁺ monocytes can be distinguished. In erysipelas we found strongly increased numbers of CD14⁺CD16⁺ monocytes on the day of diagnosis (day 1) in 11 patients with an average of 150.5±76.0 cells/μl, while 1 patient had low levels (35 cells/μl, control donors 48.8±19.8 cells/μl). The classical monocytes were only moderately elevated in the erysipelas patients (factor 1.7 as compared to controls). Patients exhibited increased body temperature, erythrocyte sedimentation rate and increased serum levels for C-reactive protein (CRP), IL-6 and macrophage-colony-stimulating factor. Among these, body temperature and CRP showed a significant correlation to the numbers of CD14⁺CD16⁺ monocytes. In 4 of 4 patients with high levels of CD14⁺CD16⁺ monocytes, these levels returned to that seen in controls by day 5 of antibiotic therapy. Determination of intracellular TNF was performed by three-color immunofluorescence and flow cytometry after *ex vivo* stimulation with lipoteichoic acid, a typical constituent of streptococci. Here, patient CD14⁺DR⁺⁺ pro-inflammatory monocytes showed a twofold lower level of intracellular TNF. By contrast, expression of TNF was unaltered in the classical CD14⁺⁺ monocytes. These data show that in erysipelas the pro-inflammatory CD14⁺CD16⁺DR⁺⁺ monocytes are substantially expanded and selectively tolerant to stimulation by streptococcal products.

Received	20/3/01
Revised	6/12/01
Accepted	1/2/02

Key words: Monocyte / Subpopulation / TNF / Inflammation / Infection

1 Introduction

Erysipelas is an infectious disease of the skin that typically is caused by β-hemolytic streptococci group A. The painful lesions are characterized by areas of intense inflammation with sharp edges and they are usually located in the face or the lower limb. After proper diagnosis antibiotic treatment will control infection but the disease tends to recur in spite of an immune response as evidenced by increasing antibody titers. The mechanisms leading to inflammation in the skin are understood only in part. Here the streptococcal pyrogenic exotoxin (SPE) superantigens or lipids like lipoteichoic acid (LTA) may be crucial for activation of T cells and monocytes, respectively. The cytokines released by these cells may induce local inflammation but they may also be responsible for the systemic response that includes increased C-reactive protein (CRP) and fever. The release of cytokines can lead to activation of the different types of leukocytes that can then be recruited to the site on infection.

Among the monocytes in human blood we recently described the novel population of CD14⁺CD16⁺DR⁺⁺ monocytes [1, 2]. Based on their high expression of the pro-inflammatory cytokine TNF and their low expression of the anti-inflammatory cytokine IL-10 these cells were termed pro-inflammatory monocytes. These cells have been shown to be expanded in systemic inflammatory diseases including sepsis and acute HIV infection [3–6]. Based on their pro-inflammatory properties and their high capacity to present antigen to T cells [7] the CD14⁺CD16⁺ monocytes may be instrumental in fighting infection.

In the present report we have asked whether in a localized infection like erysipelas the pro-inflammatory mono-

[I 21870]

Abbreviations: BFA: Brefeldin A **CRP:** C-reactive protein **ESR:** Erythrocyte sedimentation rate **LPS:** Lipopolysaccharide **LTA:** Lipoteichoic acid **PMB:** Polymyxin B **SPE:** Streptococcal pyrogenic exotoxin **WPB:** Whole peripheral blood

 0014-2980/02/0505-1319$17.50+.50/0

cytes may also be expanded. The strong increase of the pro-inflammatory $CD14^+CD16^+$ monocytes that we report herein for most erysipelas patients may be part of an effective immune response against the streptococcal skin infection.

Still, the pro-inflammatory monocytes show a decreased TNF production in erysipelas patients whereas in the classical monocytes the expression of this cytokine is unaltered.

This is the first report to show a selective state of tolerance in the pro-inflammatory monocytes in patients with infectious disease, indicating that the activity of these cells is stringently controlled.

2 Results

2.1 Monocyte populations in erysipelas at the day of admission

We have studied 12 patients with erysipelas with the diagnosis based on typical skin inflammation, increased erythrocyte sedimentation rate (ESR) and antibody titers against *S. pyogenes* antigens. Nine patients had skin infection of the lower limb and 3 infection of the facial skin (Table 1). Eight patients had primary disease and 4 patients had recurrent erysipelas. In these patients we studied monocyte subpopulations by two-color flow cytometry at the day of admission (day 1). As shown in Fig. 1 the patient with erysipelas (right panel) had strongly increased numbers of $CD14^+CD16^+$ monocytes (182 cells/μl) as compared to a control donor (34 cells/μl, left panel). Numbers of $CD14^+CD16^+$ monocytes were increased in 11 of 12 patients. Only 1 patient (p3) showed low $CD14^+CD16^+$ monocyte numbers of 35/μl which is within 1 standard deviation of the control values (29–69 cells/μl, Table 1). In average of all patients the $CD14^+CD16^+$ monocytes were increased to 140.8±74.1 cells/μl (Fig. 2) which was significantly higher than the values in control donors (48.8±19.8 cells/μl, $p < 0.05$). This threefold expansion of the $CD14^+CD16^+$ monocytes was much stronger than what was seen for the classical $CD14^{++}$ monocytes which increased from 285±96 to 480±197 cells/μl (factor 1.7, $p < 0.05$). Therefore, when looking at the percentage among all monocytes ($CD14^+CD16^+$ plus $CD14^{++}$) the $CD14^+CD16^+$ cells were increased from 13.6±3.5% in controls to 21.5±8.0% in erysipelas patients ($p < 0.05$).

All patients tested showed an increased ESR and CRP and some showed increased body temperature (Table 1). Among these parameters CRP and body temperature significantly correlated with the number of $CD14^+CD16^+$ monocytes ($p < 0.05$), while ESR did not.

2.2 Monocyte populations in erysipelas over time

We determined the numbers of $CD14^+CD16^+$ monocytes for up to 5 days of antibiotic therapy in five patients with three examples shown in Fig. 3. While in one example the patients $CD14^+CD16^+$ monocytes decreased immediately, another one showed some increase on day 2, but in both examples numbers of $CD14^+CD16^+$ monocytes reached the control range by day 5. The same pattern was seen for two additional patients (not shown). The

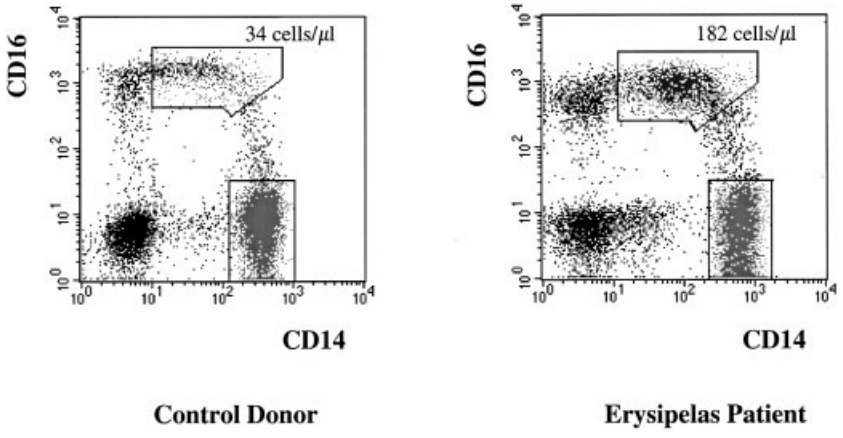

Fig. 1. Example of increased $CD14^+CD16^+$ monocytes in erysipelas. After lysis of erythrocytes whole-blood samples were stained with CD14 and CD16 antibodies and monocyte subsets were analyzed by FACS. Shown is an example from a control donor and from an erysipelas patient p1 on day 1. The absolute number of $CD14^+CD16^+$ monocytes is given in the upper right corner of each histogram.

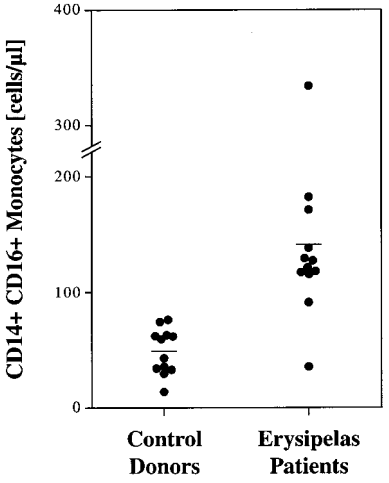

Fig. 2. Absolute numbers of CD14⁺CD16⁺ monocytes in control donors and in erysipelas patients on day 1. CD14⁺CD16⁺ monocytes were determined by FACS analysis in 12 control donors and patients each. —: mean value. The difference is significant at $p < 0.05$.

patient with low numbers of CD14⁺CD16⁺ monocytes to begin with remained low for the entire time of observation (Fig. 3).

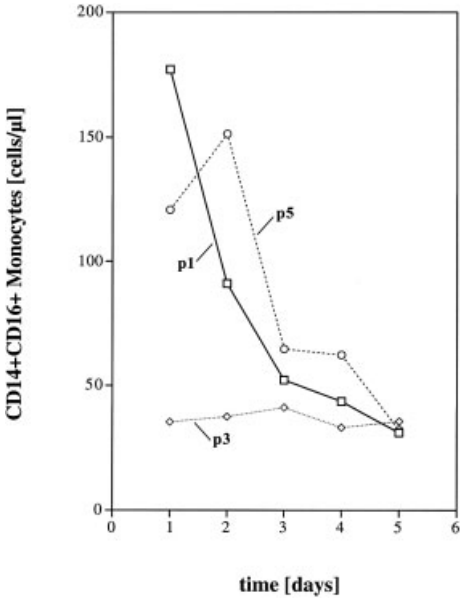

Fig. 3. CD14⁺CD16⁺ monocytes in erysipelas patients. Given are CD14⁺CD16⁺ monocyte numbers in three patients that were studied daily for 5 days. Of the two additional patients not shown, one had high numbers of CD14⁺CD16⁺ monocytes on day 2 while the other showed a continuous decrease in time and in both patients the numbers of CD14⁺CD16⁺ monocytes returned into the control range by day 5.

2.3 Serum cytokines in erysipelas

To analyze which mediators might be involved in the pronounced expansion of the CD14⁺CD16⁺ monocytes in erysipelas we studied serum samples from all 12 patients taken on day 1 for IFN-γ, IL-10, IL-6 and M-CSF. In these studies no serum IFN-γ and IL-10 was detected (detection limit 28 pg/ml and 18 pg/ml, respectively). By contrast, serum M-CSF was 652±319 pg/ml, which was significantly higher as compared to control donors (355±135 pg/ml, $p < 0.05$). The levels of IL-6 were found increased (66±135 pg/ml) as compared to control (5±3 pg/ml) as well, but the difference was not significant ($p = 0.08$).

When serum cytokines were plotted against CD14⁺ CD16⁺ numbers then we found no significant correlation for both M-CSF and IL-6 ($p > 0.05$).

2.4 Cytokine production by monocyte populations

We next asked whether the expanded CD14⁺CD16⁺ monocytes would respond to *ex vivo* stimulation. For this, whole-blood samples were stimulated with LTA at 100 µg/ml for 4 h in the presence of BFA and cells were then stained for cell surface markers and for intracellular TNF. In these experiments cells were stained with CD14 and HLA-DR antibodies since the CD16 antigens disappear with *in vitro* stimulation and therefore cannot be used for identification of the monocyte subpopulations [8].

When gating on the CD14⁺DR⁺⁺ monocytes 95.4 % were found positive for CD16 as shown for patient p1 in Fig. 4. In average of 6 patients 90.2±4.1% of the CD14⁺DR⁺ cells were CD16 positive. For comparison among the CD14⁺⁺DR⁺ monocytes in the example in Fig. 4 only 3.2% were positive for CD16 (average of 6 patients: 8.6±2.5%). Thus, the majority of CD14⁺DR⁺⁺ monocytes are identical to the CD14⁺CD16⁺ monocytes.

Using CD14-DR-staining for identification of the pro-inflammatory monocytes we found that in control donors LTA induced a strong TNF production in the CD14⁺DR⁺⁺ monocytes (401 channels) but in the erysipelas patient TNF production in these cells was substantially reduced (170 channels), reflecting a 2.4-fold decrease (Fig. 5). Treatment with polymyxin B (PMB) for neutralization of LPS had no effect on LTA-induced TNF expression, demonstrating that the stimulation was not due to contaminant lipopolysaccharides (LPS) (data not shown). In aver-

Table 1. Characteristics of erysipelas patients and control donors

Patient	Age (years)	Gender	ESR 1 h (mm)	Temp °C	CRP (mg/100 ml)	Serum antibodies to ASL/ASDNaseB	Affected site	$CD14^+$ $CD16^+$ (cells/ μl)	Concurrent diseases
p1	65	m	72	< 37.0	14.5	Decrease[a] (ASDNaseB)	Leg	182	None
p2	60	m	40	< 37.0	2.7	Increase (ASDNaseB)	Leg	118	None
p3	71	f	n.d.	< 37.0	3.7	Decrease (ASL)	Leg, rec[b]	35	Skin ulcer
p4	71	m	78	39.4	29.7	Increase (ASL)	Leg	345	Adipositas, venous insufficiency, fungus infection of the feet
p5	64	m	50	37.8	26.9	Increase (ASL)	Leg	121	Deep vein thrombosis, adipositas
p6	65	m	70	37.4	3.7	Increase (ASL)	Leg, rec	116	Skin ulcer, diabetes, hypertension
p7	31	m	52	< 37.0	4.8	> 1000 (ASDNaseB)	Leg, rec	91	Eczema, planta pedis contact dermatitis both feet, adipositas
p8	56	m	n.d.	< 37.0	6.2	Decrease (ASL)	Face	129	None
p9	59	f	50	< 37.0	8.9	Increase (ASDNaseB)	Face	117	Streptococcal folliculitis of the Leg
p10	64	m	34	< 37.0	3.2	> 1000 (ASL)	Leg, rec	127	Pediculosis corporis
p11	44	m	78	38.4	6.0	> 1000 (ASDNaseB)	Leg, rec	171	Lymphangitis of the Leg, skin ulcer, rosacea
p12	37	f	22	< 37.0	5.4	Increase (ASL)	Face	138	None
	57 ± 13[c]	3f/9m						140.8 ± 74.1[c]	
Control donors									
	57 ± 7.1[c]	5f/7m						48.8 ± 19.8[c]	

a) Increase or decrease by more than factor 3 over up to 3 months.
b) Reccurent disease.
c) Average ± SD

age, the LTA-induced TNF-production of 5 patients with expanded $CD14^+DR^{++}$ monocytes was reduced by factor 2 ($p < 0.05$). At the same time TNF production by the classical $CD14^{++}$ monocytes was similar for patients and controls (Fig. 6). Also, unstimulated cells did not show any TNF expression (Fig. 6, left columns). Taken together, LTA-induced TNF production is selectively reduced in the $CD14^+DR^{++}$ monocytes.

When looking at other cytokines we observed a similar phenomenon for IL-1β. The example in Fig. 7 shows a 3.7-fold lower expression of this cytokine in the patient cells as compared to the control donor. Data summarized in Table 2 show an average 2-fold lower level while IL-1β production by the classical monocytes is similar for patients and controls. The analysis of IL-6 production did, however, not show any decrease in cytokine expression in patient monocyte populations (Table 2). Hence, the decreased response to *ex vivo* LTA stimulation is seen only for TNF and IL-1β but not for IL-6.

3 Discussion

In human peripheral blood we have discovered some years ago a novel type of monocyte that is characterized by expression of CD16 and low levels of CD14 [1, 9]. These cells, termed $CD14^+CD16^+$ monocytes, produce high amounts of the pro-inflammatory cytokine TNF and little if any of the anti-inflammatory cytokine IL-10 [10]. We therefore consider them to be pro-inflammatory monocytes. Consistent with this concept, the $CD14^+$

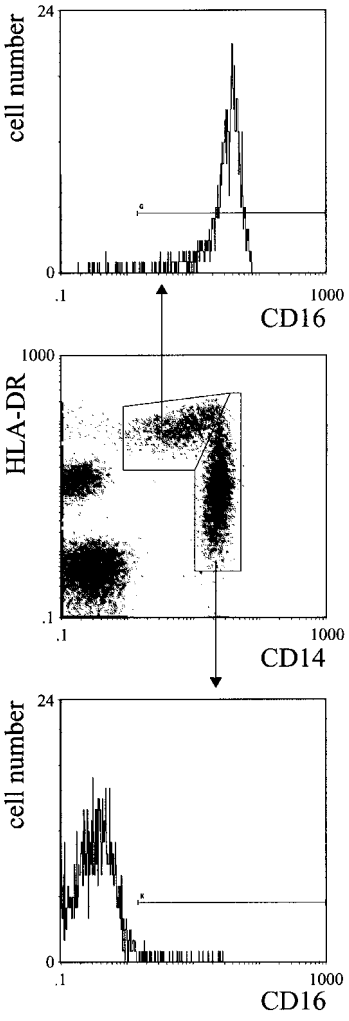

Fig. 4. Three-color-analysis of monocyte subpopulations in erysipelas patients. Whole blood samples were stained with monoclonal antibodies against CD14, CD16 and DR. Shown is the sample from patient p1 representative of six patients. The $CD14^{+}DR^{++}$ monocytes in the middle panel are 95.4% positive for CD16 (upper panel). The $CD14^{++}DR^{+}$ monocytes in the middle panel are 3.2% positive for CD16 (lower panel).

$CD16^{+}$ monocytes are depleted by anti-inflammatory glucocorticoid therapy [11]. Conversely, they have been shown to expand in severe systemic inflammatory disease like sepsis and post-surgical inflammation [3, 5, 12]. In the present study we have asked whether in a local type of infection the $CD14^{+}CD16^{+}$ monocytes will increase, as well, and we have studied the capacity of these cells to produce cytokines.

Our study demonstrates a pronounced increase of the $CD14^{+}CD16^{+}$ monocytes in 11/12 patients with erysipelas. The single patient without an increase still showed an inflammatory response with increase of CRP, but she

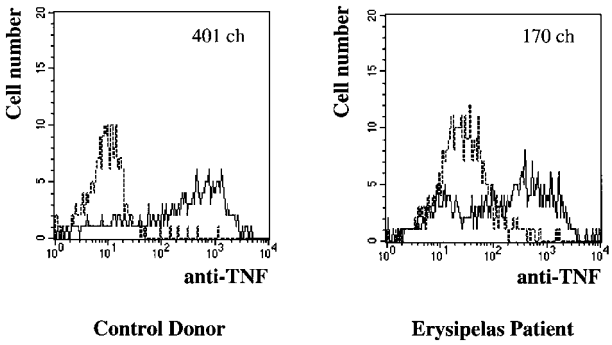

Fig. 5. TNF expression by $CD14^{+}DR^{++}CD16^{+}$ monocytes in a control donor and an erysipelas patient. Whole-blood samples were stimulated with LTA in the presence of BFA for 6 h followed by cell surface staining with CD14 and anti-DR antibodies. Then cells were permeabilized and reacted with anti-TNF (———) or with anti-TNF plus a tenfold molar excess of recombinant TNF (·····). Shown are the histograms for the $CD14^{+}DR^{++}$ monocyte from a control donor and a patient. Specific staining is given in channels in the upper right corner of each graph.

had recurrent disease. The additional patients with recurrent disease also showed less of an increase of the $CD14^{+}CD16^{+}$ monocytes (see Table 1).

On the other hand we observed one patient with extremely high numbers of $CD14^{+}CD16^{+}$ monocytes (345 cells/μl) who had the highest febrile response (39.4°C). We therefore suggest that the increase in the

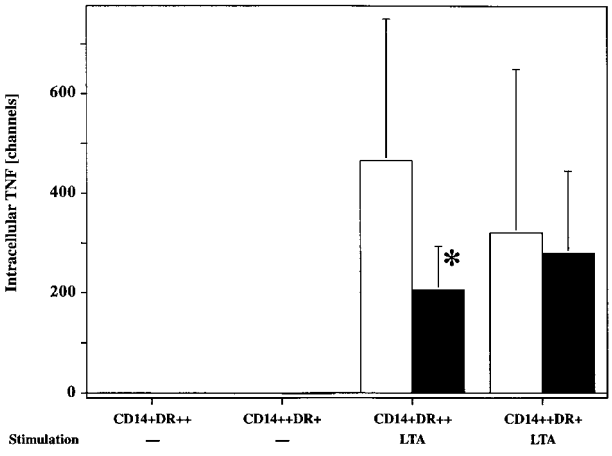

Fig. 6. TNF expression by monocyte subpopulations in control donors and erysipelas patients. Given are the data for specific anti-TNF staining in the $CD14^{+}DR^{++}$ pro-inflammatory monocytes and the $CD14^{++}DR^{+}$ classical monocytes without and with LTA stimulation from five patients (solid bars) and five control donors (open bars). * $p < 0.05$ as compared to the control group.

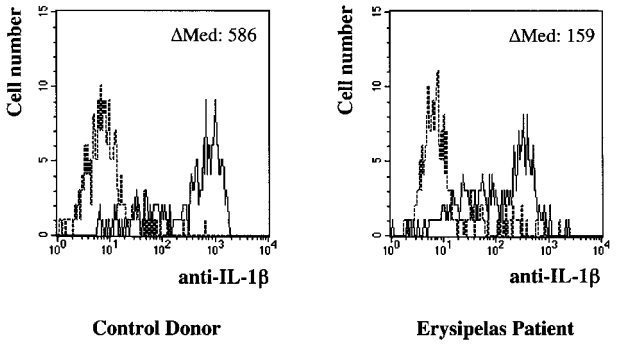

Fig. 7. IL-1β expression by $CD14^+DR^{++}CD16^+$ monocytes in a control donor and an erysipelas patient. Whole-blood samples were stimulated with LTA in the presence of BFA for 6 h followed by cell surface staining with CD14 and anti-DR antibodies. Then cells were permeabilized and reacted with anti-IL-1β (———) or with anti-IL-1β plus a tenfold molar excess of recombinant IL-1β (·····). Shown are the histograms for the $CD14^+DR^{++}$ monocyte from a control donor and a patient. Specific staining is given in channels in the upper right corner of each graph.

$CD14^+CD16^+$ monocytes does reflect the degree of the inflammatory response. To this end we found a positive correlation between the number of pro-inflammatory monocytes and body temperature as well as CRP.

This does not mean to say that CRP is involved in the induction of the $CD14^+CD16^+$ monocytes. We rather favor a role for M-CSF in this process. This is based on three reports that demonstrated an expansion of the $CD14^+CD16^+$ monocytes in the patients who were infused with M-CSF [13–15]. In addition, Saijoni et al. [16] found a significant correlation between M-CSF levels and the number of $CD14^+CD16^+$ monocytes in patients undergoing hemodialysis. In our study such a correlation was found not to be significant, but we speculate that it will become significant when a larger cohort of patients is being studied. Also, we have to keep in mind that the patients seen in the hospital on day 1 of the study have already had ongoing infection for 2–4 days. Therefore, analysis of cytokines at an earlier point in time may be required to identify a cause-effect-relationship.

An important question is whether an increase of the $CD14^+CD16^+$ monocytes is specific to erysipelas or whether it will also occur in other localized infections with group A streptococci like tonsillitis.

We then looked at the cytokines produced by the pro-inflammatory $CD14^+CD16^+$ monocytes. With *ex vivo* stimulation the CD16 antigen would be down-regulated and therefore this marker cannot be used for identification of the cells anymore. We have used instead a combination of CD14 and DR antibodies for identification of the pro-inflammatory monocytes based on the observation that prior to culture 96% of the $CD14^+DR^{++}$ monocytes carry the CD16 antigen (Belge et al., in press). Herein we could confirm this finding for the expanded pro-inflammatory monocytes of erysipelas patients. When looking at TNF production in the pro-inflammatory $CD14^+DR^{++}$ monocytes we noted a clearly reduced level of intracellular protein in patients as compared to control donors (Fig. 5). The down-regulation of cytokines was not restricted to TNF; we also found decreased levels of expression of IL-1β (Fig. 7 and Table 2). By contrast, no such decrease was seen for IL-6.

Table 2. Production of IL-1β and IL-6 in monocyte populations in erysipelas[a)]

	IL-1β		IL-6	
	$CD14^+DR^{++}$	$CD14^{++}DR^+$	$CD14^+DR^{++}$	$CD14^{++}DR^+$
Patients (n = 3)	264 ± 129*	500 ± 318	76 ± 46	85 ± 82
Controls (n = 3)	534 ± 66	597 ± 131	116 ± 65	146 ± 92

a) Whole-peripheral blood was stimulated with LTA in the presence of BFA and intracellular cytokines were measured with directly conjugated anti-cytokine antibodies after 4 h. Given is specific fluorescence intensity in channels. *The difference between patient and controls was p = 0.08 (not significant).

Such a down-regulation of cytokine expression has been described earlier in patients with severe sepsis [17–19]. This down-regulation, termed tolerance, is induced by prior *in vivo* activation of the cells by microbes and their products and it is meant to protect the host from the detrimental effects of excessive amounts of cytokines [20]. We show here that in erysipelas this tolerance is specific since it is found for TNF and IL-1β but not for IL-6. It will be of interest to study other pro-inflammatory cytokines like IL-12, which is associated with Th1 responses.

Such a tolerance induction with respect to cytokines like TNF may ensure that damage to local tissue due to excessive inflammation is limited.

We assume that the low levels of cytokines produced by the $CD14^+CD16^+$ monocytes in erysipelas are a result of a primary *in vivo* exposure of the cells to *S. pyogenes* and its products including LTA. The mechanisms involved may include blockade of MAP kinases [21] and of NF-κB mobilization [22].

On the other hand it could involve up-regulation of p50 homodimers, which bind to promoters without transactivation. This then prevents access of the prototypic p50p65 NF-κB and thereby blocks gene expression [23–26]. Whatever the mechanism which is operative in erysipelas monocytes, this mechanism has to be selective in that it only operates in the $CD14^+CD16^+DR^{++}$ pro-inflammatory monocytes.

Such a selective tolerance induction has not been reported before and we assume that this may be a more common phenomenon as compared to the global tolerance seen in diseases like sepsis. Since the $CD14^+CD16^+$ monocytes with their pro-inflammatory capacity could be very harmful, tolerance induction in these cells may be of importance for protection of the host. While we show that tolerance to LTA stimulation is specific for the pro-inflammatory $CD14^+CD16^+DR^{++}$ monocytes as compared to the classical monocytes, it is unclear at present whether other cells like granulocytes are rendered tolerant as well in patients with erysipelas.

Recently, we reported that the majority of the $CD14^+CD16^+$ monocytes resides in the marginal pool and that these cells can be mobilized in a catecholamine dependent fashion [27]. Increases in the number of $CD14^+CD16^+$ monocytes may also be due in part to such a mobilization but we favor a mechanism of expansion that results from a net increase of these cells due to cytokine driven differentiation. Still, mobilization studies in erysipelas patients with localized infection will be required in order to determine whether the $CD14^+CD16^+$ monocytes in the central pool are expanded at expense of the cells in the marginal pool.

Taken together, we have discovered in patients with localized skin infection a pronounced increase of the $CD14^+CD16^+$ monocytes. These pro-inflammatory monocytes, which express lower levels of cytokines, may be instrumental in immune defense against streptococcal infection.

4 Materials and methods

4.1 Patients and control donors

Twelve consecutive patients admitted to the Department of Dermatology at the University of Munich with the diagnosis of erysipelas were studied after informed consent had been obtained. Diagnostic criteria were typical skin lesion (painful, sharp-edged swelling, and redness of the skin) and antibodies against Streptolysin (ASL) or Streptococcal DNase B (ASDNaseB) being either 1,000 U/ml or showing upon follow up for up to 3 months an increase or a decrease of antibodies by more than factor 3. Patients were treated with either Cefotaxim (3×2 g i.v./day in 8 patients), Flucloxacillin (3×1g i.v./day in 2 patients), Ciprofloxacin (2×200 mg orally/day in 1 patient) or Penicillin G ($3\times1\times10^6$ U i.v./day in 1 patient).

Control donors were 12 age-matched volunteers recruited from staff, relatives and friends.

4.2 Determination of monocyte subpopulations

Whole-blood staining was done on 200 μl samples of EDTA-blood. After lysis of erythrocytes with ammonium chloride buffer (0.83% w/v) and one wash step, samples were incubated with directly conjugated monoclonal antibodies at saturating concentrations for 20 min on ice. The antibodies used were CD14-FITC (322A-1, clone My-4, #6603511, Beckman-Coulter, Krefeld, Germany) and CD16-PE (Leu11c, clone B73.1, #347617, BD, Heidelberg, Germany).

FACS analysis was done on a FACScan (BD) by gating in the light scatter histogram on monocytes plus the upper portion of lymphocytes and recording fluorescence signals of 15,000 cells in the monocyte gate (see: www.monocytes.de). Absolute numbers were calculated based on the percentage of cells among all leukocytes with the leukocyte count being determined in a Celldyn 3000 hematology analyzer (Abbott, Wiesbaden, Germany).

4.3 Detection of intracellular cytokines

4.3.1 Cell stimulation

Whole peripheral blood (WPB, 600 μl) anti-coagulated with heparin at 10 U/ml wase incubated for 4–6 h at 37°C in 15 ml polypropylene-tubes (#188 261, Greiner, Frickenhausen, Germany) in the presence of brefeldin A (BFA) at 10 μg/ml final concentration (#B 7651, Sigma, Muenchen, Germany). Samples were left untreated or were stimulated with LTA (# L-3140, Sigma) at 100 μg / ml. For control of LPS contamination cultures were set up with and without PB (Pfizer, Karlsruhe, Germany) at 10 μg/ml.

4.3.2 Cell surface staining

The stimulated samples were treated with ammonium chloride buffer (0.83% w/v) for about 3 min until erythrocytes were lysed. After washing the leukocytes with PBS/2% FCS (staining buffer) cells were resuspended in 100 μl of staining buffer and antibodies to CD14-FITC at 18 μg/ml final and HLA-DR-PC-5 (clone Immu357, at 0.06 μg/ml final, #2659, Beckman-Coulter) were added followed by incubation on ice for 20 min. After washing the samples with staining buffer the cells were fixed with paraformaldehyde at 4% in PBS for 20 min on ice followed by two wash steps.

4.3.3 Intracellular staining

Paraformaldehyde-fixed samples were permeabilized with Perm/Wash solution (#2097 KZ, BD, Heidelberg, Germany) for 5 min at RT. For detection of intracellular TNF we added phycoerythrin-conjugated anti-TNF antibody (MP9–20A4, #RHTNFA04, Caltag via Medac, Hamburg, Germany) or as isotype control phycoerythrin-conjugated rat IgG1 (#R104, Caltag) both at 10 μg/ml for 20 min on ice. Samples were then washed twice and were resuspended in PBS/0.5% paraformaldehyde and analyzed within 24 h.

For detection of IL-1β we used a phycoerythrin-conjugated anti-IL-1β antibody (#IC2018, R&D Systems, Wiesbaden, Germany). For detection of IL-6 we used a phycoerythrin-conjugated rat antibody (#20655A, Becton Dickinson, Heidelberg, Germany).

4.3.4 Specificity control

The specificity of TNF staining was determined by incubating the phycoerythrin-conjugated anti-TNF for 10 min at room temperature with a tenfold molar excess of recombinant human TNF (kindly provided by BASF-Knoll, Ludwigshafen, Germany) followed by addition to the permeabilized cells. Specificity control for IL-1β and IL-6 was with tenfold molar excess of IL-1β (#IL-1β-10, Strathmann Biotech, Hamburg, Germany) and IL-6 (#200–06, Peprotech, London, GB), respectively.

4.4 ELISA for serum cytokines

ELISA for serum cytokines were performed according to manufacturers' instructions. The following assays were used: IL-6, PeliKine, Amsterdam, Netherlands # M1916; detection limit 4.4 pg/ml; IL-10, PeliKine, # M1910; detection limit 18.2 pg/ml; IFN-γ, PeliKine, # M1933; detection limit 28.0 pg/ml; M-CSF: R&D Systems, Wiesbaden-Nordenstadt, Germany; # DMC00; detection limit 157.5 pg/ml.

4.5 Statistics

For comparison of leukocyte numbers from patients and controls Student's *t*-test was applied. Wilcoxon test was used for comparison of data on intracellular cytokine expression. For correlation analysis polynomial regression analysis was used. All average values are given as mean ± standard deviation.

Acknowledgements: The authors acknowledge the excellent technical support by S. Multhaup and the expert advice provided by M. Frankenberger, Gauting, Germany. We also thank R .D. R.Camp, Leicester, GB for critically reading the manuscript. This work was supported by the DFG-Graduierten-Kolleg "Infektion und Immunität" and by DFG-Grant Zi 288/2.

References

1 **Passlick, B., Flieger, D. and Ziegler-Heitbrock, H. W. L.,** Identification and characterization of a novel monocyte subpopulation in human peripheral blood. *Blood* 1989. **74:** 2527–2534.

2 **Ziegler-Heitbrock, H. W. L.,** Heterogeneity of human blood monocytes: the $CD14^+$ $CD16^+$ subpopulation. *Immunol. Today* 1996. **17:** 424–428.

3 **Fingerle, G., Pforte, A., Passlick, B., Blumenstein, M., Ströbel, M. and Ziegler-Heitbrock, H. W. L.,** The novel subset of $CD14^+$/$CD16^+$ blood monocytes is expanded in sepsis patients. *Blood* 1993. **82:** 3170–3176.

4 **Blumenstein, M., Boekstegers, P., Fraunberger, P., Andreesen, R., Ziegler-Heitbrock, H. W. L. and Fingerle-Rowson, G. R.,** Cytokine production precedes the expansion of $CD14^+CD16^+$ monocytes in human sepsis: a case report of a patient with self-induced septicemia. *Shock* 1997. **8:** 73–75.

5 **Nockher, W. A. and Scherberich, J. E.,** Expanded $CD14^+$ $CD16^+$ monocyte subpopulation in patients with acute and chronic infections undergoing hemodialysis. *Infect. Immun.* 1998. **66:** 2782–2790.

6 **Otani, I., Akari, H., Nam, K. H., Mori, K., Suzuki, E., Shibata, H., Doi, K., Terao, K. and Yosikawa, Y.,** Phenotypic changes in peripheral blood monocytes of cynomolgus monkeys acutely infected with simian immunodeficiency virus. *AIDS Res. Hum. Retroviruses* 1998. **14:** 1181–1186.

7 **Thomas, R. and Lipsky, P. E.,** Human peripheral blood dendritic cell subsets. Isolation and characterization of precursor and mature antigen-presenting cells. *J. Immunol.* 1994 **153:** 4016–4028.

8 **Belge, K.-U., Dayyani, F., Horelt, A., Siedlar, M., Frankenberger, M., Frankenberge, B., Espevik, T. and Ziegler-Heitbrock, H. W. L.,** The pro-inflammatory $CD14^+$ $CD16^+$ DR^{++} monocytes are a major source of tumor necrosis factor. *J. Immunol.* 2002, in press.

9 **Ziegler-Heitbrock, H. W. L., Passlick, B. and Flieger, D.,** The monoclonal antimonocyte antibody My4 stains B lymphocytes and two distinct monocyte subsets in human peripheral blood. *Hybridoma* 1988. **7:** 521–527.

10 **Frankenberger, M., Sternsdorf, T., Pechumer, H., Pforte, A. and Ziegler-Heitbrock, H. W. L.,** Differential cytokine expression in human blood monocyte subpopulations: a polymerase chain reaction analysis. *Blood* 1996. **87:** 373–377.

11 **Fingerle-Rowson, G., Angstwurm, M., Andreesen, R. and Ziegler-Heitbrock, H. W. L.,** Selective depletion of $CD14^+$ $CD16^+$ monocytes by glucocorticoid therapy. *Clin. Exp. Immunol.* 1998. **112:** 501–506.

12 **Fingerle-Rowson, G., Auers, J., Kreuzer, E., Fraunberger, P., Blumenstein, M. and Ziegler-Heitbrock, H. W. L.,** Expansion of $CD14^+CD16^+$ monocytes in critically ill cardiac surgery patients. *Inflammation* 1998. **22:** 367–379.

13 **Munn, D. H., Garnick, M. B. and Cheung, N.-K. V.,** Effects of parenteral recombinant human macrophage colony-stimulating factor on monocyte number, phenotype, and antitumor cytotoxicity in nonhuman primates. *Blood* 1990. **75:** 2042–2048.

14 **Weiner, L. M., Li, W., Holmes, M., Catalano, R. B., Dovnarsky, M., Padavic, K. and Alpaugh, R. K.,** Phase I trial of recombinant macrophage colony-stimulating factor and recombinant gamma-interferon: toxicity, monocytosis, and clinical effects. *Cancer Res.* 1994. **54:** 4084–4090.

15 **Schmid, I., Baldwin, G. C., Jacobs, E. L., Isacescu, V., Neagos, N., Giorgi, J. V. and Glaspy, J. A.,** Alterations in phenotype and cell-surface antigen expression levels of human monocytes: differential response to *in vivo* administration of rhM-CSF or rhGM-CSF. *Cytometry* 1995. **22:** 103–110.

16 **Saionji, K., and Ohsaka, A.,** Expansion of CD14⁺CD16⁺ blood monocytes in patients with chronic renal failure undergoing dialysis: possible involvement of macrophage colony-stimulating factor. *Acta Hematol.* 2001. **105:** 21–26.

17 **Munoz, C., Carlet, J., Fitting, C., Bisset, B., Blériot, J.-P. and Cavaillon, J.-M.,** Dysregulation of *in vitro* cytokine production by monocytes during sepsis. *J. Clin. Invest.* 1991. **88:** 1747–1754.

18 **Ertel, W., Kremer, J.-P., Kenney, J., Steckholzer, U., Jarrar, D., Trentz, O. and Schildberg, F. W.,** Downregulation of proinflammatory cytokine release in whole blood from septic patients. *Blood* 1995. **85:** 1341–1347.

19 **McCall, C.E., Grosso-Wilmoth, L.M., LaRue, K., Guzman, R.N., and Cousart, S.L.,** Tolerance to endotoxin-induced expression of the interleukin-1 beta gene in blood neutrophils of humans with the sepsis syndrome. *J. Clin. Invest.* 1993. **91:** 853–861.

20 **Ziegler-Heitbrock, H. W. L.,** Molecular mechanism in tolerance to lipopolysaccharide. *J. Inflamm.* 1995. **45:** 13–26.

21 **Durando, M. M., Meier, K. E. and Cook, J. A.,** Endotoxin activation of mitogen-activated protein kinase in THP-1 cells; diminished activation following endotoxin desensitization. *J. Leukoc. Biol.* 1998. **64:** 259–264.

22 **Kohler, N. G. and Joly, A.,** The involvement of an LPS inducible I kappa B kinase in endotoxin tolerance. *Biochem. Biophys. Res. Commun.* 1997. **232:** 602–607.

23 **Ziegler-Heitbrock, H. W. L., Wedel, A., Schraut, W., Ströbel, M., Wendelgass, P., Sternsdorf, T., Bäuerle, P., Haas, J. G. and Riethmüller, G.,** Tolerance to lipopolysaccharide involves mobilization of nuclear factor kappa B with predominance of p50 homodimers. *J. Biol. Chem.* 1994. **269:** 17001–17004.

24 **Kastenbauer, S. and Ziegler-Heitbrock, H. W.,** NF-kappaB1 (p50) is upregulated in lipopolysaccharide tolerance and can block tumor necrosis factor gene expression. *Infect Immun.* 1999. **67:** 1553–1559.

25 **Liu, H., Sidiropoulos, P., Song, G., Pagliari, L. J., Birrer, M. J., Stein, B., Anrather, J. and Pope, R. M.,** TNF-alpha gene expression in macrophages: regulation by NF-kappa B is independent of c-Jun or C/EBP beta. *J. Immunol.* 2000. **164:** 4277–4285.

26 **Fujihara, M., Wakamoto, S., Ito, T., Muroi, M., Suzuki, T., Ikeda, H. and Ikebuchi, K.,** Lipopolysaccharide-triggered desensitization of TNF-alpha mRNA expression involves lack of phosphorylation of IkappaBalpha in a murine macrophage-like cell line, P388D1. *J. Leukoc. Biol.* 2000. **68:** 267–276.

27 **Steppich, B., Dayyani, F., Gruber, R., Lorenz, R., Mack, M. and Ziegler-Heitbrock, H. W. L.,** Selective mobilization of CD14⁺CD16⁺ monocytes by exercise. *Am. J. Physiol. Cell Physiol.* 2000. **279:** C578-C586.

Correspondence: Löms Ziegler-Heitbrock, Department of Microbiology and Immunology, University of Leicester, University Road, Leicester, LE1 9HN, GB
Fax: +44-116-252-30-50
e-mail: LZH1@le.ac.uk