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## Research Article

# Identification of factor XIII-A as a marker of alternative macrophage activation

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**Abstract.** Factor XIII subunit A of blood coagulation (FXIII-A) is known to be synthesized but not secreted by the monocyte/macrophage cell line. On the basis of its intracellular localization and substrate profile, FXIII-A is thought to be involved in certain intracellular processes. Our present study was designed to monitor the changes in FXIII-A gene expression and protein production in long-term culture of human monocytes during their differentiation into macrophages in the presence of activating agents (interleukin-4, interferon-γ, *Mycobacterium bovis* BCG) inducing classical and alternative activation pathways. By using quantitative RT-PCR and fluorescent image analysis at the single-cell level we demonstrated that the expression of FXIII-A both at the mRNA as well as at the protein level is inversely regulated during the two activation programmes. Here we conclude that FXIII-A expression is an intracellular marker for alternatively activated macrophages, while its absence in monocyte-derived macrophages indicates their classically activated state.

**Key words.** Factor XIII-A; macrophage; gene expression; alternative activation; classical activation.

Factor XIII (FXIII) is well known as the fibrin-stabilizing factor of blood coagulation, and is present in the circulation in tetrameric form consisting of two enzymatic A and two inhibitory B subunits [1]. Work over the last two decades has revealed that the role of FXIII is not restricted to haemostasis but it also acts as an intracellular transglutaminase.

In addition to megakaryocytes/platelets [2, 3], which are considered to be the source of plasma FXIII [4, 5], the A subunit of FXIII (FXIII-A) can also be detected in monocytes/macrophages [6–9]. FXIII-A has been convincingly shown to be synthesized but not secreted by this cell type, and is also accumulated and activated at the cellular level [10–13]. FXIII-A shows a characteristic distribution in monocytes/macrophages; it accumulates around cytoplasmic vacuoles and in pseudopods [4, 8] in association with the microfilament structure but can never be detected either in phagocytic or in secretory vesicules [4]. In accordance with these findings, as well as with the fact that major cytoskeletal proteins such as actin [14], myosin [15] and vinculin [16] are substrates of activated FXIII-A, a reasonable supposition is that FXIII-A plays (a) role(s) in intracellular process(es) involving cytoskeletal remodelling. Recently, we have found that phagocytosis of certain particles (sensitized erythrocytes and complement-coated yeast particles) via Fcγ and complement receptors is strongly diminished in monocytes of FXIII-A-deficient patients, and the phagocytic functions of cultured monocytes/macrophages show a change in parallel with FXIII-A mRNA expression and protein synthesis [17].

These results strongly suggest that FXIII-A plays a role in the phagocytic activities of monocytes/macrophages

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but are not supported by clinical reports [18–20] on increased susceptibility to infectious diseases due to impaired phagocytic elimination of microbes in FXIII-A-deficient patients.

Phagocytosis is a key event among functional changes of macrophages in response to microenvironmental exposures inducing their activation. A broad range of macrophages can be activated by a number of humoral and particle-like (among microbial) agents via different surface receptors and signalling pathways, which makes this single lineage both morphologically and functionally very heterogeneous. Despite this heterogeneity, the extremes of the potential functional states, the fully polarized classically (type I) and alternatively (type II) activated macrophages, represent the two main types of activated macrophages [for a review see ref. 21].

The classical activation of macrophages is induced by pro-inflammatory stimuli, such as Th1 cytokines [such as interferon-γ (IFN-γ)] and microbes or microbial products, and via this pathway, their ability to kill intracellular microbes, such as *Mycobacterium tuberculosis*, is greatly enhanced [22].

Alternative activation is a response to Th2 cytokines, such as interleukin 4 (IL-4) and results in the expression of a broad range of surface receptors and receptor antagonists which bestow them with specific functions, such as disposal of soluble and particulate (typically cell/tissue debris) matter [23, 24].

Very little is known about the effector mechanisms and molecules utilized by these two activation states to fulfil their distinct biological functions. Therefore, we decided to characterize the expression of the FXIII-A gene during classical and alternative activation of macrophages. Here we first demonstrate that FXIII-A is inversely regulated in the two major activation programmes and that, consequently, the detection of FXIII-A expression can be considered as a marker for alternatively activated macrophages while its absence in monocyte-derived macrophages indicates their classically activated state. We also provide evidence that the upregulation of FXIII-A gene expression by IL-4 is inhibited when activated cells are exposed to stimuli of the classical activation pathway.

## Materials and methods

**Monocyte separation.** Monocytes were obtained from platelet- free buffy coats isolated from healthy donors by Ficoll-Hypaque (Pharmacia, Freiburg, Germany) gradient centrifugation and immunomagnetic cell separation using anti-CD14-conjugated microbeads (VarioMACS; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturers' protocols. Cell viability was at least 98%, as determined by trypan blue exclusion, and monocyte purity varied between 95%–98% as judged by morphology and CD14 expression.

**Macrophage and dendritic cell generation**. To obtain macrophages, human-blood-derived monocytes separated as described earlier were cultured in RPMI 1640 supplemented with 10% FBS (Invitrogen, Karlsruhe, Germany) for 5 days. This untreated cell line served as a control in our studies.

To obtain dendritic cells (DCs) 500 U/ml IL-4 (Peprotech, Frankfurt am Main, Germany) and 800 U/ml granulocyte/macrophage long-stimulating-factor (GM-CSF) (Peprotech) were added to the culture medium at the starting point, and again on the third day of cultivation as described elsewhere [25].

**Activation of macrophages.** The following activating agents were added to the monocyte suspensions at the 0 time point of cultivation. For the induction of the classical activation pathway: 500 U/ml IFN-γ (Peprotech) as described [26] previously and *M. bovis* BCG (Danish Strain 1331) at a dose of 0.3 bacteria/cell. *M. bovis* BCG was purchased in lyophilized form (Statens Serum Institut, Copenhagen, Denmark) and dissolved in culture medium right before infection, and clumps were removed by a 10-min centrifugation at 20 g. The density of bacteria in the cultures was adjusted to a dose of 0.3 bacteria/cell assuming that an OD 600 of 0.2 equals a concentration of $10^7$ bacteria/ml [27]. For the induction of the alternative activation pathway: 500 U/ml IL-4 (Peprotech) as described before [26].

**Fluorescence-activated cell sorting analysis.** To verify the differentiation and the classical and alternative activation of cells, fluorescence-activated cell sorting (FACS) analysis was carried out using FITC-conjugated monoclonal antibody (Serotec, Oxford, UK) for DC-SIGN (a DC differentiation marker [28]), for the mannose receptor (an alternative-activation marker [23]) and Fcγ RI (a classical activation marker [22]). Results obtained were evaluated by WinMDI software (J. Trotter, TSRI, La Jolla, Calif.).

**Infection of DCs with M. bovis BCG.** DCs were infected with *M. bovis* BCG on the fifth day as described earlier. The response of DCs to BCG infection was verified by detecting CD80 and CD86 expression by FACS analysis using FITC-conjugated monoclonal antibodies as described by the manufacturer (Serotec).

**Sampling.** To examine the changes in the expression of FXIII-A, samples were collected from the cultures of monocytes/macrophages every 24 h for 5 days both for cytospin preparation and extraction of total RNA. Samples were collected for RNA isolation from the *M. bovis*-infected DC cultures 24 and 48 h after infection.

**Cytospin preparations.** Cytospin preparations were made by cytocentrifugation (Shandon, Pittsburgh, UK) on each day of culturing at 800 rpm for 3 min. Slides were air-dried and stored at –20 °C until use.

**RNA isolation**. To obtain total RNA, cells ($2 \times 10^6$) were centrifuged at the indicated times, the cell pellet was resuspended in 1 ml TRIZOL (Invitrogen Life Technology, Carlsbad, Calif.), and RNA was extracted following the manufacturer's instructions. The purity of the RNA preparation was checked by measuring the absorbance ratio at 260/280 nm.

**Real-time RT-PCR.** Samples containing equal amounts (2 μg) of RNA were used for reverse transcription, which was carried out at 42 °C for 30 min and 72 °C for 5 min using Superscript II reverse transcriptase (Invitrogen) and random hexamers (Invitrogen) according to the recommendation of the manufacturer. The cDNA obtained was used for real-time quantitative PCR (ABI PRISM 7900; Applied Biosystems, Darmstadt, Germany), 40 cycles of 95 °C for 10 sec and 60 °C for 1 min. All PCR reactions were done in triplicate with one control reaction containing no reverse transcription enzyme. The comparative Ct method was used to quantify transcripts and to normalize for cyclophilin. Taqman probes were used both for FXIII-A and for cyclophilin (Applied Biosystems). On samples isolated from control, IL-4- and IFN-γ-activated macrophages, real-time RT-PCR reactions were carried out also for tissue transglutaminase type 2 (TGase 2) as described previously [29].

**Immunofluorescent detection of FXIII-A and fluorescent image analysis.** Cytospin preparations were fixed in 4% paraformaldehyde at room temperature for 30 min. Incubation with 5% normal human serum in phosphate-buffered isotonic saline, pH 7.4 (PBS) was performed for 15 min to prevent non-specific IgG binding. FXIII-A was detected by an indirect immunofluorescent reaction on samples incubated with rabbit anti-human FXIII-A polyclonal antiserum diluted 1:200 for 12 h at 4 °C (Centeon Pharma, Stuttgart, Germany) and subsequently with swine FITC-conjugated anti-rabbit antibody diluted 1:40 for 45 min at room temperature (DAKO A/S, Glostrup, Denmark). For negative controls, identical dilution of non-immune rabbit serum was substituted for rabbit anti-human FXIII-A serum. Slides were washed and mounted in an antifade containing DAPI (Vector Laboratories, Burlingame, Calif.). The relative amount of FXIII-A was determined at a single-cell level by measuring integrated fluorescence intensity values using an Axioplan fluorescence microscope (Zeiss, Oberkochen, Germany) connected to a black-and-white intensified charge coupled device (CCD) IMAC camera (Sony, Tokyo, Japan) and a computer system (ISIS fluorescence imaging system; Metasystems, Altlussheim, Germany) as described previously [30]. Each fluorescence image was obtained through a ×63, NA 1.25 oil immersion objective with fixed capture time of 0.04 s. Quantitative fluorescent image analysis was carried out on 150-200 cells/slide. Statistical analysis and figures were made using Microsoft Excel software.

**Tissue samples.** Sections (6 μm) from laryngeal tumors (n = 3) and a lymph node sample with tuberculotic granulomas archived after histopathological diagnosis (n = 1) were kindly provided by Z. Szentirmay of the National Cancer Institute (Budapest, Hungary). Tissue samples were fixed in neutralized formalin and embedded in paraffin for diagnostic histopathological (including immunohistochemical) examination.

**Immunohistochemical detection of FXIII-A and macrophage marker antigen on tissue sections.** Sections were dewaxed, rehydrated and treated with the Biogenex (San Ramon, Calif.) antigen retrieval solution, according to the manufacturer's instructions. Endogenous peroxidase activity was blocked by 1% $H_2O_2$ in absolute methanol for 30 min at room temperature. Non-specific IgG binding was prevented by preincubation of the sections in 20% normal goat serum. Rabbit immune serum to human FXIII-A (Centeon Pharma) or anti-human CD68 monoclonal antibody (DAKO A/S) recognizing a glycosylated lysosomal membrane protein strongly expressed by macrophages [31] was used as primary immunoreagent. Antigen-antibody reactions were detected using biotinylated anti-rabbit IgG or anti-mouse IgG and avidin-biotinylated peroxidase complex, according to the instructions of the Vectastain ABC kit (Vector Laboratories). The specific peroxidase activity was visualized by 0.05% 3,3'diaminobenzidine in 0.1 mol/l TRIS-HCl buffer, pH 7.2. Counterstaining was performed by Mayer's haematoxylin. Sections were dehydrated in graded alcohol and mounted with Canada balsam.

## Results

**Changes in FXIII-A gene expression during macrophage differentiation and activation.** mRNA levels were measured to determine the expression levels of FXIII-A during the differentiation and activation of macrophages derived from monocytes. In line with our previous observation, the mRNA level of FXIII-A increased in parallel with the differentiation of macrophages. A significant change in expression was detected on the second day and the highest level was observed on the third day which was followed by a slight drop.

To characterize and compare the changes in expression during the classical and the alternative activation of mac-

rophages, IL-4 and IFN-γ or *M. bovis* BCG were used to trigger activation.

The expression of FXIII-A was found to be changed in the opposite way in the two activation pathways. In comparison with the control macrophages, the induction of FXIII-A in the IL-4- treated cells was more than twofold higher even after 48 h than the highest induction obtained during differentiation of control cells. The further increase in gene expression by IL-4 treatment resulted in a peak on the fourth day, when the expression level of FXIII-A was about 45-fold more than the highest level detected in control macrophages. During classical activation of macrophages, the expression of the FXIII-A gene was inhibited if compared to the control (fig. 1). In contrast, the expression of the TGase 2 gene showed no considerable change during activation by either IL-4 or IFN-γ compared to the control. Even the maximum change observed in the case of IFN-γ treatment on the second day had not exceeded the control level by fourfold.

**Change in the mRNA level of FXIII-A results in a parallel change in protein expression.** To determine that changing mRNA levels resulted in changing protein expression, immunofluorescent detection for FXIII-A and image analysis were carried out. IL-4-activated monocytes/macrophages showed continuously increasing fluorescent intensity during culture. From the third day, a significant increase in fluorescent intensity was observed, showing a fourfold higher fluorescent intensity on the third day in culture, sevenfold higher on the fourth and 11-fold higher on the fifth day, while BCG- or IFN-γ-activated macrophages showed a significantly lower fluorescent intensity than the control at all stages (figs 2, 3). These data suggest that protein expression follows the mRNA expression during macrophage activation.

**FXIII-A is expressed in monocyte-derived dendritic cells and BCG infection inhibits its expression.** Beside alternative activation, the cytokine IL-4 is also implicated in the in vitro differentiation of monocytes into DCs. Therefore we decided to examine the expression of FXIII-A in this cell type.

Our results showed that the IL-4 + GM-CSF-treated cells showed a very high level of FXIII-A mRNA on the fifth day in culture (fig. 4), suggesting that not only alternatively activated macrophages but also professional antigen-presenting DCs express high levels of this protein. Then we decided to test the effect of a pathogenic microorganism (BCG) on this expression level. As shown in figure 4, as a result of BCG infection, levels of FXIII-A were significantly suppressed even after 24 h; further decrease was detected 48 h after infection when the FXIII-A mRNA level decreased to values seen in macrophages

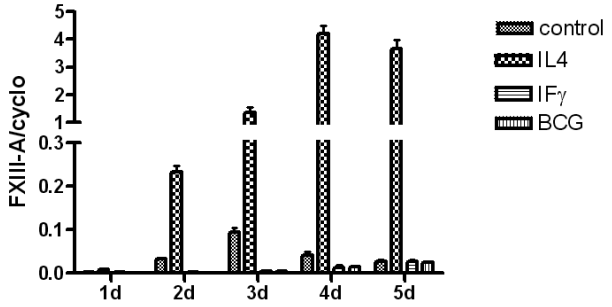


Figure 1. FXIII-A mRNA expression by monocytes/macrophages in different culture conditions.

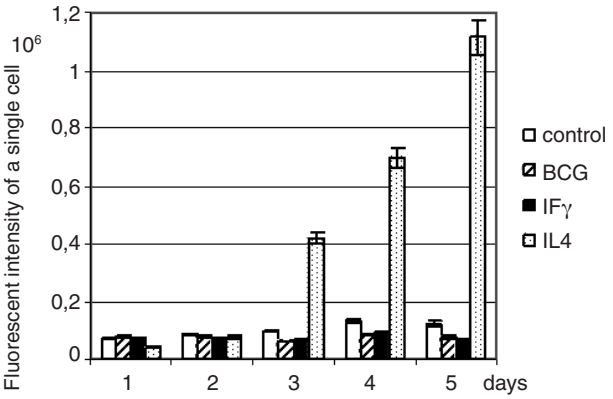


Figure 2. Average fluorescence intensity representing FXIII-A of a single cell in association with days of culturing monocytes/macrophages under different conditions.

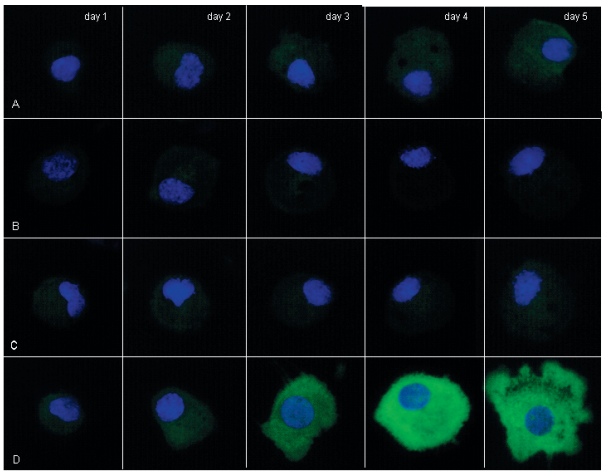


Figure 3. Digitized images of immunofluorescently labelled monocytes/macrophages on days 1–5 of culture in control and different activating conditions (×63, NA 1.3, oil immersion). (A) Control. (B) BCG. (C) IFN-γ. (D) IL-4 activation.

without IL-4 stimulation (fig. 4). These data indicate that the expression level of FXIII-A changes dynamically during dendritic cell differentiation and activity. Depending on the context and the extracellular stimuli it can be

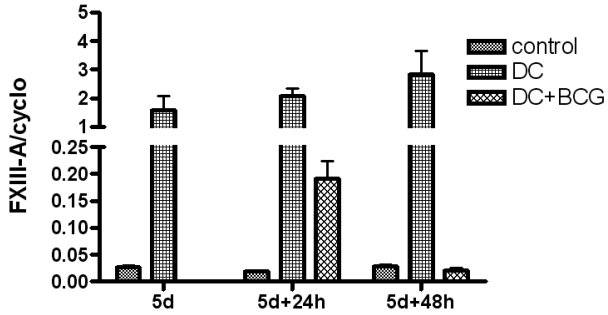


Figure 4. FXIII-A gene expression changes as the effect of BCG infection on DCs.

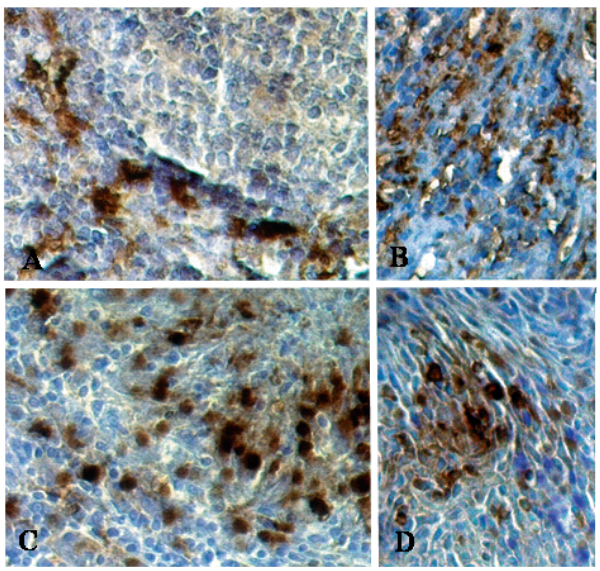


Figure 5. Immunohistochemical detection of FXIII-A (A, B) and CD68 (C, D) in laryngeal tumour (B, D) and a lymph node with a tuberculotic granuloma (A, C). Haematoxylin counterstain (×40, NA 1.3).

up- or downregulated. These experiments also showed that not only can the induction be inhibited, but also that already high levels of FXIII-A can be downmodulated by an infectious agent such as BCG.

**FXIII-A can be detected only in alternatively activated tissue macrophages by immunohistochemistry.** Finally, we wanted to see if the inverse regulation of FXIII-A is also reflected in in-vivo-differentiated and activated macrophages. For this we used tissue sections of laryngeal tumours and a lymph node with tuberculotic granulomas.

In laryngeal tumours, FXIII-A could be detected throughout the tissue in tumor-associated macrophages. Similarly, macrophages of the lymph node parenchyma were intensely labelled for FXIII-A, but no reaction could be seen in CD68+ cells of tuberculotic granulomas of the lymph node (fig. 5). These findings indicate that macrophages considered alternatively activated (i. e. tumour associated) expressed FXIII-A, whilst classically activated ones from granulomas were negative for this protein.

## Discussion

The presence of FXIII-A in circulating blood monocytes was first reported 20 years ago [6, 7] and in subsequent studies, FXIII-A could be detected in early monoblastic precursor cells in the bone marrow [3] as well as in different subsets of monocyte-derived mobile and fixed macrophages throughout the body [8, 11, 32–37] as a result of direct synthesis [4]. On the basis of results showing that (i) FXIII-A has no signal sequence and is not secreted into the culture medium when it is expressed in baby hamster kidney cells [10], (ii) it has a characteristic intracellular distribution [8, 11, 12] and (iii) it can be activated intracellularly [13], to interpret monocytes/macrophages as a source of the plasma FXIII-A subunit would be an oversimplification. Consequently, the possible role of intracellular FXIII-A became an open and widely discussed question. Today, FXIII-A is strongly believed to be involved in certain so far poorly defined cytoplasmic and nuclear processes [for a reviewed see ref. 38].

FXIII-A is a member of the family of transglutaminase (TG) enzymes which catalyse the formation of ε(γ-glutamyl)lysyl cross-links between different substrate proteins. Actin and myosin, the two major elements of the cell cytoskeletal locomotory system, were first identified as substrates of intracellular FXIII-A [14, 15], as was also later verified for vinculin [16, 39], the small heatshock protein HSP27 [40] and thymosin beta4 [41], which have a significant role in cytoskeletal remodelling. In addition, certain adhesive glycoprotein components of the extracellular matrix involved in tissue repair, such as fibronectin, vitronectin, osteopontin and thrombospondin [42–45] are substrates for FXIII-A.

Phagocytosis is one of the most characteristic functions of macrophages, in which the cytoskeletal system is involved. Monocytes of patients with FXIII-A deficiency show an impaired capacity to phagocytose sensitized erythrocytes, as well as complement-coated and uncoated yeast particles [17]. In long-term cultures of monocytes/macrophages, FXIII-A expression increased in parallel with the phagocytic activity, in accordance with a previous study on a non-phagocytic, FXIII-A-negative, myelomonocytic cell line (DD), in which FXIII-A production and phagocytic activity could be restored by phorbol ester treatment [46]. On the basis of these findings, the possible role of FXIII-A in phagocytosis by monocytes/macrophages seems rather obvious at a first glance. However, such a straightforward interpretation is limited by the fact that among the clinical symptoms

reported in connection with FXIII-A deficiency, alterations in host immune responses (including phagocytic elimination of microbes) to infections have not been reported so far.

On the other hand, impaired wound healing is frequently observed in FXIII-A-deficient patients [18–20] and the same observation was made in studies on FXIII-A gene knock-out mice [47]. FXIII-A treatment is also known to facilitate wound healing in different clinical and experimental conditions [48, 49]. To elucidate the contradiction between the above observations, one must hypothesize that FXIII-A is not necessarily required for all types of phagocytosis.

Cytokines and microbes (microbial products) profoundly affect the function of macrophages and trigger them for intense phagocytosis. Stimulation by microbes (microbial products), like the cytokines (of which the best-known is IFN-γ) released by activated Th1 cells, initiates the classical activation pathway. The important role of IFN-γ in the regulation of the classical activation of macrophages has been demonstrated in mice with distrupted IFN-γ [50] or interferon receptor [51] genes showing increased susceptibility to intracellular pathogens, such as *Listeria monocytogenes*. IFN-γ initiates the rapid JAK/STAT1a-mediated priming response and induces the synthesis of a set of IFN regulatory transcription factors [52].

During the last decade, it became evident that anti-inflammatory molecules such as Th2 cytokines, among them IL-4, are not simply inhibitors of macrophage activation, but induce a distinct alternative activation program via their receptors by initiating the STAT6-dependent signaling pathway [23, 24].

Classically activated macrophages are considered potent effector cells in killing microorganisms, while alternatively activated ones are interpreted as cells tuning inflammatory responses and adaptive immunity, scavenging debris and promoting angiogenesis, tissue remodelling and repair [21].

In our studies, FXIII-A gene expression was investigated in monocytes/macrophages triggered for classical and alternative activation, and clearly inverse alterations in FXIII-A expression were observed both at the mRNA and protein levels. In macrophages activated by the classical path using IFN-γ and BCG, gene expression became strongly downregulated and, consequently, the protein product could be detected only in trace amounts. In contrast, during alternative activation with IL-4, FXIII-A gene expression became upregulated to an almost unprecedented scale by two orders of magnitude compared to the peak value in untreated differentiating macrophages. The fact that in DCs the very high level of gene expression was almost completely, hyperresponsively reduced by BCG raises the possibility that FXIII-A gene expression is tightly controlled at the transcriptional level. In agreement with these findings, a very intense reaction for FXIII-A could be detected in tumour-associated macrophages which are known to be driven by tumour- and T-cell-derived cytokines to acquire the characteristic properties of fully polarized type 2 (alternatively activated) macrophages. In our previous studies, the presence of FXIII-A+ macrophages was always a characteristic finding in various types of tumour [53–56]. However, no reaction for FXIII-A was observed in macrophages of granulomas formed as a result of *M. tuberculosis* infection, although FXIII-A+ macrophages were present in the lymph node parenchyma surrounding them. Probst-Cousin et al. [57] published the same observation on six tuberculotic lesions. Theoretically, the lack of FXIII-A may be compensated by the overproduction of another TG, but in our study, the expression level of the most common tissue TG (TGase 2) did not show considerable change to the activating stimuli of IL-4 or IFN-γ. On the basis of these findings, it is not reasonable to suppose that the TGase 2 has a role in classically activated macrophages similar to that of FXIII-A in alternatively activated ones, although it may have a complementary role, when FXIII-A gene expression is inhibited.

Our results are in line with clinical observations on FXIII-A-deficient patients, as well as with experimental findings on their monocytes. Wound healing was frequently found to be impaired in these individuals, but no increased susceptibility to microbial infections was observed [18–20]. Our present findings create a meaningful link between the observations on alternatively activated macrophages and FXIII-A in wound healing. It is reasonable to predict that the local source of FXIII-A in healing wounds is the alternatively activated macrophage population.

The interpretation of our findings is limited by the fact that presently we do not know much about the regulation of FXIII-A gene expression. Further studies are required to analyse how the alternative activation pathway is linked to the regulation of FXIII-A gene expression. On the basis of our findings, it is important to emphasize that the opposite regulation of FXIII-A gene expression in the classical and alternative macrophage activation pathways can be utilized in diagnostic histopathology, considering FXIII-A expression as a marker of alternatively activated mobile or fixed macrophages. Histopathological studies to test this hypothesis are now in progress in our laboratory.

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