Granulocyte colony-stimulating factor (G-CSF) plays an important role in immune complex-mediated arthritis

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Keywords: G-CSF, K/BxN serum-transfer arthritis model, immune-complex driven arthritis, neutrophils, chemokines

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/eji.201546185.

List of abbreviations

AUC = Area under the curve

CIA = Collagen-induced arthritis

CCL = Chemokine (C-C motif) ligand

CXCL = Chemokine (C-X-C motif) ligand

G6PI = Glucose-6-phosphate isomerase

Hi = High

i.p. = intraperitoneal

Lo = Low

RA = Rheumatoid arthritis

rIgG = rat IgG

SEM = Standard error of the mean

SSC = Side scatter

STA = Serum-transfer arthritis

Abstract

Neutrophils are an abundant cell type in many chronic inflammatory diseases such as rheumatoid arthritis (RA); however, their contribution to the pathology of RA has not been widely studied. A key cytokine involved in neutrophil development and function is granulocyte-colony stimulating factor (G-CSF). In this study we used the K/BxN serum-transfer arthritis (STA) model, mimicking the effector phase of RA, to investigate the importance of G-CSF in arthritis development and its relation to neutrophils. Here, we show for the first time in this model that G-CSF levels are increased both in the serum and in inflamed paws of arthritic mice and importantly that G-CSF blockade leads to a profound reduction in arthritis severity, as well as reduced numbers of neutrophils in blood. Moreover, CXCL1 and CXCL2 levels in the arthritic joints were also lowered. Our data demonstrate that G-CSF is a pivotal driver of the disease progression in the K/BxN STA model and possibly acts in part by regulating neutrophil numbers in the circulation. Therefore, our findings suggest that G-CSF might be a suitable target in RA, and perhaps in other immune complex-driven pathologies.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterised by chronic inflammation of the distal joints. The affected joints are characterised by synovial hyperplasia and extensive infiltration of inflammatory cells, such as neutrophils, macrophages, fibroblasts, T cells and dendritic cells (DCs), into the synovial and peri-articular regions [1]. Complement deposition, high levels of pro-inflammatory cytokines and remodelling of the cartilage and bone are found in the affected joints [1]. Until recently the contribution of neutrophils to the pathology of RA was thought to be mainly through the release of cytotoxic products [2]. However, it has been shown that neutrophils can modulate the inflammatory response and regulate the function of other immune cells [3]. RA synovial fluid neutrophils potentially secrete an array of cytokines and chemokines and can also express MHC class II molecules and hence present antigen to T cells [2]. Evidence also suggests that some therapeutic drugs, such as methotrexate and tumor necrosis factor (TNF) inhibitors, downregulate neutrophil activation, migration and function which are associated with clinical improvement in disease activity [4-6]. Thus, targeting neutrophil-associated pathways could potentially represent a novel therapeutic strategy for RA.

Granulocyte-colony stimulating factor (G-CSF) is the major regulator of granulocyte production and can be produced by bone marrow stromal cells, endothelial cells, macrophages, chondrocytes and fibroblasts in response to inflammatory stimuli such as IL-1 and TNF [7, 8]. G-CSF acts through the G-CSF receptor (G-CSFR) which is expressed on early myeloid progenitors and mature neutrophils [9]. G-CSF is also tightly regulated by IL-17 released from tissue-resident γδ T cells, natural killer (NK) T cells and $CD4^{+}$αβ T cells

[10, 11]. During an inflammatory response neutrophils are mobilised from the bone marrow to the circulation by G-CSF indirectly by inducing the release of the neutrophil chemoattractants, CXCL1 and CXCL2, from bone marrow-resident cells [12]. In addition to promoting the egress of neutrophils from the bone marrow, CXCL1 and CXCL2 are also released locally in an inflamed tissue and can potentially attract neutrophils to such tissue [12].

In the clinic, G-CSF has been widely used to treat neutropenia associated with chemotherapy and to mobilise hematopoietic stem cells for transplantation [13]. Moreover, elevated levels of G-CSF have been found in the serum and synovial fluid of RA patients and correlate with disease activity and severity [14] which suggests that G-CSF might play a role in the pathogenesis of RA. We showed previously that G-CSF can exacerbate arthritis when injected into mice which are sub-optimally primed to develop collagen-induced arthritis (CIA) [15]. Arthritis was ameliorated upon G-CSF neutralisation/deletion in two T cell-dependent models [16, 13] _ENREF_6. We have shown that G-CSF is upregulated both locally and systemically in hapten-induced skin inflammation and that G-CSF blockade suppressed the inflammatory response [17].

In the K/BxN serum-transfer arthritis (STA) model, serum from arthritic K/BxN mice induces a synchronized, joint-specific inflammatory reaction in normal mice which is driven by autoantibodies against the ubiquitously expressed glucose-6-phosphate-isomerase (G6PI) [18, 19] and which is independent of B- and T cells [20]. The arthritic manifestations in this model are similar to RA and it is a useful one to study the effector phase of arthritis [21]. The innate immune system, including neutrophils [22], a blood monocyte subpopulation [23], mast cells [24], Fcγ receptor [25] and C5a [25], have all been shown to be crucial to the development of this arthritis. We found that blockade of granulocyte macrophage-colony stimulating factor (GM-CSF) could suppress arthritis severity [26].

In the current study, we investigated for the first time whether G-CSF is involved in the pathogenesis of the K/BxN STA model and additionally examined the association between G-CSF activity and neutrophils. We found that G-CSF is a key driver of this arthritis and its blockade, in addition to arthritis abrogation, also reduced neutrophil numbers in the circulation and chemokine levels in the joint tissue.

Results

G-CSF, CXCL1 and CXCL2 levels are increased in the K/BxN serum-transfer arthritis model

Seeing that neutrophils have been implicated in the K/BxN STA model we chose to measure some mediators based on their involvement in neutrophil function (G-CSF, CCL5, CXCL1, and CXCL2); we also measured other mediators due to their known role in the K/BxN STA model (CXCL1, CXCL2, IL-1β and TNF) [27, 28] and on their general involvement in inflammatory responses (IFNγ, CXCL10, IL-10 and IL-4). We found that the levels in the paws of only G-CSF and the two neutrophil chemoattractants, CXCL1 and CXCL2, were significantly increased (figs. 1A-C). CCL5, IL-1β, TNF, IFNγ, CXCL10, IL-10 and IL-4 were either not detectable or found at levels not significantly different from those in control mice (data not shown). As depicted in figs. 1A-C, paw levels of G-CSF, CXCL1 and CXCL2 all rose by day 4, the earliest time point examined, and decreased at days 7 and 10. The levels in naive mice are included by way of a comparison.

G-CSF is known to be produced locally in inflamed tissue and thereafter be released into the circulation leading to neutrophil mobilisation from the bone marrow or even affecting their numbers in the bone marrow [12]. We therefore measured G-CSF in the serum of arthritic mice over a 10 day period after the first serum treatment and, as can be seen in fig. 1D, G-CSF serum levels were significantly increased at day 2, peaking at around days 3-4.

G-CSF plays an important role in the K/BxN serum-transfer arthritis model

After having demonstrated increased levels of G-CSF both locally and systemically in the K/BxN STA model, we next investigated its importance for arthritis development. G-CSF activity was blocked by injecting anti-G-CSF monoclonal antibody (mAb) one day prior to the first serum treatment (i.e. day -1). Control mice were treated with either rIgG1 (isotype control) or with phosphate buffered saline (PBS). Figs. 2A-D show paw swelling and clinical score over a 10 day period after the first serum treatment (A+C) and summarized as area under the curve (AUC) (B+D). For the paw swelling there was an earlier onset for the rIgG1-treated group compared with the PBS control; however, by day 7 the degree of swelling was similar between the two control groups. There was no difference in clinical score between these two control groups. However, paw swelling and clinical score were significantly decreased in mice treated with anti-G-CSF mAb, both when compared with its isotype control as well as to the PBS-treated group. At days 3 and 7, the numbers of neutrophils and monocytes were measured in peripheral blood. In the FACS analysis we used high expression of CD88 as an additional marker to LyG/C (Gr-1) to identify neutrophils since neutrophils, as opposed to monocytes, have been shown to express high levels of CD88 [29]. Fig. 2E shows a representative FACS plot of the neutrophil population, defined as $CD45^{+}TCR\beta^{-}CD19^{-}Ly6G/C^{+}CD11b^{+}CD88^{hi}$ cells. From figs. 2F+G it can be seen that G-CSF blockade results in a reduced number of circulating neutrophils compared with both the PBS- and the rIgG1-treated groups, consistent with G-CSF being capable of mobilising neutrophils into the circulation [12]. The neutrophil numbers in the PBS-treated (control) group in figs. 2F+G are similar to those found in naive mice (data not shown). For monocytes, defined as $CD45^{+}TCR\beta^{-}CD19^{-}Ly6G/C^{+}CD11b^{+}CD88^{lo}$ cells, anti-G-CSF mAb lowered also their numbers at day 7 (figs. 2H+I), suggesting that in this arthritis model G-CSF can also mobilise monocytes from the bone marrow for which there is evidence [30]. The above data indicate that G-CSF is an important mediator in K/BxN STA model possibly in part by regulating neutrophil numbers (see Discussion).

Blockade of G-CSF results in reduced CXCL2 in inflamed K/BxN serum-transfer arthritis joints

We next investigated whether G-CSF blockade would affect CXCL1 and CXCL2 levels in the inflamed joint. CXCL1 and CXCL2 were measured in homogenates of inflamed paws from mice treated with anti-G-CSF mAb, rIgG1, and PBS, at day 10 after the first serum injection. From figs. 3A+B it can be seen that CXCL1 and CXCL2 levels after G-CSF blockade were indeed suppressed when compared with PBS treatment. When compared with the isotype control (rIgG1), treatment with anti-G-CSF mAb also led to lower levels of CXCL2 with a trend towards lower CXCL1.

These results indicate that G-CSF blockade, in addition to reducing the number of neutrophils in peripheral blood, also leads to reduced levels in the joints of CXCL2 and potentially CXCL1 in the K/BxN STA model (see Discussion).

Depletion of neutrophils inhibits progression in the K/BxN serum-transfer arthritis model

Previously, it has been shown that depletion of neutrophils in the K/BxN STA model with anti-Ly6G/C (Gr-1) mAb (clone RB6.8C5) results in complete abrogation of arthritis [22]; however, this antibody not only depletes neutrophils but also other Gr-1^+ cells including a subset of monocytes [31]. The more specific anti-Ly6G mAb (clone 1A8) has also been successfully tested in the K/BxN STA model in a study designed to investigate Ly6G function and not neutrophil depletion *per se* [32]. Since anti-G-CSF mAb lowers circulating neutrophil numbers we compared its efficacy in the K/BxN STA model to that found following specific neutrophil depletion with the anti-Ly6G mAb. Briefly, C57BL/6 mice were injected with serum from K/BxN mice on days 0 and 2, whereupon arthritis, monitored by swelling and redness of paws, developed from day 1 and peaked on day 10. The depleting Ly6G antibody and its

isotype control, rIgG2a, were administered on days -1 and 2, respectively. Additionally, groups were treated with anti-Ly6G/C mAb and its isotype control, rIgG2b, respectively while a group treated with PBS at days -1 and 2 served as a negative control. A multiple dosing protocol has previously been used to deplete neutrophils in this model by Wipke *et* al. (2001) [22]. At days 1 and 7 after KBxN serum treatment, cell depletion was monitored by flow cytometry on blood samples. With this approach neither Ly6G nor LyG/C could be used as surface markers to identify neutrophils; instead neutrophils were gated as $CD45^{+}TCR\beta^{-}CD19^{-}CD11b^{+}$SideScatter$(SSC)^{hi}$ cells [33] with $CD45^{+}TCR\beta^{-}CD19^{-}CD11b^{+}SSC^{lo}$ cells most likely being monocytes [33]. Two days after the first treatment with the depleting antibody (i.e. day 1), the absolute number of neutrophils was significantly decreased compared with the control group (fig. 4A). By comparison, the anti-Ly6G/C mAb depleted neutrophils more efficiently than the anti-Ly6G mAb (fig. 4A). Furthermore, it was confirmed [31] that the anti-Ly6G/C mAb, in addition to depleting neutrophils, also depleted a fraction of the monocytes (fig. 4B). Similar results were obtained at day 7 for the relative effects of both depleting antibodies (figs. 4C+D). Representative FACS plots of populations of neutrophils and monocytes from each of the different groups are shown in fig. 4E.

Arthritis progression was measured as paw swelling and as a clinical score at 10 days after the first serum treatment. Figs. 5A-D depict that the anti-Ly6G mAb significantly suppressed arthritis development as assessed by both parameters (figs. 5A+B and 5C+D, respectively) when compared with its isotype control and to the PBS-treated group. The data for neutrophil depletion with the anti-Ly6G mAb are similar to what we found above after neutralisation of G-CSF. A trend towards a more effective suppression by the anti-Ly6G/C mAb compared with anti-Ly6G mAb was observed.

Neutrophil depletion lowers the levels of CXCL1 and CXCL2 in K/BxN serum-transfer arthritis joints

After having demonstrated an upregulation of CXCL1, CXCL2 and G-CSF in the arthritic paws in the K/BxN STA model and, based on the fact that neutrophils may participate in their own recruitment via the local release of both CXCL1 and CXCL2 in the joint [28], we examined if neutrophil depletion led to lower levels of joint CXCL1 and CXCL2, as well as of G-CSF. On day 7 homogenates of arthritic paw tissue from mice treated with anti-Ly6G mAb, rIgG2a, and PBS, respectively, were analysed. From figs. 6A+B it is evident that treatment with the anti-Ly6G mAb resulted in significantly reduced levels of CXCL1 when compared with its isotype control, rIgG2a, as well as a significant suppression of CXCL2 when compared with the PBS-treated mice. Mice treated with anti-Ly6G/C mAb had lower levels of both CXCL1 and CXCL2, both when compared with mice treated with PBS or the isotype control, rIgG2b (figs. 6A+B). As can be seen in fig. 6C, G-CSF could be detected in inflamed tissue but was not significantly reduced after treatment with either mAb.

On day 10 anti-Ly6G mAb treatment led to significantly diminished levels of CXCL1 when compared both to PBS and rIgG2a (fig. 6D) and significantly reduced levels of CXCL2 when compared with rIgG2a (fig. 6E). Levels of both CXCL1 and CXCL2 in inflamed paws were again found to be significantly decreased in the mice treated with anti-LyG/C mAb when compared with both the PBS- and rIgG2b-treated mice (figs. 6D+E).

Overall these results suggest that neutrophils are involved in the induction of CXCL1 and CXCL2 in the K/BxN STA model.

Discussion

Increased levels of G-CSF, in addition to CXCL1 and CXCL2, were found in homogenates of inflamed whole paws and its levels rose early in the response and with similar kinetics to the two chemokines. We also measured G-CSF levels in the serum during arthritis progression and found them to be significantly increased at days 2, 3, 4 and 7 correlating with the onset

of arthritis. In the murine collagen-induced arthritis model, non-hematopoietic cells, such as synovial fibroblasts, endothelial cells and chondrocytes [7], have been shown to be the major sources of G-CSF [13]. In the K/BxN STA model we hypothesize that very early in the induction of arthritis, cells in the joint release G-CSF in response to inflammatory mediators such as IL-1β and TNF [34, 8], which have been linked with this model [27]. G-CSF is then released from the joint into the blood stream [35]. This is the first study to show increased levels of G-CSF both systemically and locally during progression of arthritis in the K/BxN STA model.

G-CSF neutralisation/deletion has been shown to be effective in ameliorating inflammatory arthritis in two lymphocyte-dependent models [16, 13]. We extended these observations by demonstrating that G-CSF neutralisation is also effective in an immune complex-driven, lymphocyte-independent model. Our findings support the concept that G-CSF might be a suitable target in RA [16, 13] and perhaps in other immune complex-driven pathologies. Furthermore, the results presented here also support our previous data investigating the role of G-CSF in a hapten-induced inflammation model where we showed that G-CSF levels are increased both locally in the inflamed tissue and systemically during the inflammatory response. We further showed before that blockade of G-CSF led to a reduced ear-swelling response and lower number of neutrophils in peripheral blood [17]. These findings together highlight the potential important role of G-CSF not only in arthritis but also in other inflammatory conditions.

One of the effects of G-CSF is to mobilise neutrophils from the bone marrow [7]; however, it is unknown whether the dramatic effect of G-CSF-blockade on arthritis progression shown above can be entirely explained by the reduction in neutrophil number observed or whether G-CSF has other functions in this model. We found that blockade of G-CSF led to reduced expression of both CXCL1 and CXCL2 in the inflamed paws. Whether this is an indirect effect caused by, for example, reduced infiltration of neutrophils or whether G-CSF can

directly stimulate release of CXCL1 and CXCL2 from cells in the joint is currently unknown. In this context it has been shown that G-CSF stimulates release of CXCL1 and CXCL2 from bone marrow cells [36]; also endothelial cells express G-CSFR [37] and are capable of secreting CXCL1 and CXCL2 [38] suggesting that G-CSF might stimulate CXCL1 and CXCL2 formation by this cell type. It has been described that, apart from neutrophil mobilisation from the bone marrow, G-CSF also can have pro-inflammatory activities such as modulating leukocyte adhesion molecules [39, 40], enhancing angiogenesis [41], inducing other neutrophil chemoattractants, such as CXCL5 and CXCL6 [39], and prolonging neutrophil survival [42, 43]. Additionally, it has been found that G-CSF increases expression of CD44 [16] and CD11b [13] on neutrophils but decreases their CD62L (L-selectin) expression [13] with implications for a role for G-CSF in regulating neutrophil trafficking into inflamed tissue. Indeed, based mainly on data obtained following G-CSF administration, it has been suggested in the murine collagen-induced arthritis model that G-CSF may have a role in the promotion of neutrophil trafficking [13]; there could be such a role for G-CSF in the K/BxN STA model although there could also be a role in regulating blood monocyte function in this model [23] as indicated above. Dose-response studies with anti-G-CSF mAb would be of interest to address whether G-CSF has other functions, such as local neutrophil trafficking or neutrophil activation, in addition to controlling systemic neutrophil numbers. Additionally, an analysis of the links between G-CSF and other cytokines implicated in this model, such as IL-1β [27], TNF [27] and GM-CSF [26], would also be of interest.

The receptor for CXCL1 and CXCL2, namely CXCR2, together with CCR1, have been reported to act non-redundantly to mediate all neutrophil chemokine activity in the K/BxN STA model [28]. It has been suggested in this model that CXCL1 is produced by resident joint cells, especially endothelial cells, in response to IL-1β released by neutrophils infiltrating the joint [28]; CXCL2, on the other hand, was demonstrated to be produced by neutrophils in the joint and not by resident tissue cells. Thus, neutrophils can potentially participate in their own recruitment by directly or indirectly mediating the release of CXCL1

and CXCL2 [28]. When analysing levels of CXCXL1 and CXCL2 after neutrophil depletion we found them both to be significantly reduced, data consistent with a role for neutrophils in their expression.

This study confirmed that neutrophils are a crucial cell type in the K/BxN STA model and provided a direct comparison between the effects of specific neutrophil depletion by the anti-Ly6G mAb and anti-G-CSF mAb blockade. The anti-Ly6G/C (Gr-1) mAb (clone RB6.8C5) has been used traditionally to deplete neutrophils, but which more recently has been shown also to deplete some monocytes [31]. We here confirmed that the anti-Ly6G mAb reduces only neutrophils in the blood while anti-LyG/C mAb, in addition to lowering neutrophil numbers, also reduces blood monocytes in the K/BxN STA model. We found only a partial neutropenia with anti-Ly6G mAb which was associated with a significant arthritis inhibition while complete depletion of neutrophils and a partial depletion of monocytes after treatment with anti-Ly6G/C mAb led to a particularly effective suppression. These results confirm the essential role of neutrophils in this model [32, 22] but additionally highlight the differences between the two antibodies [31]. The data with the anti-Ly6G/C mAb suggest that $Ly6C^+$ blood monocytes are also playing a part. Very recently it has been reported that the $Ly6C^-$ blood monocyte subpopulation is both necessary and sufficient for the initiation and propagation of the K/BxN STA model although an important role for neutrophils and $Ly6C^+$ monocytes could not be dismissed [23]; a role for CSF-1-independent macrophages has also been claimed [44, 45]. However, the anti-Ly6G/C mAb may also deplete other cell types such as plasmacytoid DCs [46].

Taken together, our data demonstrate that G-CSF is a pivotal driver of the disease progression in the K/BxN STA model. The similar data obtained for reduced severity, reduced CXCL1/CXCL2 levels, and lower neutrophil numbers upon both G-CSF neutralisation and neutrophil depletion suggest that G-CSF acts in part by regulating

neutrophil numbers in the circulation and hence in the inflamed joint. This study also emphasises the potential for targeting G-CSF and neutrophils in inflammatory arthritis.

Materials and methods

Induction of arthritis

For induction of arthritis, 50 µl K/BxN serum + 150 µl PBS were injected intraperitoneally (i.p.) into 8-10 week old male C57BL/6 mice (from Walter and Elizabeth Hall Institute of Medical Research, Parkville, Australia) on days 0 and 2. A group of control mice were administered 200 µl PBS i.p. at the same time. On days 0-10 after the first serum transfer, arthritis was assessed by a clinical score given to all four paws as well as a measurement of paw swelling in rear paws [47, 26]. The clinical score was given based on the degree of swelling and joint distortion and each paw was scored from 0-4 as follows: 0) normal, 1) slight swelling and/or erythema, 2) moderate swelling, 3) extensive swelling, 4) joint distortion and/or rigidity. The maximum score per mouse was 16. The experiments were all approved by the Ethical Review Committee at Novo Nordisk, as well as by the University of Melbourne, Animal Ethics Committee (id: 1212506.1.)

Measurement of inflammatory mediators in inflamed paws

Where indicated, mice were euthanized and the left and right rear paws removed, snap-frozen in liquid nitrogen and stored at -80°C until use. After being thawed each paw was weighed and placed on ice in 1.25 ml buffer (0.9% saline with 0.01% Triton X-100 (Sigma, St Louis, MO, USA) + a protease inhibitor cocktail tablet (Complete EDTA-free, Roche, Basel, Switzerland)) [17]. While kept cold the paws were subsequently homogenised using a disperser (IKA ultra turrax T25 basic, IKA®-Werke GmbH & Co, Staufen, Germany) with the disperser element (IKA 1024200 S25 N – 8G, IKA®-Werke GmbH & Co). Homogenates were then centrifuged for 15 min. for 10000 g at 4˚C and the supernatants centrifuged once more (15 min. for 10000 g at 4˚C) before being frozen at -80°C [17]. For the multiplex analysis, supernatants were analysed with MILLIPLEX MAP Mouse Cytokine/Chemokine Panel (Millipore, Billerica, MA, USA) by the Luminex detection method for the following analytes: IFNγ, IL-10, IL-1β, IL-4, CXCL10, CXCL2, TNFα, CXCL1, CCL5 and G-CSF. To measure G-CSF levels in serum and tissue, as well as levels of CXCL1 and CXCL2 in inflamed paws, mouse ELISA kits (R&D systems, Minneapolis, MN, USA) were used according to the manufacturer's recommendations.

Preparation of serum samples

Serum samples were prepared on days 1, 2, 3, 4, 7 and 10 after the first K/BxN serum treatment. Briefly, blood samples were taken by puncture of the submandibular vein with a lancet and 100 µl of blood collected into an Eppendorf tube (Eppendorph, Hamburg, Germany). The samples were left at 4°C overnight to clot and stored at -80°C.

Blockade of G-CSF activity

G-CSF activity was blocked by i.p. injection of a rat mouse anti-G-CSF mAb (clone 67604, IgG1, R&D systems) at a dose of 0.5 mg/mouse one day prior to the first K/BxN serum treatment (i.e. day -1) as previously described in [48]. As negative controls, one group of mice was treated i.p. with 0.5 mg/mouse of rat IgG1 (rIgG1, clone HRPN, BioXcell) and

another group with 200 µl PBS. On days 3 and 7, blood analysis was performed by flow cytometry to quantify the absolute number of neutrophils in the circulation as below but included the following anti-mouse mAbs: CD45-Efluor450 (eBiosciences), TCRβ-PECy7 (Biolegend), CD19-PerCP-Cy5.5 (BD Biosciences), CD88-PE (Biolegend), Ly6G/C-FITC (BD Biosciences) and CD11b-APC-Cy7 (Biolegend).

Neutrophil depletion

To deplete neutrophils the antibodies, rat anti-mouse Ly6G mAb (clone 1A8, IgG2a, BioXcell, West Lebanon, NH, USA), as well as the rat anti-mouse Ly6G/C (Gr-1) mAb (clone RB6.8C5, IgG2b, BioXcell), were injected at 1 mg/mouse on days -1 and 2 in relation to the first K/BxN serum treatment. The isotype control for anti-Ly6G mAb, rat IgG2a (rIgG2a, clone: 2A3, BioXcell), and the isotype control for anti-Ly6G/C mAb, rat IgG2b (rIgG2b, clone: LTF-2, BioXcell), were injected at the same doses at the same time points and served as negative controls. Additionally, a group was treated with 200 µl PBS on days -1 and 2 and additionally served as a negative control. On days 1 and 7, neutrophil depletion was confirmed by flow cytometry. Briefly, 100 µl of blood was collected into Eppendorf tubes containing 10 µl of heparin (5000U). Subsequently, each blood sample was re-suspended in 4 ml of cold ACK-lysing buffer to lyse red blood cells and incubated on ice for 5 min. To stop the reaction, cold PBS was added and samples centrifuged. Thereafter samples were blocked with anti-CD32/CD16 mAb (Fc block, BD Biosciences, San Jose, CA, USA) for 10 minutes and surface-stained with the following anti-mouse mAbs: CD45-Efluor450 (eBiosciences, San Diego, CA, USA), TCRβ-PECy7 (Biolegend, San Diego, CA, USA), CD19-PerCP-Cy5.5 (BD Biosciences) and CD11b-APC-Cy7 (Biolegend) before being analysed. To estimate absolute number of cells, BD Trucount beads (BD Biosciences) were included in the flow cytometric analysis [17]. We choose to measure and show absolute neutrophil number instead of neutrophil percentage since percentage would be misleading when part of the cell population is depleted.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism version 6 (GraphPad Software, Inc., La Jolla, CA, USA). To estimate the total response over time for each animal, data were summarised in a composite area under the curve (AUC) score, both for the clinical score and for the paw swelling. To compare the AUC of the clinical score between the different groups, the non-parametric statistical test, Kruskal-Wallis test with Dunn's correction for multiple comparisons, was used. When comparing AUC for paw swelling between the different groups, as well as levels of protein in the different groups, the parametric test, one-way ANOVA with Bonferroni's correction, was applied.

Acknowledgements

The authors wish to thank Melody Chang, University of Melbourne, for assistance with the animal work and Cynthia Louis, University of Melbourne, for assistance with the FACS analysis. The research is the result of a PhD project funded by the Novo Nordisk & Life In Vivo Pharmacology Centre (LIFEPHARM), Denmark (to A.D. Christensen). C.H. is employed by Novo Nordisk A/S. This work was also supported by a Senior Principal Research Fellowship (to J.A.H.) and a Project Grant (to A.D. Cook) from the National Health and Medical Research Council of Australia.

Conflict of interest

A.D.Christensen and C.H. are employees at Novo Nordisk A/S. The authors declare that they have no non-financial conflicts of interest.

Authors' contribution

A.D.Christensen participated in design of the study, carried out the experimental work and drafted the manuscript. C.H. participated in design and coordination of the study and helped draft the manuscript. A.D.Cook participated in design and coordination of the study and helped draft the manuscript. J.A.H. provided intellectual support and helped draft the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1: Levels of G-CSF, CXCL1 and CXCL2 in arthritic tissue and of G-CSF in serum during K/BxN serum-transfer arthritis.

Mice were injected with serum from arthritic K/BxN mice on days 0 and 2 while naive control mice were injected with PBS on the same days. At days 4, 7 and 10 after the first serum treatment, homogenates of arthritic paws were prepared and analysed for their content of G-CSF, CXCL1 and CXCL2 by a multiplex analysis. At days 1, 2, 3, 4, 7 and 10 after the first serum treatment, serum samples were taken and analysed for G-CSF by ELISA. ***A:*** *Tissue levels of G-CSF.* ***B:*** *Tissue levels of CXCL1.* ***C:*** *Tissue levels of CXCL2.* ***D:*** *Serum levels of G-CSF. (A-D) Each dot represents an individual mouse and mean is depicted for each group, n=5-6/group from one experiment performed, *p<0.05, **p<0.01 and ***p<0.001 vs naive controls (one-way ANOVA followed by Bonferroni's correction for multiple comparisons).*

Figure 2: Effect of G-CSF neutralisation on K/BxN serum-transfer arthritis progression and systemic neutrophil number.

Mice were injected i.p. with 0.5 mg/mouse anti-G-CSF monoclonal antibody (mAb), 0.5 mg/mouse rat IgG1 (rIgG1, isotype control) or phosphate-buffered saline (PBS), respectively, one day prior to the first K/BxN serum treatment (i.e. day -1). Mice were injected with serum from arthritic K/BxN mice on days 0 and 2 and progression of arthritis was followed by measuring paw swelling and assigning a clinical score for 10 days after the first serum treatment. ***A:*** *Paw swelling after the first serum treatment.* ***B:*** *Paw swelling summarized as area under curve (AUC).* ***C:*** *Clinical score after the first serum treatment.* ***D:*** *Clinical score summarized as AUC. On days 3 and 7 blood samples were taken, stained for relevant markers and analysed by flow cytometry. Absolute numbers of neutrophils and monocytes were quantified using BD Trucount beads and neutrophils were defined as* $CD45^+TCR\beta^-CD19^-Ly6G/C^+CD11b^+CD88^{hi}$ *cells while monocytes were defined as* $CD45^+TCR\beta^-CD19^-Ly6G/C^+CD11b^+CD88^{lo}$ *cells. (A-D) Data are depicted as mean ±SEM, n=10/group from one experiment performed.* ***E:*** *Representative FACS plots showing the neutrophil- and monocyte population, respectively, in a rIgG1-treated control mouse (upper) as well as in a mouse treated with anti-G-CSF mAb (lower) on day 7. Cells shown in the plot are* $CD45^+TCR\beta^-CD19^-Ly6G/C^+$*. Plots are representative of one experiment performed.* ***F:*** *Number of neutrophils/ml blood on day 3.* ***G:*** *Number of neutrophils/ml blood on day 7.* ***H:*** *Number of monocytes/ml blood on day 3.* ***I:*** *Number of monocytes/ml blood on day 7.*

(F-I) *Each dot represents an individual mouse and mean is depicted for each group Data are representative of one experiment performed with n=10/group. p<0.05, **p<0.01 and ***p<0.001 for the following comparisons: anti-G-CSF vs. PBS, anti-G-CSF vs. rIgG1 and PBS vs. rIgG1. (A+B+F-I: one-way ANOVA followed by Bonferroni's correction for multiple comparisons, C+D: Kruskal-Wallis test with Dunn's correction for multiple comparisons*).

Figure 3: Impact of G-CSF neutralisation on CXCL1 and CXCL2 levels in arthritic tissue

Mice were injected i.p. with 0.5 mg/mouse anti-G-CSF monoclonal antibody (mAb), 0.5 mg/mouse rat IgG1 (rIgG1, isotype control) or phosphate-buffered saline (PBS), one day prior to the first K/BxN serum treatment (i.e. day -1). Mice were injected with serum from arthritic K/BxN mice on days 0 and 2 and arthritis progression was followed by measuring paw swelling and assigning a clinical score for 10 days after the first serum treatment. On day 10 after the first serum transfer homogenates of arthritic paws were prepared and analysed for their content of ***(A)*** *CXCL1 and* ***(B)*** *CXCL2 by ELISA. Data are depicted as mean ±SEM, n=10/group from one experiment performed, *p<0.05, **p<0.01 and ***p<0.001*

for the following comparisons: anti-G-CSF vs. PBS and anti-G-CSF vs. rIgG1 (one-way ANOVA followed by Bonferroni's correction for multiple comparisons).

Figure 4: Neutrophil and monocyte reduction after administration of different neutrophil-depleting antibodies in the K/BxN serum-transfer arthritis model

Mice were injected with serum from arthritic K/BxN mice on days 0 and 2. Blood samples were taken on days 1 and 7 after the first K/BxN serum transfer from mice treated with either anti-Ly6G monoclonal antibody (mAb) (clone 1A8), anti-Ly6G/C (Gr-1) mAb (clone RB6.8C5), rat IgG2a (rIgG2a, isotype control for anti-Ly6G mAb), rat IgG2b (rIgG2b, isotype control for anti-Ly6G/C mAb) or phosphate-buffered saline (PBS), stained for relevant markers and analysed by flow cytometry. The depleting mAbs and isotype controls were injected i.p. at 1 mg/mouse on days -1 and 2, respectively. Neutrophils were defined as $CD45^+TCR\beta^-CD19^-CD11b^+SideScatter(SSC)^{hi}$ cells ($CD11b^+SSC^{hi}$ cells) while monocytes were defined as $CD45^+TCR\beta^-CD19^-CD11b^+SSC^{lo}$ cells ($CD11b^+SSC^{lo}$ cells). Absolute number of neutrophils and monocytes was quantified using BD Trucount beads. ***A:*** *Number of neutrophils/ml blood on day 1.* ***B:*** *Number of monocytes/ml blood on day 1.* ***C:*** *Number of neutrophils/ml blood on day 7.* ***D:*** *Number of monocytes/ml blood on day 7. For the day 1 data the control group uses data from the two isotype groups as well as from the PBS group. For the day 7 only data from the PBS group was analysed. Each dot represents an individual mouse and mean is depicted for each group. n=4-12/group from one experiment performed,* ***E:*** *Representative FACS plots for mice treated with PBS (control), anti-Ly6G mAb or anti-Ly6G/C mAb, respectively on day 1. The plots show $CD11b^+$ cells.*

**p<0.05, **p<0.01 and ***p<0.001 for the following comparisons: control vs. anti-Ly6G, control vs. anti-Ly6G/C and anti-Ly6G vs. anti-Ly6G/C (A-D: one-way ANOVA followed by Bonferroni's correction for multiple comparisons).*

Figure 5: Paw swelling and clinical score after depletion of neutrophils in the K/BxN serum-transfer arthritis model

Mice were injected with anti-Ly6G monoclonal antibody (mAb) (clone 1A8), anti-Ly6G/C mAb (clone RB6.8C5), rat IgG2a (rIgG2a, isotype control for anti-Ly6G mAb), rat IgG2b (rIgG2b, isotype control for anti-LyG/C mAb) or PBS, respectively on days -1 and 2 in relation to the first K/BxN serum treatment. The depleting antibodies and isotype controls were injected i.p.

at 1 mg/mouse. Mice were injected with serum from arthritic K/BxN mice on days 0 and 2 and arthritis progression was followed by measuring paw swelling and assigning a clinical score for 10 days after the first serum transfer. ***A:*** *Paw swelling after the first serum treatment.* ***B:*** *Paw swelling summarized as area under the curve (AUC).* ***C:*** *Clinical score after the first serum treatment.* ***D:*** *Clinical score summarized as AUC.*

Data are depicted as mean ±SEM, (A+C), n=8/group from days 0-7 and n=4/group from days 7-10 (four mice from each group of eight mice were euthanized on day 7 and the remaining four mice in each group continued until termination of the study on day 10); (B+D), n=4 (only the four mice that continued until day 10 are included in the AUC score). Data shown are representative of one experiment performed.

**p<0.05, **p<0.01 and ***p<0.001 for the following comparisons: anti-Ly6G vs. PBS and rIgG2a, anti-Ly6G/C vs. PBS and rIgG2b. (A+B: one-way ANOVA followed by Bonferroni's correction for multiple comparisons, C+D: Kruskal-Wallis test with Dunn's correction for multiple comparisons*).

Figure 6: Levels of CXCL1, CXCL2 and G-CSF in arthritic tissue after depletion of neutrophils in the K/BxN serum-transfer arthritis model

Mice were injected with anti-Ly6G monoclonal antibody (mAb) (clone 1A8), anti-Ly6G/C mAb (clone RB6.8C5), rat IgG2a (rIgG2a, isotype control for anti-Ly6G mAb), rat IgG2b (rIgG2b, isotype control for anti-LyG/C mAb) or PBS, respectively, on days -1 and 2 in relation to the first K/BxN serum treatment. The depleting antibodies and isotype controls were injected i.p. at 1 mg/mouse. Mice were injected with serum from arthritic K/BxN mice on days 0 and 2 and arthritis progression was followed by measuring paw swelling and assigning a clinical score for 10 days after the first serum transfer. On days 7 and 10 after the first K/BxN serum treatment homogenates of arthritic paws were prepared and analysed for their content of CXCL1, CXCL2 and G-CSF by ELISA. ***A:*** *CXCL1, day 7.* ***B:*** *CXCL2, day 7.* ***C:*** *G-CSF, day 7.* ***D:*** *CXCL1, day 10.* ***E:*** *CXCL2, day 10.*

*Data are depicted as mean ±SEM, n=4/group from one experiment performed, *p<0.05, **p<0.01 and ***p<0.001 for the following comparisons: anti-Ly6G vs. PBS and rIgG2a, anti-Ly6G/C vs. PBS and rIgG2b (one-way ANOVA followed by Bonferroni's correction for multiple comparisons).*