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The quinoline-3-carboxamide paquinimod (ABR-215757) reduces leukocyte recruitment during sterile inflammation: leukocyte- and context-specific effects.

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Running title: Paquinimod selectively reduces leukocyte recruitment

Abstract

Quinoline-3-carboxamides (Q-compounds) are currently in clinical development for both autoimmune disease and cancer. We have previously shown that the Q-compound paquinimod (ABR-215757) significantly ameliorates disease symptoms in several mouse models of human inflammatory disease. Considering that recruitment of inflammatory cells into tissue is a common denominator of these models, we have in this report investigated whether paquinimod would interfere with cell accumulation during sterile peritoneal inflammation. To mimic the cell recruitment elicited by tissue injury, we used necrotic cells to induce the acute inflammatory response. We show that per oral treatment with paquinimod significantly reduced the accumulation of Ly6C^{hi} inflammatory monocytes and eosinophils, but not neutrophils, in this model, and that this correlated with reduced number of such cells also in the omentum. Treatment also reduced the accumulation of these cell populations at a subcutaneous site of inflammation. In alum-induced inflammation, however, neutrophils were the dominant cell population and paquinimod failed to reduce the accumulation of inflammatory cells. Taken together, our results indicate that paquinimod selectively inhibits cell recruitment during acute sterile inflammation, but that this effect is context-dependent. These data have important implications for the understanding of the mechanism of action of Q-compounds in both pre-clinical and clinical settings.

Keywords:

Quinoline-3-carboxamide, monocyte, inflammation, peritoneum, omentum, immunomodulation

1. Introduction

Both tissue injury and infection can induce an acute inflammatory response. The application of irritants or bacterial products in the peritoneal cavity has long been used as a standard method for collecting macrophages from mice. The induction of experimental peritonitis in such settings has been widely used as a model to study the accumulation of inflammatory cells during sterile conditions.

The steady state peritoneal cavity contains a self-renewing resident macrophage population[1, 2]. These cells are activated upon injury and infection and contribute significantly to the recruitment of inflammatory leukocytes during peritoneal inflammation[3, 4]. Mesothelial cells express Toll-Like Receptors (TLRs) and are also engaged in the response by producing various inflammatory cytokines and chemokines (reviewed in[5]). The inflammatory cells entering the peritoneal cavity originate from blood vessels in milky spots in the greater omentum, mesenteric blood vessels and blood vessels at other mesothelial sites[6, 7]. In the initial phase, neutrophils and CX_3CR1^+ patrolling monocytes[8] are recruited. These cells are followed by influx of inflammatory monocytes and eosinophils. Upon induction of the inflammatory response, the resident macrophage population is reduced and this cell population is replenished by proliferation when the inflammatory response is resolved[1, 9].

Experimental peritonitis can be induced by a wide variety of stimuli such as various irritants, infection, particulate antigens and dead cells. The induction of sterile peritoneal inflammation by necrotic cells involves the stimulation of both resident macrophages and radioresistant cells[10] such as mesenchymal cells[11]. The necrotic cells will release cellular components known as Damage-Associated Molecular Patterns (DAMPs)[12, 13]. It is well established that DAMPs can bind to and stimulate both TLRs and Receptor of Advanced Glycation End products (RAGE) and thus induce a sterile inflammatory response. RAGE-deficient mice display reduced thioglycollate-induced peritonitis[14] and complementation of RAGE expression in endothelial cells reversed this phenotype. Stimulation of RAGE on endothelial cells induces VCAM-1 expression and promotes leukocyte transmigration[15-18]. Interestingly, peritonitis induced by necrotic cells is largely independent of TLRs[19]. Rather, the inflammatory response is mediated by uric acid released by necrotic

cells[20] and involves the production of IL-1α and IL-1β[10]. IL-1 production in response to cellular necrosis involves activation of the NLRP3 inflammasome[21, 22], which in turn can also be activated by monosodium urate crystals[23]. Alum adjuvant crystals can induce cell damage[24] thereby causing the release of uric acid[25]. It is known that alum adjuvant can induce IL-1β production in an NLRP3 inflammasome-dependent way[26]. However, alum can also stimulate innate immune mechanisms through NLRP3 inflammasome-independent mechanisms[27, 28]. Because of the particulate nature of alum adjuvant, it will most likely also provide more extended immune stimulation as compared to necrotic cells. These two agents would therefore be expected to induce partially different inflammatory responses.

Our laboratory, as well as other investigators, has investigated the impact of quinoline-3-carboxamides (Q-compounds) on inflammatory conditions. These compounds have shown efficacy in several mouse models of inflammatory autoimmune disease[29-33] and they are currently in clinical development for multiple sclerosis[34-37], systemic sclerosis and prostate cancer[38, 39]. Recently, the S100A9 protein was identified as one molecular target of the Q-compound paquinimod (ABR-215757)[40]. We have previously shown that paquinimod interferes with development of disease both in mouse models of multiple sclerosis[40, 41] and systemic lupus erythematosus[42]. Further, our previous work indicated that this compound interfered with the accumulation of myeloid cells during inflammation[43]. Due to the efficacy of Q-compounds in several models of inflammatory disease, we reasoned that these compounds most likely target a mechanism common to these diseases. In here, we have therefore used sterile peritoneal inflammation as a model to determine whether paquinimod would interfere with the accumulation of inflammatory cells. Our results presented in this report indicate that this is indeed the case.

2. Materials and Methods

2.1 Mice and treatment

Wild type C57Bl/6 mice were purchased from Taconic Europe (Ry, Denmark). All animal experiments were performed with the permit of the local committee on the

ethics of animal experiment of Malmö and Lund (permits M4-11 and M12-13). To study the effects of the Q-compound paquinimod, mice at the age of 7-9 weeks were treated with paquinimod dissolved in drinking water at a concentration of 140 μg/ml (corresponding to a daily dose of about 25 mg/kg body weight/day) for 24 hrs prior to any other procedures. Paquinimod was provided by Active Biotech, Lund, Sweden.

2.2 Induction of peritonitis

EG7 cells (OVA-transfected EL4 lymphoma cell line)[44] were cultured in RPMI medium (RPMI-1640 supplemented with 10% fetal calf serum, 10 mM HEPES, 1 mM sodium pyruvate, 100 U/ml penicillin-streptomycin and 50 μM β-mercaptoethanol (all supplements from Invitrogen Life Technologies, Paisley, UK)) at 37°C, 5% CO_2. The EG7 cells were obtained from Dr Clotilde Thery, Institute Curie, INSERM U932, Paris, France.

Necrosis was induced using the protocol from a previous study[19]. Briefly, the cells were harvested, washed twice with PBS (Invitrogen Life Technologies) and heat-shocked at 45°C in water bath for 10 min and subsequently incubated at 37°C for 4 hrs prior to use. Mice were injected intraperitoneally (i.p.) with 10^7 heat-shocked necrotic cells. Peritoneal cells were lavaged after 20 hrs by injection of 7 ml RPMI medium. The volume of recovered lavage solution was determined such that the total number of peritoneal cells could be calculated. Omenta were also collected. To prepare omental cells, we used the "walk-out" method previously reported by Carlow et al[45]. In brief, omenta from similar cohorts of mice were pooled and placed in wells of flat-bottom 96-well plates in RPMI medium and incubated at 37°C overnight. Cells migrating out from the omenta were collected and wells were washed with 10 mM EDTA (Millipore, Billerica, MA) to collect adherent cells.

Alternatively, mice were injected i.p. with 1 mg Imject alum (Thermo Scientific, Waltham, MA). In this setting, peritoneal cells and omenta were collected 4 hrs or 20 hrs after immunization. Peritoneal and omental cells were quantified using the Sysmex KX-21N automated hematology analyzer (Kobe, Japan).

2.3 Matrigel plugs

Growth factor-reduced matrigel, purchased from BD Biosciences (San Diego, CA), was injected subcutaneously (200 µl) in the flank. Matrigels were either substituted with PBS (3:1 vol/vol) or with PBS containing 1 mg Imject alum. Plugs were removed from mice 48 hrs later, cut into pieces with a scalpel and incubated on ice for 1 hr in cell recovery solution (BD Biosciences). Finally, pieces were mashed through a 70 µm cell strainer. The cells obtained from matrigels were quantified using AccuCount beads (Spherotech, Lake Forest, IL).

2.4 Antibodies and flow cytometry

The following antibodies were purchased from Biolegend (Nordic Biosite, Täby, Sweden): CD11b-Alexa700, CD11c-APC-Cy7, F4/80-PE-Cy7, Ly6G-FITC and I-A/I-E (MHCII)-Pacific Blue. The following antibodies were purchased from BD Biosciences: CD19-PerCP-Cy5.5, Ly6C-biotin, streptavidin-BD Horizon V500 and SiglecF-PE. CD115-APC was purchased from eBioscience (Nordic Biosite, Täby, Sweden). Cells were stained with the above antibodies in FACS buffer (PBS supplemented with 5% fetal calf serum and 0.05% NaN_3 (Sigma-Aldrich, St. Louis, MO). Propidium iodide (PI) (Invitrogen, Carlsbad, CA) was used to detect dead cells. Analysis of stained cells was performed using the LSRII flow cytometer (BD Biosciences).

2.5 Statistical analyses

Statistical analyses were performed using the Mann-Whitney *U* test.

3. Results

3.1 Paquinimod reduces accumulation of CD11b$^+$ cells during peritoneal inflammation

To study the impact of paquinimod on the recruitment of leukocytes to a site of inflammation, we used a mouse model of sterile peritoneal inflammation. The inflammation was elicited by injecting necrotic tumor cells[19] and this led to increased number of CD11b$^+$ myeloid cells in the peritoneal lavage obtained 20 hrs after immunization (Figure 1B). Similar to observations in other peritonitis models[46], immunization with necrotic cells also caused the loss of resident CD11b$^+$ F4/80$^+$ peritoneal macrophages (Figure 1A). The accumulation of CD11b$^+$ cells was

significantly reduced in paquinimod-treated mice (Figure 1B), suggesting that the compound might interfere with cell recruitment to the peritoneum during this inflammatory condition.

Peritoneal immunization with TLR agonists reduces peritoneal B cell numbers[47]. This involves CXCL13-dependent migration of B1 cells to the greater omentum[48] and may involve exit via efferent lymphatics[49], but paquinimod treatment had no effect on B cell numbers (Figure 1C). In addition, it did not interfere with the immunization-induced loss of peritoneal DCs (Figure 1C) and macrophages (Figure 1A) either. Thus, while paquinimod treatment displayed significant effects on myeloid cell populations, it did not affect the dynamics of peritoneal B cells, DCs and macrophages. Taken together, these data indicate that paquinimod selectively affects cell dynamics in this model of peritoneal inflammation. Finally, paquinimod did not significantly influence the number of steady state peritoneal CD11b^{+} cells in normal non-immunized mice (Supplementary Figure 1A), indicating that the compound does not have toxic effects on resident myeloid cells.

3.2 Paquinimod treatment reduces accumulation of inflammatory cells in the omentum

Inflammatory cells that enter the peritoneal cavity during inflammation at least partially originate from the greater omentum[6, 7]. We therefore wanted to investigate whether paquinimod would also reduce the accumulation of inflammatory cells at this site. To prepare omental cells, we used a method previously reported by Carlow et al[45]. As compared to using enzymatic treatment to release the cells from omental tissue, the cell viability and number of cells recovered is enhanced using this protocol. Omental inflammatory leukocytes, but not resident macrophages, can be efficiently recovered using this protocol[45] (Supplementary Figure 1B). However, because of the low cell recovery, we had to pool the omental cells from individual mice in the experimental groups to enable robust analysis of minor cell populations.

As shown in Figure 1B, the peritoneal immunization with necrotic cells increased the number of CD11b^{+} myeloid cells in the omentum. Importantly, paquinimod treatment reduced the number of CD11b^{+} cells also at this site. Taken together, the correlation between reduced numbers of CD11b^{+} cells in peritoneum and omentum in

paquinimod-treated mice provided further support to the hypothesis that at least part of the cells accumulating in the peritoneum may originate from the omentum.

3.3 Selective effect of paquinimod on recruitment of CD11b$^+$ subpopulations

We next investigated whether paquinimod treatment would selectively reduce accumulation of certain subpopulations of the CD11b$^+$ cells. Peritoneal immunization with necrotic cells elevated the numbers of Ly6C^{hi} inflammatory monocytes, Ly6G$^+$ neutrophils and SiglecF$^+$ eosinophils both in the peritoneum (Figure 2A, Supplemenary Figure 2) and in the omentum (Figure 2B). The reduced number of CD11b$^+$ cells in the peritoneum of paquinimod-treated mice correlated with reduced number of inflammatory monocytes and eosinophils. In contrast, paquinimod failed to significantly reduce the number of neutrophils at this site. A similar reduction of inflammatory monocytes and eosinophils was observed in the omentum of the treated mice, while there was little effect on neutrophils. Thus, in this model, paquinimod selectively reduced the accumulation of the same CD11b$^+$ subpopulations both in peritoneum and omentum.

However, the composition of the CD11b$^+$ cell population was different in peritoneum and omentum of the immunized mice (Figure 2C). Thus, while inflammatory monocytes were the dominant population in the peritoneum, these were only a minor fraction of the omental cell population in which eosinophils were dominant. This difference might indicate that the cellular composition in the peritoneum reflects the net cell accumulation over time. Cellular influx from other sources such as mesenteric vessels could also contribute to the peritoneal cell content. Nevertheless, the same CD11b$^+$ cell populations are selectively reduced in both peritoneum and omentum of the paquinimod-treated mice, suggesting a common mechanism of action of paquinimod at these two sites.

3.4 Paquinimod selectively reduces accumulation of inflammatory cells at a subcutaneous site of injury

We next wanted to elucidate whether paquinimod would also inhibit migration of leukocytes to a subcutaneous site of injury. Subcutaneously injected matrigel is a well-established model to study angiogenesis and tumor development. Shortly after the injection, monocytes are recruited to the matrigel and participate in the angiogenic

process[50-52]. This model therefore allowed us to more directly address a possible impact of paquinimod on leukocyte influx to a site of injury. As shown in Figure 3A, also in this model paquinimod treatment significantly reduced the number of myeloid cells recovered from the matrigel 48 hrs after the injection. Macrophages and inflammatory monocytes were the dominant CD11b$^+$ cell populations amongst the recovered cells (Figure 3B). Most importantly, the treatment selectively reduced the number of inflammatory monocytes and eosinophils also in this model (Figure 3A). We have previously investigated the impact of paquinimod treatment on various myeloid cell populations in the EAE model. In that model we could show that the CD115$^+$ subpopulation of the inflammatory monocytes was selectively reduced in treated mice [41]. We therefore wanted to investigate whether paquinimod treatment would reduce this particular sub-population also in the current model of subcutaneous inflammation, and we found that this was indeed the case (Figure 3A). Only very few neutrophils could be recovered, but in this model the number of these cells was significantly increased in the treated mice. We currently do not know the reason for this observation, but this effect of paquinimod appears to be peculiar to the matrigel model as it was not seen in the peritonitis model. We conclude that paquinimod selectively reduces the influx of subpopulations of CD11b$^+$ cells to sites of injury/inflammation.

3.5 Contextual effect of paquinimod in damage-induced peritoneal inflammation

Adjuvant alum displays cytotoxic activity and molecules released from the injured cells induce an inflammatory response[24, 25]. We therefore wanted to determine whether paquinimod would also reduce cell recruitment during alum-induced peritoneal inflammation. Administration of alum, similarly to necrotic cells, significantly increased the total number of peritoneal cells, the majority of which were CD11b$^+$ cells (Figure 4A and 4B). This increase was observed at both 4 hrs and 20 hrs after induction and also correlated with increased numbers of omental CD11b$^+$ cells. Neutrophils were the dominant peritoneal CD11b$^+$ population at 4 hrs after alum injection, whereas as expected, the proportion of both eosinophils and inflammatory monocytes increased at the later time point (Figure 4C). However, when compared to the response induced by necrotic cells (Figure 2C), the frequency of neutrophils was strongly elevated in the alum-induced peritoneal response still 20 hrs after induction. Thus, necrotic cells and alum induce partially distinct inflammatory responses in

peritoneum, even though both involve the release of some common mediators such as uric acid[20, 25].

When analyzing mice 4 hrs after alum injection, paquinimod treatment clearly reduced the number of CD11b$^+$ cells in the peritoneum (Figure 4A). However, unexpectedly the treatment failed to reduce the number of omental CD11b$^+$ cells in these mice. At this time point, the treatment only marginally influenced the composition of the peritoneal and omental CD11b$^+$ populations (Figure 4C, Supplementary Figure 3A). Unexpectedly, at 20 hrs after immunization, the number of CD11b$^+$ cells was elevated both in peritoneum and omentum of the paquinimod-treated mice (Figure 4B). In the peritoneum, this increase correlated with a shift in the eosinophil to neutrophil ratio of 1.7 to 0.4 (Figure 4C). This observation suggests that in alum-immunized mice, paquinimod treatment may prolong the early phase of the acute inflammatory response with dominant neutrophil influx into the peritoneum. However, in absolute numbers not only peritoneal neutrophils, but also eosinophils and inflammatory monocytes increased in these mice (Supplementary Figure 3B).

To address whether this effect of paquinimod treatment might be particular to alum-induced inflammation, we repeated the experiments shown in Figure 3, but this time we included adjuvant alum in the matrigel. The influx of neutrophils was strongly increased by the inclusion of alum (Supplementary Figure 3C) as compared to matrigel alone (Figure 3A). Similarly to the data obtained in the peritonitis model, the accumulation of CD11b$^+$ cells and in particular neutrophils was slightly elevated in matrigel plugs isolated from paquinimod-treated mice. Thus, the elevated accumulation of inflammatory cells upon paquinimod treatment (Figure 4B, Supplementary Figure 3C) appears to be a general effect of exposing mice to paquinimod in combination with alum.

4. Discussion

In this report, we show that upon sterile peritoneal inflammation induced by necrotic cells, the number of inflammatory myeloid cells was increased both in the peritoneal lavage and in the omentum. The increase in cell number at both these sites was significantly reduced in paquinimod-treated mice and correlated with the selective

loss of inflammatory monocytes and eosinophils but not of neutrophils. Further, the compound did not affect the numbers of peritoneal B cells and DCs.

Formally, the reduction in peritoneal cell number could be caused in several different ways. First, the reduced cell number could be due to reduced influx of inflammatory cells. The omentum is believed to be a major site of entry for leukocytes during peritoneal inflammation[6, 7]. Thus, the finding that paquinimod treatment had similar effects on omental and peritoneal cell numbers can be taken in support of this possibility. Second, paquinimod could selectively kill certain subpopulations of the inflammatory cells. We think this possibility is rather unlikely as elevated rather than reduced number of these cells was observed in alum-immunized mice. Further, paquinimod did not reduce the number of steady state $CD11b^+$ peritoneal cells. We also have previously reported that paquinimod treatment does not influence the production of myeloid cells in the bone marrow[41]. Third, the reduced cell number in the paquinimod-treated mice could be caused by increased efflux of recruited cells, i.e. accelerated resolution of inflammation. Monocyte-derived macrophages are known to emigrate from the peritoneum during the resolution phase of acute inflammation. This involves macrophage interaction with mesothelial cells followed by emigration via efferent lymphatics[53-55]. Also eosinophils may emigrate from the peritoneum[56]. We have not formally excluded this possibility. However, we think it is rather unlikely to be a major mechanism of action, mainly because the effect of paquinimod on inflammatory cells in peritoneum and omentum correlate well. Thus, one would then have to postulate that paquinimod enhances exit of cells both from omentum and peritoneum, which involves distinct routes, i.e. blood and lymphatic vessels, respectively.

We also administered matrigel subcutaneously in mice and analyzed the accumulation of inflammatory cells at this site. In this model, the inflammatory cells harvested from the matrigel must have been recruited from local blood vessels. Also in this case, paquinimod treatment significantly reduced the accumulation of inflammatory monocytes and eosinophils. These results provided compelling evidence that paquinimod indeed might interfere with the recruitment of inflammatory cells from blood vessels. We therefore also speculate that the efficacy of paquinimod in the peritonitis model, may also involve interference with the recruitment of inflammatory

cells from local blood vessels in omentum and potentially other peritoneal sites. Further evidence in support of this hypothesis is that the Q-compound Linomide was previously shown to reduce leukocyte extravasation in blood vessels in an *in vivo* model[57]. Thus, we conclude that paquinimod treatment reduces the recruitment of inflammatory monocytes and eosinophils to sites of inflammation and injury.

Paquinimod might potentially interfere with any stage of the complex process, which is common to the emigration of both eosinophils and inflammatory monocytes from blood vessels and subsequent recruitment into tissue. Future experiments will address whether the compound interferes with leukocyte adhesion to endothelial cells or with the transmigration mechanism *per se*.

Alum causes rapid histamine release from mast cells. This response, together with mast cell-produced IL-5, is important for eosinophil recruitment to the peritoneum[58]. Further, alum also elicits CXCL1, CCL2 and CCL11 production in the peritoneum and these chemokines recruit neutrophils, inflammatory monocytes and eosinophils, respectively[58]. As shown in this report, neutrophils were still numerous relatively late (20 hrs post induction) in the alum-induced acute peritoneal response. This was clearly distinct from the response induced by necrotic cells, in which neutrophils were only a minor population at this time point. Contrary to our expectations, paquinimod treatment in parallel cohorts of alum-injected mice enhanced the accumulation of inflammatory cells. The number of neutrophils was most prominently increased in these mice, as if the recruitment of this population of inflammatory cells was even further prolonged upon treatment with paquinimod.

We show that this enhancement was time-dependent since in the early acute response (4 hrs post induction), similarly to the effect seen in mice immunized with necrotic cells, paquinimod treatment reduced the number of peritoneal eosinophils and inflammatory monocytes. Importantly, however, also in this case the treatment failed to significantly reduce neutrophil numbers. The reducing effect of paquinimod treatment on omental and peritoneal $CD11b^+$ cell numbers correlated in the mice immunized with necrotic cells, but that correlation was not detected early in the alum-induced response. However, later in the response the treatment also enhanced the number of omental $CD11b^+$ cells well in correlation with the enhanced peritoneal cell

numbers. At present we do not know the reason for this temporal discrepancy. The data might suggest, however, that the paquinimod-induced pro-inflammatory effect at least partially could operate at the level of the omentum and that it might involve temporal accumulation of inflammatory cells at this site.

We observed a similar effect of paquinimod when adjuvant alum was inoculated subcutaneously. Thus, taken together our data reveal a contextual effect of paquinimod treatment on cell recruitment during sterile inflammation. We speculate that this differential outcome of paquinimod treatment could be a consequence of the higher and prolonged influx of neutrophils in the alum-immunized mice. As shown in this paper, irrespective of the agent used to elicit the inflammation, paquinimod treatment did not interfere with the accumulation of neutrophils. The relatively high number of neutrophils entering the peritoneum unopposed by paquinimod might cause increased tissue injury in addition to that caused by the particulate alum itself (reviewed in[59]). The increased injury might in turn prolong the acute neutrophil-dominated phase of the response resulting in a slight but transient pro-inflammatory effect. Since inflammation is a complex process with a delicate balance between competing pro-inflammatory and anti-inflammatory components already early after its induction (reviewed in[60]), manipulation with the immunomodulatory compound paquinimod might, depending on the conditions, display such opposing effects.

Taken together, these data provide important implications for the understanding of the beneficial effects of Q-compounds observed both in autoimmune disease[30, 40, 41] and cancer[61, 62]. Both autoimmune diseases and the growth of solid tumors involve inflammation mediated by myeloid cells. In both situations, myeloid cells will be recruited to tissues involved in the pathogenesis. We propose therefore that the efficacy of Q-compounds in both autoimmune disease and cancer could be due to that they interfere with recruitment of myeloid cells such as monocytes.

Disclosure statement

TL is a part-time employee and holds shares in Active Biotech AB. FI has a research grant from Active Biotech AB.

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Figure legends

Figure 1. Paquinimod treatment reduces the accumulation of CD11b^{+} cells in necrotic cell-induced peritonitis. Mice were injected i.p. with necrotic cells and one cohort of these mice was treated with paquinimod for 24 hrs prior to the injection. Mice injected i.p. with PBS served as controls. Twenty hrs after injection, peritoneal cells were collected from the mice and analyzed by FACS. To determine the frequency of resident peritoneal macrophages (gated population) amongst the collected cells, single, viable (PI^{-}), CD19^{-} cells were analyzed for expression of the CD11b and F4/80 markers (A). Omenta were also collected, pooled from individual mice in the experimental groups and omental cells prepared as described in Materials and Methods. The results shown represent calculated mean cell numbers for an individual omentum. Peritoneal (PEC) and omental (OM) cells were counted, analyzed by FACS and the number of single, viable, CD19^{-} CD11b^{+} cells calculated (B). Peritoneal MHCII^{+} CD19^{+} B cells and CD11b^{+} MHCII^{+} CD11c^{+} DCs were enumerated in a similar fashion (C). Representative results of three independent experiments are shown. n.s. = not significant, *P<0.05, **P<0.01, Mann-Whitney *U*-test.

Figure 2. Paquinimod treatment reduces the accumulation of monocytes and eosinophils in the peritoneum and omentum. Peritoneal and omental cells were prepared as in Figure 1 and analyzed by FACS. CD11b^{+} cells from mice immunized with necrotic cells or PBS (control) were subdivided into Ly6C^{hi} inflammatory

monocytes, Ly6G$^+$ neutrophils and SiglecF$^+$ eosinophils and the number of these cells calculated for the peritoneum (A). Omenta were pooled from individual mice in the experimental groups and the results shown represent calculated mean cell numbers for an individual omentum (B). The frequencies of F4/80$^+$ macrophages, Ly6C^{hi} inflammatory monocytes, Ly6G$^+$ neutrophils and SiglecF$^+$ eosinophils in the total CD11b$^+$ peritoneal (left panel) and omental (right panel) cell populations are shown (C). Representative results of two independent experiments are shown. n.s. = not significant, *P<0.05, **P<0.01, ***P<0.001, Mann-Whitney *U*-test.

Figure 3. Paquinimod treatment reduces influx of monocytes and eosinophils to matrigel plugs. Mice were treated with paquinimod for 24 hrs prior to subcutaneous injection of growth factor-reduced matrigel. Forty-eight hrs later, matrigel plugs were removed and invading cells analyzed by FACS. Single, viable, CD19$^-$ CD11b$^+$ cells were calculated as well as CD115$^+$ Ly6C^{hi} inflammatory monocytes, Ly6G$^+$ neutrophils and SiglecF$^+$ eosinophils (A). The frequency of F4/80$^+$ macrophages, Ly6C^{hi} inflammatory monocytes, Ly6G$^+$ neutrophils and SiglecF$^+$ eosinophils in the total CD11b$^+$ cell population is shown (B). Pooled data from three independent experiments are shown. *P<0.05, ***P<0.001, Mann-Whitney *U* test.

Figure 4. Paquinimod treatment increases the accumulation of cells in the peritoneum in the late phase of alum-induced peritonitis. Mice were treated with paquinimod for 24 hrs prior to i.p. immunization with Imject alum or PBS (control). Peritoneal and omental cells were prepared as in Figure 1 and analyzed by FACS 4 hrs (A) and 20 hrs (B) after immunization and the number of single, viable, CD19$^-$ CD11b$^+$ cells was calculated. Omenta were pooled from individual mice in the experimental groups and the results shown represent calculated mean cell numbers for an individual omentum. The frequency of F4/80$^+$ macrophages, Ly6C^{hi} inflammatory monocytes, Ly6G$^+$ neutrophils and SiglecF$^+$ eosinophils in the total CD11b$^+$ peritoneal cell population is shown (C). Representative results of two independent experiments are shown. n.s. = not significant, *P<0.05, **P<0.01, Mann-Whitney *U* test.

Legends to supplementary Figures

Figure S1. Normal, non-immunized mice were treated with paquinimod (see Materials and Methods, section 2.1) for 24 hrs (n=7) or for 6 days (n=7), as indicated, or left untreated (n=5; control). Peritoneal cells were collected from individual mice in each group and analyzed by FACS. The data show the absolute number of peritoneal CD11b^{+} cells from individual mice (A). Omenta from normal, non-immunized mice were collected and cells were obtained either by allowing cells to migrate out from the tissue in overnight cultures or by enzymatic degradation (See Materials and Methods, section 2.2). F4/80^{+} macrophages are efficiently obtained with enzymatic degradation but not with the overnight culture method. Ly6C^{hi} and Ly6G^{+} cells were obtained with similar efficiency using both methods (B).

Figure S2: Gating strategy used for the analyses of the FACS experiments in this report. Peritoneal cells from a mouse injected i.p. with PBS were collected and analyzed.

Figure S3: Composition of the CD11b^{+} cell population in omentum. Mice were immunized with Imject alum as in Figure 4 (A) and sacrificed 4 hrs later. Representative data of two independent experiments are shown. CD11b^{+} peritoneal cells from the mice shown in Figure 4A and 4B were subdivided into Ly6C^{hi} inflammatory monocytes, Ly6G^{+} neutrophils and SiglecF^{+} eosinophils and the absolute numbers of these cells were calculated (B). Representative results of two independent experiments are shown. Mice were treated with paquinimod for 24 hrs prior to subcutaneous injection of growth factor-reduced matrigel containing Imject alum. Forty-eight hrs later, matrigel plugs were removed and invading cells analyzed by FACS. Single, viable, CD19^{-} CD11b^{+} cells as well as Ly6G^{+} neutrophils were calculated (C). Pooled data from two independent experiments are shown. n.s. = not significant, *$P<0.05$, **$P<0.01$, ***$P<0.001$, Mann-Whitney *U*-test.

Figure 1

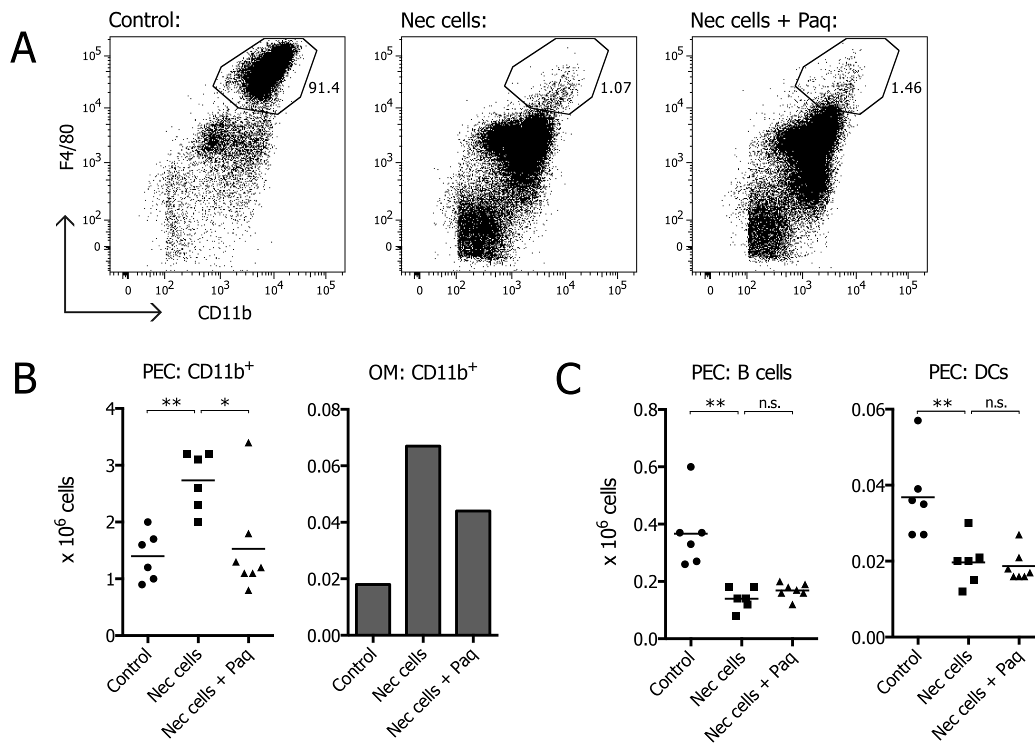

Figure 2

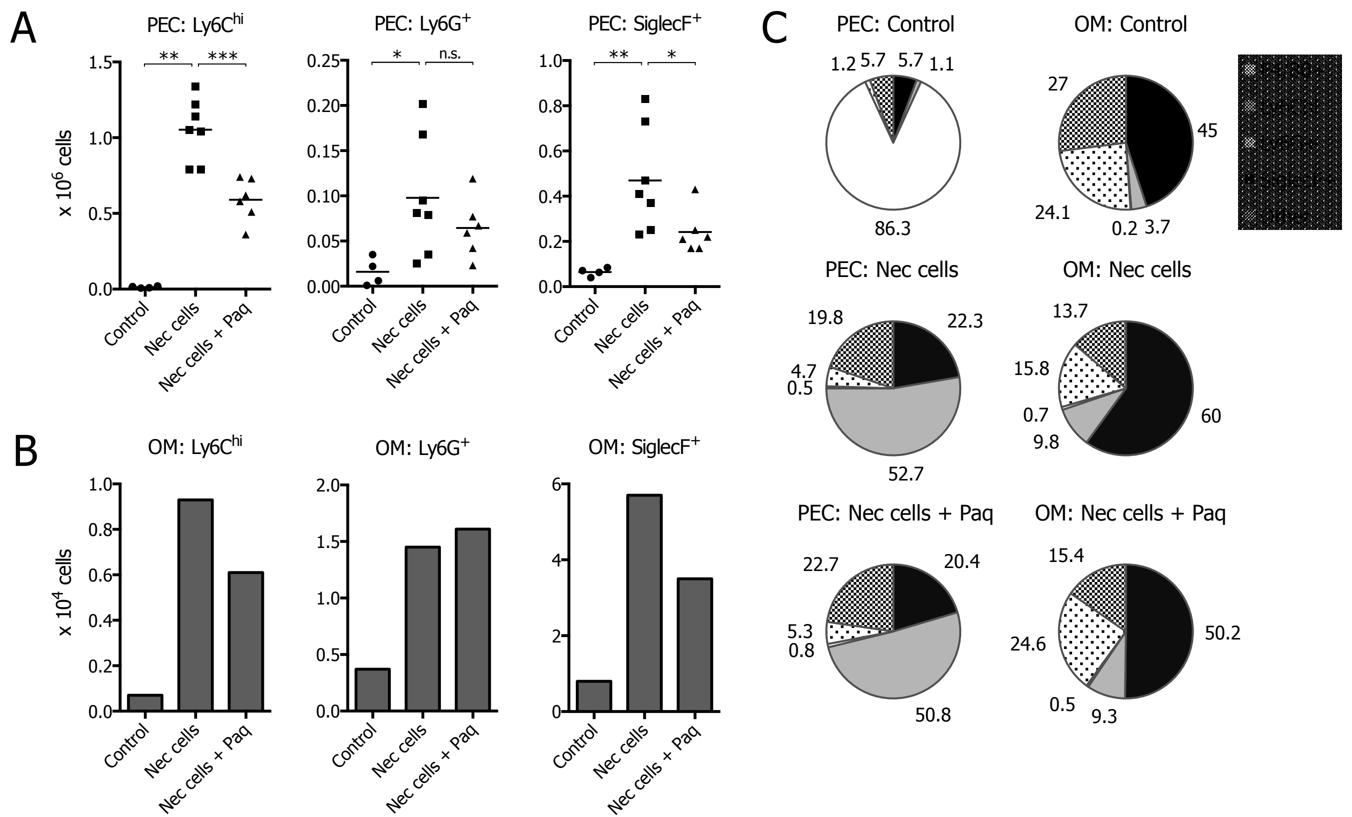

Figure 3

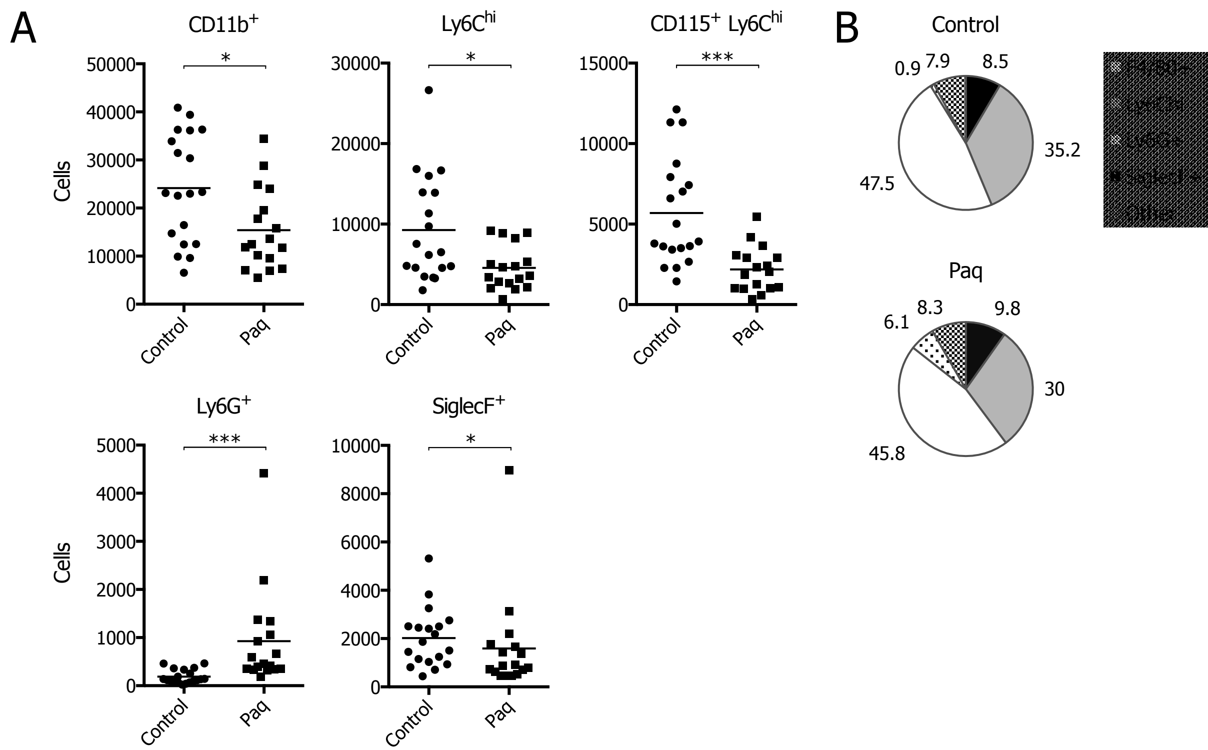

Figure 4

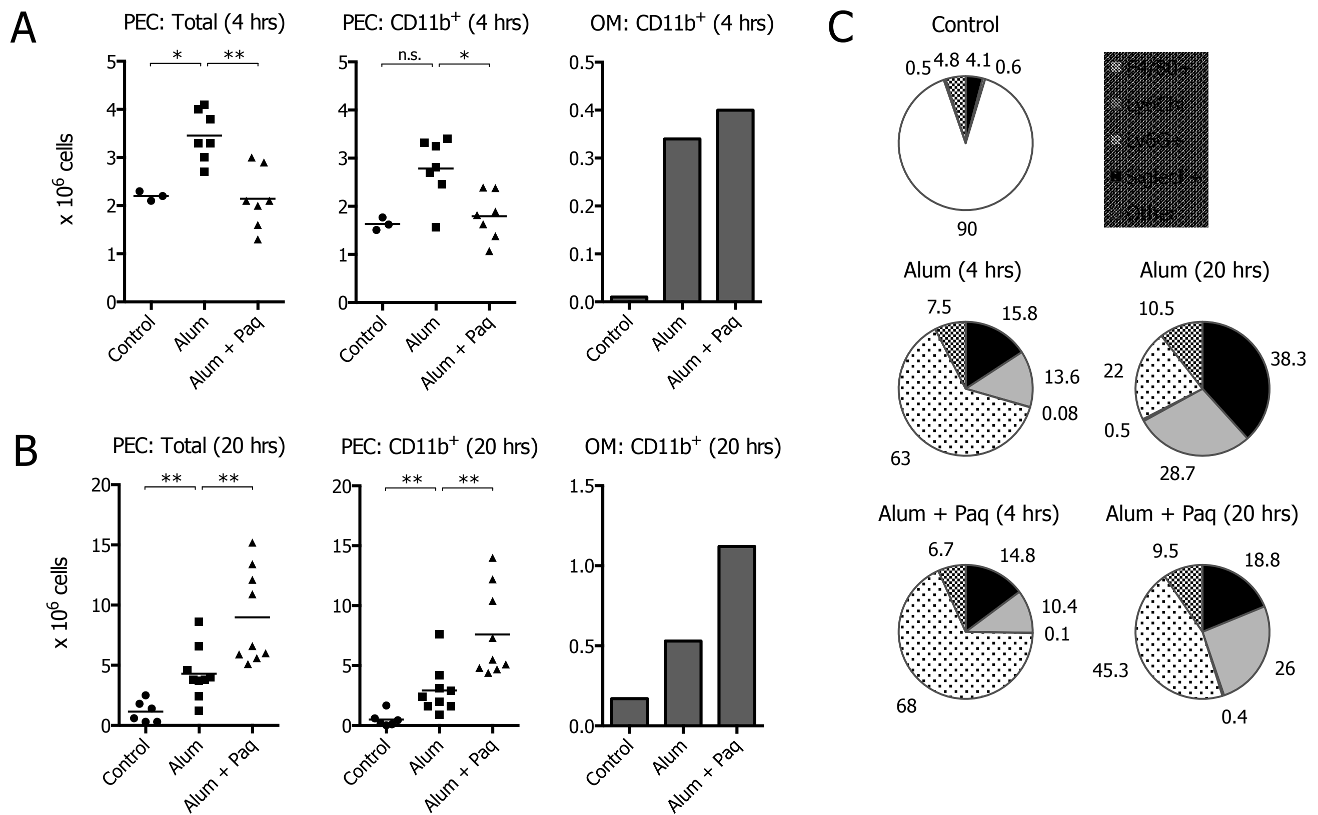

Figure S1

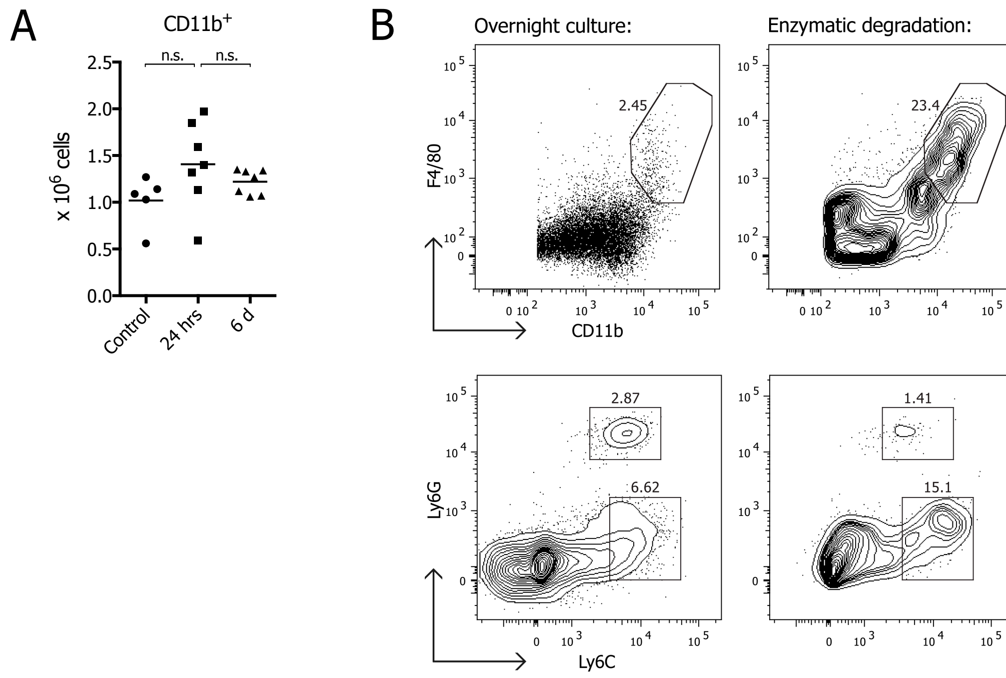

Figure S2

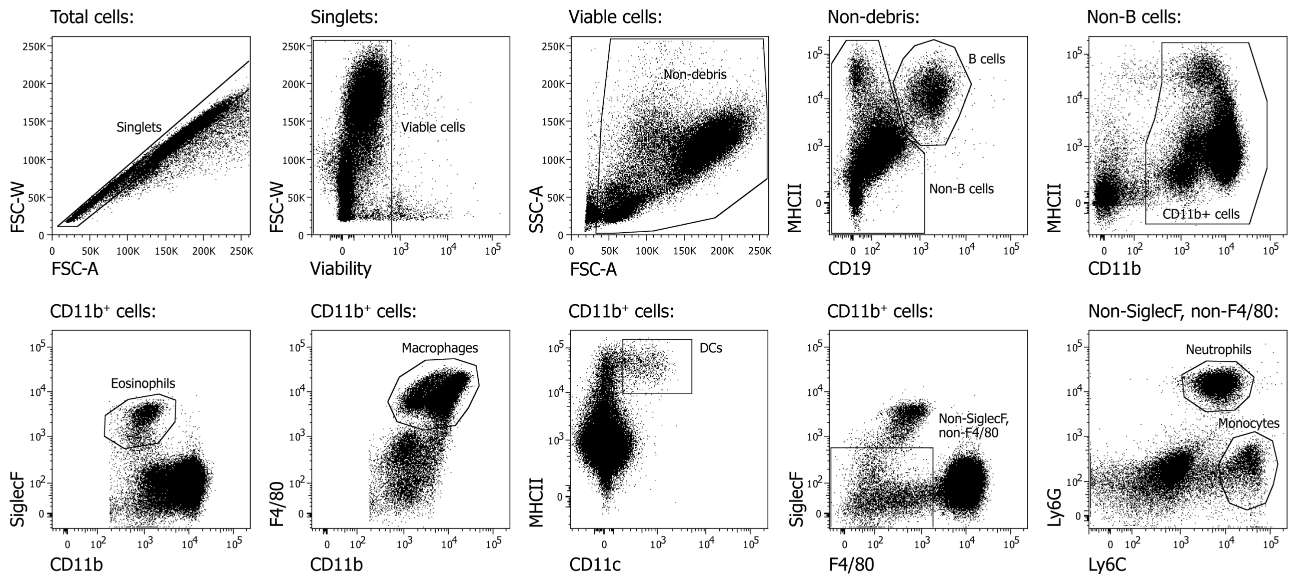

Figure S3