Specific effect of immunomodulatory quinoline-3-carboxamide ABR-215757 in GM-CSF stimulated bone marrow cell cultures: block of initiation of proliferation of Gr-1$^+$ cells

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Short title: A compound targeting Gr-1$^+$ cells

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Abstract

Quinoline-3-carboxamides are currently in clinical development for treatment of both autoimmune disease and cancer. Carboxamides such as ABR-215757 (5757) have shown efficacy in several in vivo mouse models of human inflammatory autoimmune disease. Some microbial infections in mice cause GM-CSF dependent accumulation of dendritic cells expressing TNFα and inducible nitric oxide synthase (iNOS; Tip-DCs) in lymphoid organs. Functionally similar DCs develop in GM-CSF stimulated bone marrow (BM) cell cultures and offered an in vitro model that allowed us to study the impact of 5757 on cellular development of relevance for in vivo inflammatory conditions. We show in here that addition of 5757 to such cultures, in a dose-dependent way increased the frequency of DCs, while it reduced the frequency of Gr-1^+ cells by inhibiting their proliferation. This effect was specific as the compound neither influenced DC development from myeloid progenitors, nor the development of granulocytes in G-CSF stimulated BM cell cultures. Importantly, we also show that 5757 treatment reduced the accumulation of Gr-1^+ cells during inflammation in vivo. We therefore propose that this compound may ameliorate autoimmune disease by blocking proliferation of Gr-1^+ cells during inflammation-induced mobilization of myeloid cells.

Key words: Bone marrow, GM-CSF, myeloid cell, carboxamide

1. Introduction

ABR-215757 (5757) is a quinoline-3-carboxamide currently in clinical development for systemic lupus erythematosus (SLE). In preclinical settings 5757 and structurally related compounds have shown efficacy in several mouse models of T cell-dependent human inflammatory autoimmune disease ([1-4], and unpublished data). This suggested to us that the 5757 treatment might target a common inflammatory mechanism(s) in the various disease models. Along these lines, we have detected a profound reduction of T cell priming in treated mice (our unpublished data). Further, LPS-induced production of the inflammatory cytokine TNFα was significantly reduced in 5757-treated mice [5], suggesting that early steps in T cell activation involving interactions between T cells and antigen-presenting cells might be targeted by 5757-treatment.

It was recently shown that the spleen might function as a reservoir for monocytes, which allows for their rapid recruitment into sites of inflammation [6]. Inflammatory monocytes can generate DCs both in tissue culture and under inflammatory conditions in vivo [7, 8]. In both situations DC-development from monocytes is GM-CSF-dependent and the cells generated functionally resemble the TNFα and iNOS producing DC subset (Tip-DCs), which appears in spleen of mice infected with certain pathogens [9, 10]. Monocyte-derived DCs also accumulate in peripheral lymph nodes rapidly after immunization with bacteria or LPS [11]. Interestingly, these cells were localized in the T cell zones of the lymph nodes and may potentially be involved in T cell activation.

Although in vivo effects of quinoline-3-carboxamides in disease models have been well documented, the in vitro effects of such compounds are less well studied. Because myelopoiesis is induced during states of inflammation, and treatment with quinoline-3-carboxamides ameliorates inflammatory autoimmune disease, we have here investigated whether the 5757 compound might influence myeloid cell development in vitro. We have therefore stimulated BM cells with various colony-stimulating factors and report a selective effect of 5757 on myeloid cell development in GM-CSF-stimulated cultures. We also show that 5757 influences accumulation of myeloid cells during inflammation in vivo.

2. Materials and Methods

2.1 Mice and immunization

C57BL/6 mice and OT-II OVA-specific TCR transgenic mice were used at 7-13 weeks of age. In some experiments, mice were immunized subcutaneously at the base of the tail with an emulsion (100µl) of Complete Freunds Adjuvant (CFA) in PBS. The mice were kept in the animal facility at the Biomedical Centre at Lund University. The experiments were approved of by the local ethics comity for use of animals in research.

2.2 Cell preparation

Tibia, femur and ilium bone were dissected and gently crushed in Hank's BSS supplemented with 10 mM HEPES (Invitrogen Life Technologies, Paisley, UK) using a mortar and pestle. The cell suspension was thereafter passed through a 70 µm cell strainer and cells washed in Hank's BSS. Splenic CD4 T cells were purified using CD4 magnetic beads and LS-columns (Miltenyi Biotech, Bergisch Gladbach, Germany). After washing the cells were re-suspended in complete RPMI medium (RPMI 1640 supplemented with 10% FCS, 1 mM sodiumpyruvate, 10 mM HEPES, 100 U/ml penicillin/streptomycin, 50 µM mercaptoethanol; all supplements from Invitrogen Life Technologies) and counted using Trypan blue exclusion.

2.3 Cell cultures

BM cells were cultured in 6 well plates (Falcon^{R} Becton Dickinson Labware), $2x10^{6}$ cells/well, in 2 ml complete RPMI medium supplemented with 10 % GM-CSF containing culture supernatant from GM-CSF cDNA transfected J558L cells (prepared in our laboratory), 1 ng/ml recombinant murine G-CSF (rmG-CSF; PeproTech, London, UK), or 80ng/ml M-CSF (rmM-CSF; Peprotech) to induce development of DCs, granulocytes or macrophages,

respectively. Parallel cultures were supplemented with various concentrations of ABR-215757 (Active Biotech, Lund, Sweden). CD4 T cell proliferation cultures were established in round-bottom 96 well tissue culture plates. Proliferation of CD4 T cells from normal and OTII TCR transgenic was stimulated by the addition of anti-CD3 antibodies (2,5μg/ml; 145.2C.11) or OVA323-336 peptide (Schaefer-N, Copenhagen, Denmark), respectively. The T cells were co-cultured with BM cells at indicated concentrations in complete RPMI medium at 37°C 5% CO_2 for indicated number of days. To detect proliferation 1μCi of ^{3}H-Thymidine was added to the cultures four hours before harvesting the cells.

2.4 TLR stimulation

Non-adherent cells from BM cell cultures were harvested and washed once with Hanks BSS, re-suspended in complete RPMI medium and counted. The cells ($2x10^5$cells/well) were cultured in flat bottom 96 micro well plates (Nunclone™ Surface NUNC) in the presence of indicated concentrations of LPS (Sigma-Aldrich Inc.), synthetic lipopeptide (S)-(2,3-bis(palmitoyloxy)-(2RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-Lys(4)-OH, trihydrochloride (Pam3Cys; EMC Microcollections GmbH, Tübingen, Germany) or Poly I.C. (Sigma-Aldrich Inc.).

2.5 Cell staining and flow cytometry

Cells were re-suspended in FACS buffer (5% FCS and 0.01% NaN_3 in PBS) and stained with antibodies using standard techniques. The following antibodies used in the analyses were prepared in our laboratory: B220-Alexa647, CD11c-Alexa647 and CD11c-biotin. The following antibodies were bought from eBioSciences (Nordic Biosite, Täby, Sweden): CD11b-APC and CD11b-FITC (clone: MI/70), F4/80-biotin and F4/80-PE (clone: BM8), Gr1-FITC, Gr1-APC and Gr1-PE (clone: RB6-8C5). The following antibodies/reagents were

bought from BD Pharmingen (San Diego, CA): CD11c-PE (clone: HL3), streptavidine (SA)-PE, SA-APC, SA-FITC and SA-PerCP. Analysis of stained cells was preformed using a FACS Calibur or LSR II flow cytometers (BD Biosciences) using CellQuest (BD Biosciences) or FlowJo (Tree Star Inc.) analysis softwares. Various cell populations were sorted from cultured BM cells using FACSAria cell sorter (BD Biosciences). To enrich for myeloid progenitors, BM cells were first depleted of Lineage$^+$ (B220, Ter119 and Gr-1$^+$ and CD11b) cells and CD115$^+$ cells positively selected using magnetic cell sorting. Thereafter, Lineage$^-$, CD117$^+$, CD105$^-$ and CD150$^-$ cells were FACS sorted. Dead cells were excluded using 7-amino-actinomycin D (7AAD; Sigma Aldrich) or propidium iodide staining. In some experiments cells were labeled with CFSE using a standard protocol.

2.6 ELISA

Flat bottom 96 micro well ELISA plates (Corning, NY, USA) were coated with anti-TNFα antibody (eBioscience, clone: 1F3F3DH) or anti-IL-6 (Biolegend, NordicBioSite, Täby, Sweden clone: MP5-32C11). Binding of TNFα or IL-6 was detected using biotin-conjugated anti-TNFα antibody (eBioscience, clone: MP6-XT22 & MP6-XT3) and biotin-conjugated anti-IL-6 (Biolegend, clone: MP5-20F3), respectively followed by horseradish peroxidase (HRP)-conjugated SA (Southern Biotechnology, Birmingham, AL). Tetrametylbenzidine (1mM) was used as HRP-substrate and the enzymatic reaction stopped using 1M H_2SO_4. The absorbance was measured at 450 nm in a plate reader (Emax, Molecular Devices). Murine TNFα and IL-6 standard was used at concentrations between 4 ng/ml-31.25 pg/ml.

2.7 Western blot

Cells ($1x10^5$) were lysed in lysis buffer (10% SDS, 20% glycerol, 0.2M Tris-HCl pH 6.8, 0.05% bromophenolblue) containing 100 mM DTT and heated for 10 minutes at 95°C. Equal

amounts of lysates were loaded on a 12% SDS-polyacrylamidgel. To detect the S100A8 protein, biotin-conjugated rat-anti-mouse S100A8 antibody was (R&D Systems, Minneapolis, MA) and binding revealed using HRP-anti-biotin antibody (Vector Laboratories Inc., Burlingame, CA). To detect the S100A9 protein, rat-anti-mouse S100A9 antibody (R&D Systems) was used and binding revealed using HRP-conjugated rabbit-anti-rat antibody (Southern Biotechnology Associates Inc.). Actin was detected using rabbit-anti-actin (US Biologicals, Swampscott, MA) and binding revealed using goat-anti-rabbit HRP antibody (BioRad, Richmond, CA). The filters were developed using ECL regent (GE Healthcare, Buckinghamshire, UK)

2.8 Quantitative Real-Time PCR

Cells were lysed in TRIzol and total RNA was isolated with a PureLink™ RNA Mini Kit. cDNA was synthesized with a SuperScript™ III Reverse Transcriptase kit. mRNA was quantitatively measured with qRT-PCR using a Platinium ® SYBR® Green qPCR supermix-UDG kit (total volume of each sample was 20μl). All products were purchased from Invitrogen (Carlsbad, USA). qRT-PCR experiments were performed in triplicate and run on an iCycler MyIQ™ instrument (BioRad, Hercules, CA,). The qRT-PCR was performed with the following primers: S100A8-sense (5'-CCGTCTTCAAGACATCGTTTGA-3'), anti-sense (5'-GTAGAGGGCATGGTGATTTCCT-3'), S100A9-sense (5'-CAGCATAACCACCATCATCG-3'), anti-sense (5'-GCCAACTGTGCTTCCACCAT-3'), β-actin-sense (5'-ACCCACACTGTGCCCATCTA-3'), anti-sense (5'-GGCCAT CTCTTGCTCGAA-3') (obtained from SIGMA Genosys, Canada). Relative S100A8 and S100A9 expression was determined using the formula $2^{\text{gene of intrest(Ct)-β-actin(Ct)}}$.

2.9 Statistical analyses

Statistical analyses were performed using unpaired Student’s t-test.

3. **Results**

3.1 Specific effect of 5757 on cell development in BM cell cultures

To determine whether 5757 would influence development of myeloid cells in GM-CSF stimulated BM cell cultures, we supplemented the cultures with various concentrations of the compound (Figure 1A). As can be seen, the frequency of $CD11c^+$ DCs increased while the frequency of $Gr\text{-}1^+$ cells decreased in a dose-dependent way in the supplemented cultures. This effect was detected at concentrations of the 5757 compound well below those inducing non-specific cell death (≥100μM; data not shown).

We next wanted to determine whether the effect of 5757 on BM cell differentiation in GM-CSF stimulated cultures would be specific for that setting or not. BM cells were therefore enriched for immature myeloid cells by depleting cells expressing a high level of CD11b and thereafter cultured for six days either with GM-CSF, M-CSF or G-CSF in the absence or presence of 25 μM 5757. Similarly to GM-CSF stimulated cultures, 5757 also influenced the development of $CD11c^+$ DCs and $Gr\text{-}1^+$ cells in M-CSF stimulated cultures albeit to a lower extent (Figure 1B). In contrast, addition of 5757 did not influence the development of $Gr\text{-}1^+$ granulocytes in G-CSF stimulated cultures. These data suggest that 5757 has a selective effect on myeloid cell development in growth factor stimulated BM cell cultures.

3.2 5757 blocks proliferation cycle entry of $Gr\text{-}1^+$ cells

To better define the observed effect of 5757 on cell development in GM-CSF stimulated cultures, we sorted $CD117^+$ $CD105^-$ $CD150^-$ BM cells to enrich for myeloid progenitors [12]. The sorted cells proliferated extensively in the presence of GM-CSF and a significant fraction of $CD11c^+$ could be detected after 6 days of culture. Addition of 5757 to parallel cultures had

no detectable effect on the development of $CD11c^+$ cells in the cultures (Figure 2A). We also sorted monocyte populations from BM cells and cultured in the presence GM-CSF. However, under the conditions used here, the monocytes proliferated poorly and the impact of 5757 on their growth could not be addressed (data not shown).

We next investigated whether 5757 might have a direct effect on the $Gr\text{-}1^+$ cells. To this end, we labelled BM cells with CFSE and studied their proliferation in the presence or absence of 5757. As can be seen, addition of 5757 significantly reduced the frequency of $Gr\text{-}1^+$ cells that initiated proliferation (Figure 2B), while other cells in the $Gr\text{-}1^+$ population had proliferated extensively. In contrast, the proliferation of $CD11c^+$ cells was not influenced, which is line with the above data (see Figure 2A). In accordance with these results the kinetics of accumulation of $CD11c^+$ cells and reduction of $Gr\text{-}1^+$ cells was the same in the presence and absence of 5757 (Figure 2C). Taken together these data indicate that 5757 exerts its effect early in the GM-CSF stimulated BM cultures by preventing entry of $Gr\text{-}1^+$ cells into the proliferation cycle, while it does not inhibit cell division per se, neither of $CD11c^+$ DCs nor of $Gr\text{-}1^+$ cells.

3.3 Cells developing in the presence of 5757 are functionally normal

It has previously been shown that GM-CSF promotes the generation of TNFα and iNOS producing DCs from bone marrow cells in vitro [13, 14]. We next wanted to determine whether 5757 would influence the functional capacity of cells developing in the GM-CSF stimulated cultures. Cells cultured in the presence or absence of 5757 were therefore stimulated with various Toll like receptor (TLR) agonists for 24 hours and the concentration of TNFα and IL-6 was determined in cell culture supernatants. As shown in Figure 3A,

addition of 5757 had no detectable impact on TLR-induced responses of the cells developing in GM-CSF stimulated cultures.

To further confirm the functional competence of these cells, we studied their capacity to stimulate antigen-specific proliferation of OVA-specific TCR transgenic OT-II T cells. Some BM cell cultures were also exposed to LPS, to induce maturation of the $CD11c^+$ DCs. Irrespective of whether the cells were matured with LPS or not, the 5757 exposed cells were equally potent inducers of T cell proliferation as control cells grown in the absence of 5757 (Figure 3B, left panel).

BM cells grown in the presence of GM-CSF are known to contain cells capable of suppressing T cell proliferation [13, 14]. During conditions of polyclonal T cell stimulation using anti-CD3 antibody this suppression is mainly due to iNOS expression by the myeloid cells, which in turn is dependent on IFNγ production by the activated T cells ([14]; our unpublished observations). When CD4 T cells were cultured with increasing numbers of cells from GM-CSF stimulated BM-cells, there was a dose-dependent inhibition of T cell proliferation (Figure 3B, right panel). The inhibition was, however, equally potent irrespective of whether the cells had been cultured in the presence of 5757 or not. Thus, at high numbers the cells developing in 5757-supplemented GM-CSF stimulated BM cultures showed normal capacity to inhibit the T cell proliferation response.

3.4 High-level S100A8 and S100A9 expression in $Gr1^+$ cells

The S100A8 and S100A9 proteins are expressed by monocytes and neutrophils, but not by mature macrophages (reviewed in [15]). Since supplementation of the GM-CSF stimulated BM cell cultures with 5757 affected $Gr\text{-}1^+$ cells and human S100A9 is a molecular target of

5757 [5], we wanted to investigate the expression of S100A8 and S100A9 proteins in these cells. To this end, we sorted various cell populations from the GM-CSF stimulated cultures. The highest level of S100A8 and S100A9 mRNA and protein expression was detected in the Gr-1^+ population (Figure 4A, B), while the CD11c^+ population displayed low expression. Thus the Gr-1^+ cell population that was reduced in the 5757 exposed cultures was also expressing S100A8/A9 at a high level. We also compared the expression levels of S100A8 and S100A9 mRNA in sorted Gr-1^+ cells from cultures grown in the presence or absence of 5757. As shown in Figure 4C, there was no detectable effect of 5757 on expression S100A8 and S100A9 mRNA in the Gr-1^+ cells.

3.5 5757-treatment reduces accumulation of Gr-1^+ cells during in vivo inflammation

We next wanted to determine whether 5757-treatment would also have an impact on Gr-1^+ cell accumulation in vivo. Previous reports have shown that quinoline-3-carboxamides ameliorate autoimmune disease in experimental models such as experimental autoimmune encephalomyelitis (EAE) [2, 4]. It is well established that subcutaneous immunization with Complete Freunds Adjuvant (CFA) induces mobilization of myeloid cells in animals, leading to accumulation of CD11b^+ myeloid cells in the spleen (reviewed in [16]). Since EAE is induced by immunization with neural antigens emulsified in CFA, we thought that it would be relevant to study the impact of 5757 on accumulation of Gr-1^+ cells in CFA-immunized mice. As expected there was an elevated number of CD11b^+ cells, most of which are Gr-1^+, in the spleen of CFA immunized mice as compared to controls immunized with Incomplete Freunds Adjuvant (IFA; Figure 5). Importantly, the number of Gr-1^+ cells was significantly reduced in mice receiving 5757 in the drinking water from the day of immunization. These data indicate that 5757 also reduces the accumulation of Gr-1^+ cells during myelopoiesis in vivo.

4. Discussion

It is well established that monocytes can differentiate to DCs during inflammatory conditions in vivo and in GM-CSF stimulated BM cell cultures in vitro (reviewed in [17]). The DCs are denoted Tip DCs because of their production of TNFα and expression of iNOS [9] and originate from inflammatory $CCR2^+$ monocytes [8]. These cells are important for clearing microbial infections [9] and a recent publication indicated that monocyte derived DCs also accumulate in draining lymph nodes of mice infected with LPS-carrying bacteria [11]. It is tempting to speculate that these monocytes may derive from the splenic monocyte reservoir [6].

In this report we have studied the impact of the quinoline-3-carboaxamide 5757 on cell development in BM cell cultures stimulated with various growth factors for myeloid cells. We observed a significant increase in $CDllc^+$ myeloid DCs and a parallel decrease in $Gr\text{-}1^+$ cells in GM-CSF stimulated cultures. Since this was the first example of an in vitro effect of quinoline-3-carboxamides, we thought it was important to further explore this observation. It has been described before that M-CSF can drive the in vitro development of DCs [18] and we found that addition of 5757 to such cultures had a similar but lower effect. In contrast, the compound did not influence the differentiation of granulocytes in G-CSF stimulated cultures. Thus, the effect of 5757 is specific operating mainly on cell development in GM-CSF stimulated cultures. Further, the effect was dose-dependent and seen at concentrations of 5757 that had no detectable non-specific toxic effect on the BM cells. The cells developing in the presence of 5757 responded to TLR-stimulation similarly to control cells. Further, the cells induced antigen-specific T cell proliferation and in high numbers suppressed T cell

proliferation similarly to controls. Thus, by these criteria the cells developing in the presence of 5757 appear to be functionally identical to cells developing in its absence.

We reasoned that 5757 supplementation might either increase the frequency of cells responding to GM-CSF-induced signals by adopting the $CD11c^+$ developmental fate or operate directly on $Gr\text{-}1^+$ cells. We tested the first possibility by analysing cultures established using sorted $CD117^+$ BM cells that are enriched for myeloid progenitors [12]. There was, however, no significant effect of 5757 on DC development from the BM progenitors, thereby favouring the second possibility. Using CFSE labelled BM cells, we could show that 5757 reduced the fraction of $Gr\text{-}1^+$ cells initiating proliferation, while the rest of the cells in the $Gr\text{-}1^+$ population had proliferated extensively. This result, taken together with the observation that 5757 did not influence development of $Gr\text{-}1^+$ granulocytes in G-CSF stimulated BM cultures, suggests that the compound does not have a general toxic effect on $Gr\text{-}1^+$ cells. Taken together, out data indicate that 5757 has a highly specific effect on the proliferation of $Gr\text{-}1^+$ cells in GM-CSF stimulated BM cell cultures.

The EAE model of human multiple sclerosis can be induced in mice using neural antigens emulsified in CFA. The CFA immunization per se is known to induce myelopoiesis causing accumulation of myeloid cells in lymphoid organs [16]. We show in here that in vivo treatment of CFA immunized mice with 5757 significantly reduced the CFA-induced accumulation of $Gr\text{-}1^+$ cells in the spleen. Thus, 5757 influences the accumulation of $Gr\text{-}1^+$ cells during myelopoiesis both in vivo and in vitro. While we have not addressed in here whether the accumulation of $Gr\text{-}1^+$ cells in vivo in CFA immunized mice is GM-CSF-dependent, it has been shown that components of the mycobacteria in CFA do indeed induce colony-stimulating activity in vivo [19] and GM-CSF production in cells from BCG-

vaccinated humans [20, 21]. The Gr-1^+ population contains pro-inflammatory monocytes that are recruited to sites of inflammation [7] as well as other cells such as neutrophils. One would expect that reduction of the number of pro-inflammatory cells would be beneficial in an experimental inflammatory disease such as EAE. We therefore believe that our data offer a possible mechanism for the ameliorating effect of 5757 in experimental autoimmune disease.

In the in vivo treatment experiment, the animals were exposed to 25mg/kg/day of 5757 in the drinking water. It has been shown that mice exposed orally to 30mg/kg/day of 5757 have a $C_{average}$ over 24hrs of approximately 40 μM in plasma (H. Tuvesson, Active Biotech, unpublished results). Thus, the in vivo and in vitro effects of 5757 were obtained in a similar concentration range of the compound. The related compound Laquinimod has shown efficacy in EAE and its plasma concentration time profile has been reported [2]. In a clinical trial in SLE patients, a dose of 1.5mg/day of 5757 was administered orally. The plasma Cmax in these patients was about 2.5 μM [1]. The major reason why an increased 5757 dosage is required for efficacy in the mouse is the more rapid drug metabolism and much shorter half-life of the compound in that species (H. Tuvesson, Active Biotech, unpublished results).

We recently reported that in vivo administration of 5757 caused a selective reduction of splenic $CD4^+$ DCs during steady state conditions [22]. The reduction was fully reversible and 5757 had no effect on other immune cells or on DCs in other lymphoid organs. Since 5757 treatment did not influence $CD8\alpha^+$ and $CD4^-CD8\alpha^-$ splenic DCs, which develop from the same precursors as the $CD4^+$ DCs, we proposed that 5757 would not act at the common DC

[1] Bengtsson A.A., Sturfelt G., Lood C., Rönnblom L., van Vollenhoven R.F., Axelsson B., Sparre B., Tuvesson H., Wallén Öhman M., Leanderson T. Pharmacokinetics, tolerability, and preliminary efficacy of ABR-215757, a new quinoline-3-carboxamide derivative, in murine and human SLE. Submitted for publication

precursor level [22]. BM cells cultured in vitro in presence of flt3L generate DCs that phenotypically more closely resemble conventional splenic DCs than the cells generated in GM-CSF stimulated cultures [10, 23]. We have also studied the development of DCs in flt3L stimulated BM cultures. However, supplementation of such cultures with 5757 failed to influence DC development (unpublished results), which is in accordance with the lack of activity of 5757 on BM-precursor cells in vivo [22].

In a recent report we demonstrated that the human S100A9 protein is a molecular target of 5757 [5]. We show in here that the S100A8 and S100A9 genes and the corresponding proteins are mainly expressed in $Gr1^+$ cells in the GM-CSF stimulated BM cell cultures. While 5757 blocked induction of proliferation of $Gr\text{-}1^+$ cells in GM-CSF stimulated cultures, the compound had no detectable effect on the generation of such cells in cultures stimulated with G-CSF. Similarly, 5757-induced reduction of $Gr\text{-}1^+$ cells was also obtained in cultures of GM-CSF stimulated BM cells from S100A9-deficient mice. Thus, the high expression level of S100A9 in $Gr\text{-}1^+$ cells did not correlate with the effect of 5757 on these cells. At present we cannot provide an explanation to this observation, but future experiments aim at clarifying this issue.

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Disclosure statement

The authors declare that they do not have any financial or personal conflicts of interest

Figure legends

Figure 1

Selective effect of 5757 in CSF-stimulated BM cell cultures.

A) BM cells were cultured with GM-CSF and indicated 5757 concentrations for six days. B) CD11b depleted BM cells were cultured either with GM-CSF (6 days), 80 ng/ml rmM-CSF (6 days) or 1 ng/ml rmG-CSF (4 days) +/- 25 μM 5757. The cultured cells were subsequently analyzed by FACS, to define various CD11b-gated subpopulations as indicated. The data represent mean +/-SD. B) GM-CSF CD11c p=0,0055; Gr-1 p=0,0023. M-CSF CD11c p= 0,0288; Gr-1 p=0,0495. G-CSF differences were not significant. Representative results of three experiments are shown.

Figure 2

Reduced initiation of Gr-1$^+$ cell proliferation in 5757-supplemented cultures.

A) FACS-sorted CD117$^+$ CD105$^-$ CD150$^-$ BM cells cultured 6 days or C) total BM cells cultured 4-6 days, in the presence of GM-CSF +/- 25μM 5757. B) CFSE-labelled total BM cells were cultured as in A). The cultured cells were analyzed by FACS and the data show the frequency of indicated cell populations. A) Data indicates mean +/- SD of three replicate cultures. B) The frequency of non-divided (CFSE-high) cells, in the cell populations

displayed in the contour plot, in three replicate cultures is shown (*p=0,0161). C) Single bulk cultures were analysed. Data are representative of 2-4 independent experiments.

Figure 3

Cells recovered from 5757-supplemented cultures are functionally normal.

BM cells were cultured for six days with GM-CSF +/- 25 μM 5757. A) The cells were harvested and re-cultured ($2x10^5$ cells/well) in triplicate in the presence either of 1 μg/ml LPS, 100 ng/ml Pam3Cys or 20 μg/ml poly I.C. TNFα (left panel) or IL-6 concentration (right panel) in culture supernatants was determined with ELISA. Values are means of triplicates +/-SD. B) BM cells cultured with GM-CSF +/- 5757 for 6 days and +/- 1 μg/ml LPS the last 24 hours, were harvested and subsequently co-cultured ($5x10^3$) with OT-2 TCR transgenic $CD4^+$ T cells ($2,5x10^4$) in the presence of indicated concentrations of OVA-peptide (left panel). Various numbers of the same BM cells were co-cultured with $CD4^+$ T cells ($2,5x10^4$) in the presence of anti-CD3 antibody (2,5μg/ml). Proliferation in triplicate cultures at 72 h was determined using ^{3}H-Thymidine incorporation. Mean proliferation +/- SD is shown. Results are representative of three independent experiments.

Figure 4

S100A8 and S100A9 expression is not decreased in 5757 exposed BM cells.

BM cells were cultured for six days with GM-CSF +/- 25 μM 5757. Indicated cell populations were isolated by FACS sorting. A and C, S100A8 and S100A9 mRNA expression was determined using qRT-PCR and was normalized to β-actin mRNA level. Data indicate mean +/-SD of triplicate samples. B, S100A8 and S100A9 protein expression ($5x10^5$ cells/lane) was determined using Western blot. C) S100A8 and S100A9 mRNA expression in sorted $CD11b^+Gr\text{-}1^+$ cells. Representative results of two experiments are shown.

Figure 5

5757 reduces accumulation of splenic Gr-1$^+$ cells during inflammation

C57BL/6 mice were immunized with IFA/PBS (n=10) or CFA/PBS (n=10) s.c. Half of the mice received 5757 (25mg/kg/day) in their drinking water from the day of immunization. Spleen cells were analyzed by FACS 8 days thereafter. The data indicate mean +/- SD absolute number of indicated cell populations (CD11b p=0,0048; Gr-1 p=0,0077). The results are representative of three independent experiments.

References

[1] Bjork J, Kleinau S. Paradoxical effects of LS-2616 (Linomide) treatment in the type II collagen arthritis model in mice. Agents Actions. 1989;27:319-21.

[2] Brunmark C, Runstrom A, Ohlsson L, Sparre B, Brodin T, Astrom M, et al. The new orally active immunoregulator laquinimod (ABR-215062) effectively inhibits development and relapses of experimental autoimmune encephalomyelitis. J Neuroimmunol. 2002;130:163-72.

[3] Gross DJ, Weiss L, Reibstein I, Hedlund G, Dahlen E, Rapoport MJ, et al. The immunomodulator Linomide: role in treatment and prevention of autoimmune diabetes mellitus. International immunopharmacology. 2001;1:1131-9.

[4] Runstrom A, Leanderson T, Ohlsson L, Axelsson B. Inhibition of the development of chronic experimental autoimmune encephalomyelitis by laquinimod (ABR-215062) in IFN-beta k.o. and wild type mice. J Neuroimmunol. 2006;173:69-78.

[5] Bjork P, Bjork A, Vogl T, Stenstrom M, Liberg D, Olsson A, et al. Identification of human S100A9 as a novel target for treatment of autoimmune disease via binding to quinoline-3-carboxamides. PLoS Biol. 2009;7:e97.

[6] Swirski FK, Nahrendorf M, Etzrodt M, Wildgruber M, Cortez-Retamozo V, Panizzi P, et al. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. Science (New York, NY. 2009;325:612-6.

[7] Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. Immunity. 2003;19:71-82.

[8] Naik SH, Metcalf D, van Nieuwenhuijze A, Wicks I, Wu L, O'Keeffe M, et al. Intrasplenic steady-state dendritic cell precursors that are distinct from monocytes. Nature immunology. 2006;7:663-71.

[9] Serbina NV, Salazar-Mather TP, Biron CA, Kuziel WA, Pamer EG. TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. Immunity. 2003;19:59-70.

[10] Xu Y, Zhan Y, Lew AM, Naik SH, Kershaw MH. Differential development of murine dendritic cells by GM-CSF versus Flt3 ligand has implications for inflammation and trafficking. J Immunol. 2007;179:7577-84.

[11] Cheong C, Matos I, Choi JH, Dandamudi DB, Shrestha E, Longhi MP, et al. Microbial stimulation fully differentiates monocytes to DC-SIGN/CD209(+) dendritic cells for immune T cell areas. Cell.143:416-29.

[12] Pronk CJ, Rossi DJ, Mansson R, Attema JL, Norddahl GL, Chan CK, et al. Elucidation of the phenotypic, functional, and molecular topography of a myeloerythroid progenitor cell hierarchy. Cell stem cell. 2007;1:428-42.

[13] Lu L, Bonham CA, Chambers FG, Watkins SC, Hoffman RA, Simmons RL, et al. Induction of nitric oxide synthase in mouse dendritic cells by IFN-gamma, endotoxin, and interaction with allogeneic T cells: nitric oxide production is associated with dendritic cell apoptosis. J Immunol. 1996;157:3577-86.

[14] Rossner S, Voigtlander C, Wiethe C, Hanig J, Seifarth C, Lutz MB. Myeloid dendritic cell precursors generated from bone marrow suppress T cell responses via cell contact and nitric oxide production in vitro. European journal of immunology. 2005;35:3533-44.

[15] Foell D, Wittkowski H, Vogl T, Roth J. S100 proteins expressed in phagocytes: a novel group of damage-associated molecular pattern molecules. Journal of leukocyte biology. 2007;81:28-37.

[16] Billiau A, Matthys P. Modes of action of Freund's adjuvants in experimental models of autoimmune diseases. Journal of leukocyte biology. 2001;70:849-60.

[17] Naik SH. Demystifying the development of dendritic cell subtypes, a little. Immunology and cell biology. 2008;86:439-52.

[18] Fancke B, Suter M, Hochrein H, O'Keeffe M. M-CSF: a novel plasmacytoid and conventional dendritic cell poietin. Blood. 2008;111:150-9.

[19] Galelli A, Chedid L. Modulation of myelopoiesis in vivo by synthetic adjuvant-active muramyl peptides: induction of colony-stimulating activity and stimulation of stem cell proliferation. Infection and immunity. 1983;42:1081-5.

[20] Fletcher HA, Keyser A, Bowmaker M, Sayles PC, Kaplan G, Hussey G, et al. Transcriptional profiling of mycobacterial antigen-induced responses in infants vaccinated with BCG at birth. BMC medical genomics. 2009;2:10.

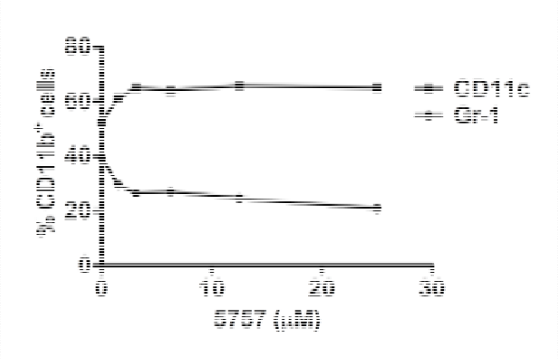
[21] Luo Y, Chen X, O'Donnell MA. Mycobacterium bovis bacillus Calmette-Guerin (BCG) induces human CC- and CXC-chemokines in vitro and in vivo. Clinical and experimental immunology. 2007;147:370-8.

[22] Stenstrom M, Anderson P, Eroukhmanoff L, Leanderson T, Ivars F. Selective depletion of splenic CD4 dendritic cells in mice treated with immunomodulatory quinoline-3-carboxamide ABR-215757. International immunopharmacology.10:837-42.

[23] Naik SH, Proietto AI, Wilson NS, Dakic A, Schnorrer P, Fuchsberger M, et al. Cutting edge: generation of splenic CD8+ and CD8- dendritic cell equivalents in Fms-like tyrosine kinase 3 ligand bone marrow cultures. J Immunol. 2005;174:6592-7.

Figure 1.

A.


CD11c
Gr-1
% CD11b+ cells
5757 (µM)

B.

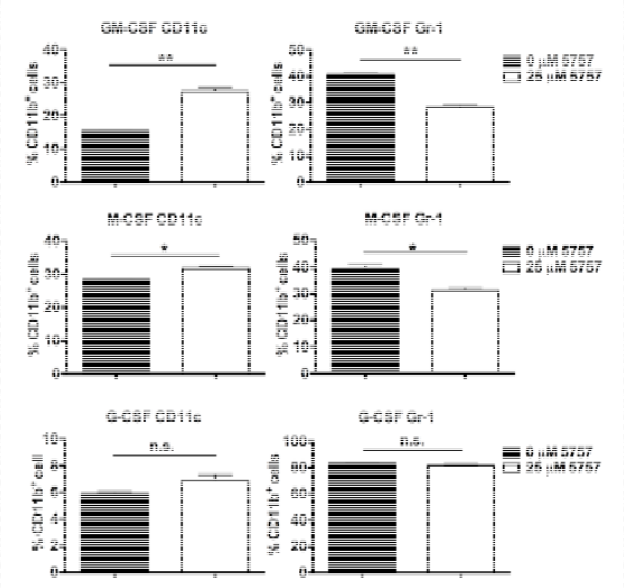
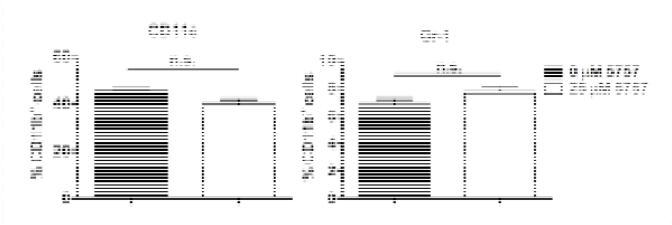
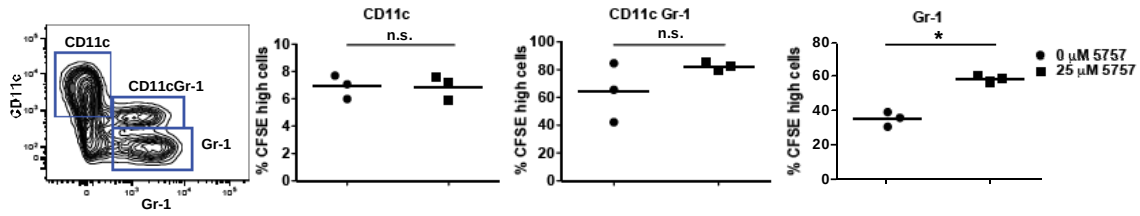

GM-CSF CD11c
GM-CSF Gr-1
M-CSF CD11c
M-CSF Gr-1
G-CSF CD11c
G-CSF Gr-1
0 µM 5757
25 µM 5757
% CD11b+ cells
n.s.

Figure 2

A.



B.


CD11c
CD11cGr-1
Gr-1
CD11c
n.s.
% CFSE high cells
CD11c Gr-1
n.s.
% CFSE high cells
Gr-1
*
% CFSE high cells
0 μM 5757
25 μM 5757

C.

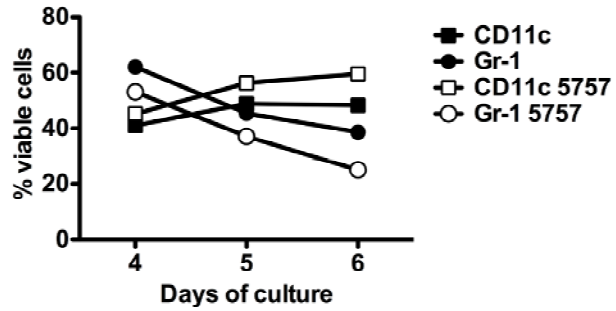
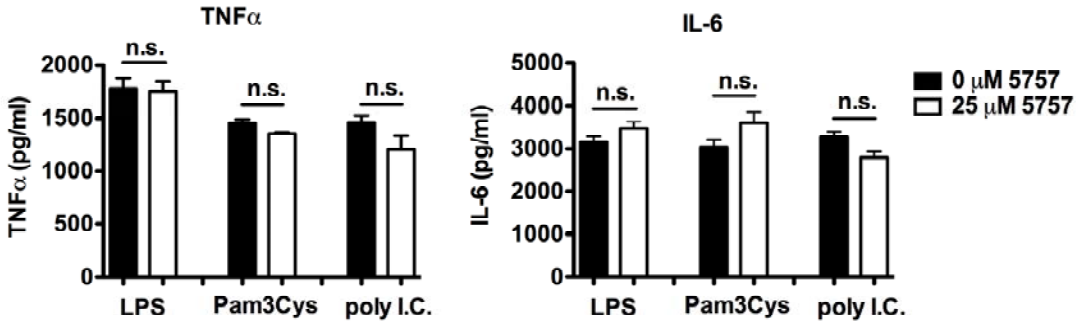

% viable cells
Days of culture
CD11c
Gr-1
CD11c 5757
Gr-1 5757

Figure 3.

A.


TNFα
IL-6
n.s.
TNFα (pg/ml)
IL-6 (pg/ml)
2000
1500
1000
500
0
5000
4000
3000
2000
1000
LPS
Pam3Cys
poly I.C.
0 μM 5757
25 μM 5757

B.

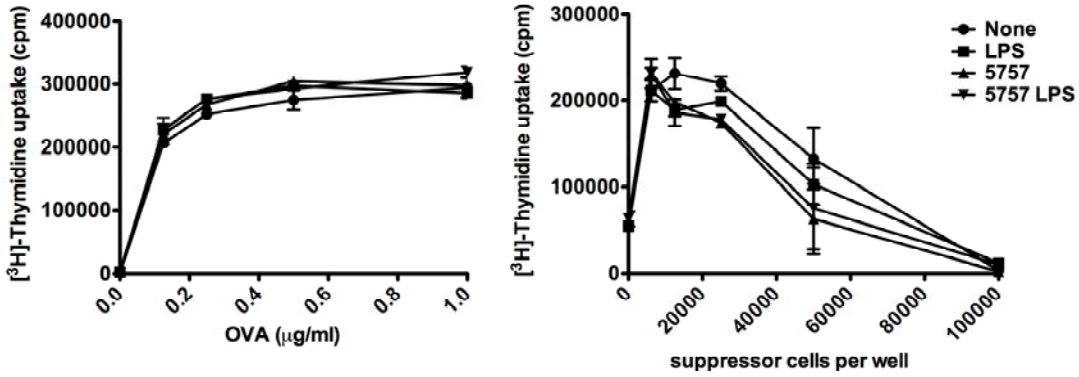
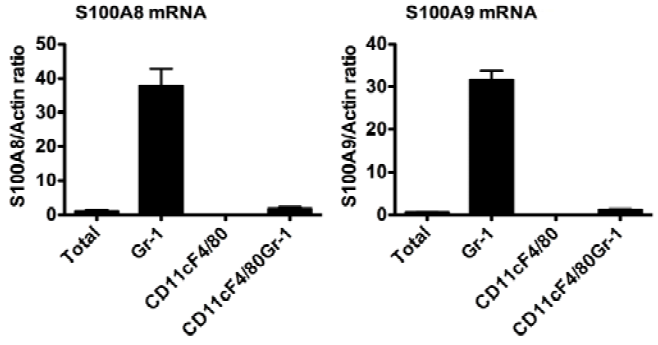
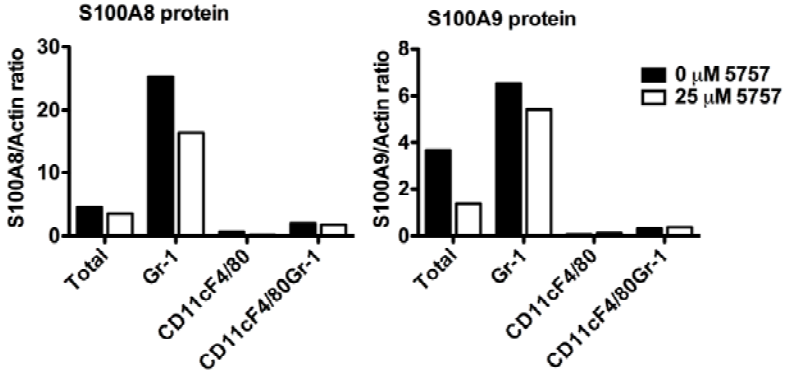

[3H]-Thymidine uptake (cpm)
400000
300000
200000
100000
0
0.0
0.2
0.4
0.6
0.8
1.0
OVA (μg/ml)
300000
200000
100000
0
20000
40000
60000
80000
100000
suppressor cells per well
None
LPS
5757
5757 LPS

Figure 4

A.


S100A8 mRNA
S100A8/Actin ratio
50
40
30
20
10
0
Total
Gr-1
CD11cF4/80
CD11cF4/80Gr-1
S100A9 mRNA
S100A9/Actin ratio
40
30
20
10
0
Total
Gr-1
CD11cF4/80
CD11cF4/80Gr-1

B.


S100A8 protein
S100A8/Actin ratio
30
20
10
0
Total
Gr-1
CD11cF4/80
CD11cF4/80Gr-1
S100A9 protein
S100A9/Actin ratio
8
6
4
2
0
0 μM 5757
25 μM 5757
Total
Gr-1
CD11cF4/80
CD11cF4/80Gr-1

C.

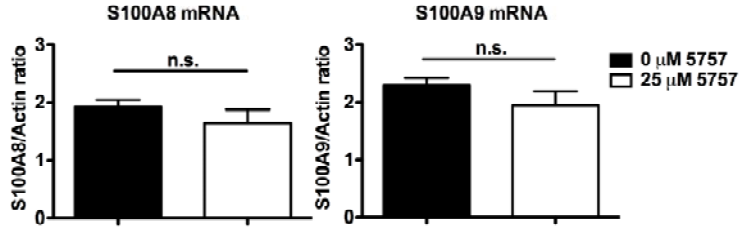
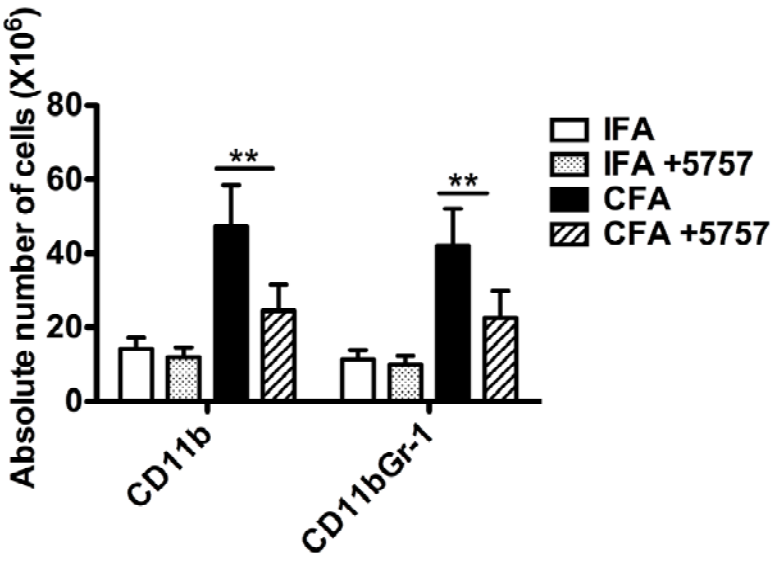

S100A8 mRNA
S100A8/Actin ratio
3
2
1
0
n.s.
S100A9 mRNA
S100A9/Actin ratio
3
2
1
0
n.s.
0 μM 5757
25 μM 5757

Figure 5


Absolute number of cells (X10^6)
80
60
40
20
0
**
**
CD11b
CD11bGr-1
IFA
IFA +5757
CFA
CFA +5757