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ORIGINAL ARTICLE

In Vitro Immunological Effects of CXCR3 Inhibitor AMG487 on Dendritic Cells

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Abstract
AMG 487 is the targeted blocker of chemokine receptor CXCR3 and improves inflammatory symptoms by blocking the inflammatory cycle. Here we investigated whether AMG 487 affects dendritic cell (DC) biology and function. The expression of co-stimulatory markers on DCs was reduced, indicating the semi-mature state of DC when AMG 487 was added throughout the in vitro differentiation period. Additionally, when added solely during the final lipopolysaccharide-induced activation step, AMG 487 inhibited DC activation, as demonstrated by a decreased expression of activation markers. AMG487 also promoted the expression of PD-L2 and impaired the ability to induce antigen-specific T cell responses. Our results demonstrated that AMG 487 significantly affects DC maturity in vitro and function leading to impaired T cell activation, inducing DCs to have characteristics similar to tolerogenic DCs. AMG 487 may directly play an immunomodulatory role during DC development and functional shaping.

Keywords AMG 487 · Dendritic cells · Development inhibition · Dysfunction

Introduction

CXCR3 (C-X-C chemokine receptor type 3) is a chemokine receptor in the CXC chemokine subfamily and can be expressed on the surface of dendritic cells, activated lymphocytes, macrophages, NK cells, and other immune cells. It is also expressed in some non-hematopoietic cells (such as vascular endothelial cells, fibroblasts, smooth muscle cells, etc.) (Struyf et al. 2011; Garcia-Lopez et al. 2001; Billottet et al. 2013). CXCR3 chemokine receptor pathway responds to CXCL9/Mig (monokine induced by IFN-gamma), CXCL10/IP-10 (interferon-gamma-inducible protein), CXCL11/I-TAC (interferon gamma-inducible T cell alpha chemoattractant) (Cole et al. 1998; Karin and Razon 2018) and plays an important role in many physiological and pathological processes (Loetscher et al. 1996).

Many studies have focused on the development of new small molecular antagonists to block the binding of CXCR3 to its active ligand CXCL9/10/11 (Wijtmans et al. 2008). CXCR3 antagonists were originally proposed for use in inflammatory and autoimmune diseases, but more and more recent studies have shown that they can be used in the treatment of a wider range of diseases (Van Raemdonck et al. 2015; Trivedi and Adams 2018; Hueso et al. 2018). At present, more than 15 kinds of CXCR3 antagonists have been identified, including not only basic molecules, such as typical chemokine receptor antagonists, but also acidic and neutral ligands. Two groups of these have been optimized—piperazinyl-piperidines, such as SCH 546738 (Jenh et al. 2012) and 8-azaquinazolinones, such as AMG 487. AMG 487 is a dihydroquinazoline analog of AMG 1237845 and it is the only small molecular antagonist that can compete with ligand CXCL9-11 to bind CXCR3 to block CXCL9-11's interaction with CXCR3. It was once used in clinical trials and entered the second phase of clinical trials for the treatment of psoriasis (Tonn et al. 2009).

Our previous in vivo experiment has found that AMG 487 combined with cyclosporine A (CsA) could alleviate murine acute graft-versus-host-disease (aGVHD) after allogeneic transplantation, compared with using CsA alone (Miao et al. 2018), implying that AMG 487 may play a immunoregulatory role as well. As the most important

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professional antigen-presenting cells, dendritic cells play an important regulatory role in aGVHD (Elze et al. 2015; Goncalves et al. 2015) and inducing T cell immune response or immune tolerance (Cools et al. 2011; Cools et al. 2007). Considering the consistent expression of CXCR3 on DCs, we speculated that AMG 487 may have an impact on DC. The effect of AMG 487 functioning as a targeted blocker has been extensively studied (Chen et al. 2019; Guo et al. 2018a; b), but whether it can modulate DCs directly is still unknown. Therefore, to deduce the impact of AMG 487 on DCs in vitro, we used GM-CSF and IL-4 to induce the differentiation and development of mouse bone marrow cells into DC, stimulated by Escherichia coli lipopolysaccharide (LPS) to induce maturation. DC were exposed to AMG 487 during both the developmental and activation stages. We detected the development, activation, and function of bone marrow-derived DCs (BMDCs) by flow cytometry.

Methods

Media and Reagents

The culture medium consisted of RPMI 1640 supplemented with 10% inactivated fetal calf serum (Gibco, Invitrogen) and 1% penicillin–streptomycin (Invitrogen). The targeted CXCR3 blocker, AMG 487, was purchased from R&D. Recombinant murine granulocyte macrophage-colony-stimulating factor (GM-CSF; PeproTech) and murine IL-4 (PeproTech) were purchased from BioLegend and prepared in advance according to the manufacturer's protocol. OVA peptide $_{323–339}$ was obtained from MedChemExpress and dissolved in aseptic water.

Generation of DCs

Murine bone marrow-derived DCs (BMDCs) were generated using GM-CSF and IL-4, as described previously (Chen et al. 2004). First, bone marrow cells were isolated from male C57BL/6 tibia and fibula. The cells were suspended in 10% fetal calf serum (FBS) RPMI-1640 supplemented with 20 ng/ml GM-CSF and 10 ng/ml IL-4 at 37 °C (37 degrees Celsius) in a humidified 5% CO_2 atmosphere. The culture medium and cytokines were 3/4 changed every 3 days. Immature BMDCs are non-adherent cells and could be harvested on day 6. For most of the experiments, to induce maturation and activation, LPS (Sigma-Aldrich) was added at 100 ng/ml for the last 24 h before harvest. For the functional experiments, ovalbumin peptide ($OVA_{323–339}$) was added simultaneously with LPS. At day 7, mature BMDCs were harvested for further experiments. One mouse can obtain 1×10^{7} bone marrow cells, and $(1–2)\times10^{5}$ DCs can be obtained through in vitro cytokine induction. A sufficient number of DCs can be obtained from 5 mice for experiments.

AMG 487 was dissolved in 20% hydroxypropyl-beta-cyclodextrin solution. For the analysis of the toxic effect of AMG 487 on bone marrow cells and the effect of AMG 487 on BMDC development, the bone marrow cells were exposed to AMG 487 (3 μM, 15 μM, 30 μM, and 60 μM) from day 0, and AMG 487 was supplemented with appropriate amounts every 3 days. The cells were washed twice before adding LPS to induce maturation. To investigate the effect of AMG 487 on BMDC activation, we added various concentrations of AMG 487 (10 μM, 30 μM, and 60 μM) only on day 6 along with the LPS. Equal amounts of the vehicle were added as a control in each case.

Isolation of Murine OT-II $CD4^+$ T Cells

The spleen and peripheral lymph nodes were removed from 6-week OT-II mice and homogenized through a 400 mesh cell strainer. After centrifugation, OT-II T cells were separated by magnetic cell sorting, utilizing the $CD4^+$ T cell negative selection kit, according to the manufacturer's protocol (BioLegend).

Cell Viability Detection

DC viability was detected by live-dead staining using 7-Amino-Actinomycin (7-AAD). Viable cells with intact membranes exclude 7-AAD whereas the membranes of dead and damaged cells are permeable to 7-AAD. Therefore, DC that are in late apoptosis or already dead are 7-AAD positive. 7-AAD was added to samples right before flow cytometry.

Phenotype Analysis by Flow Cytometry

Murine BMDCs and OT-II T cells were stained using commercial monoclonal antibodies from BioLegend, BD Biosciences, and eBioscience. Basically, cells were harvested and washed in PBS once before incubation with mAbs. TruStain FcX(anti-mouse CD16/32, BioLegend) antibodies were added for 5 min on ice to block the IgG Fc receptors II/III on the BMDCs, thus reducing the non-specific binding of immunoglobulin to the Fc receptors. Then the cells were incubated with a saturated concentration of various fluorochrome-conjugated mAbs for 30 min at 4 °C in the dark.

The stained cells were washed twice in phosphate-buffered saline (PBS) and detected by FACS. Dead cells were excluded by 7AAD staining shortly before FACS. The data were analyzed for the percentage of marker-positive cells and the mean fluorescence intensity (MFI). The mAbs used were as follows: Ly-6C (HK1.4), CD11c (N418), I-A/I-E (M5/114.15.2), CD40 (HM40-3), CD80 (16-10A1), CD86 (GL-1), CD83 (Michel-19), PD-L1 (10F.9G2), PD-L2

(TY25), CD62L (MEL-14), CD44 (IM7), DC-SIGN (MMD3), CD69 (H1.2F3), CD25 (PC61), CXCR3 (CXCR3-173), CD3 (17A2), CD4 (30-F11), IFN-γ (XMG1.2).

Intracellular Cytokine Staining

48–72 h following co-culture $CD4^+$ T cells with $OVA_{323–339}$ peptide-loaded DCs, cell activation cocktail (with Brefeldin A, biolegend) was added for the final 6 h. Then cells were stained with fluorescently labeled antibodies against CD3 and CD4 prior to fixation and permeabilization (eBioscience), and anti-IFN-γ Ab subsequently.

Induction of Ag-Specific T Cell Responses In Vitro

1 × 10^5 OT-II $CD4^+$ T cells were cocultured with the BMDCs at a ratio of 10:1 in a total volume of 250 µL per well at 37 °C in a humidified 5% CO_2 atmosphere. Before coculturing, the DCs were loaded with 10 ug/ml $OVA_{323–339}$ peptide for 4–8 h at 37 °C, followed by washout twice to wash away the peptides not loaded on DCs. Proliferation and activation of transgenic $CD4^+$ T cells were assessed by FACS 48–72 h later.

Statistical Analysis

All the experiments were performed 3 times independently. The experimental results are consistent, with representative experiments shown. Student's t test was used to analyze the statistical significance. Comparisons were made as indicated with analysis of variance (ANOVA) using Prism 5 software (Graphpad software).

Results

Exposure of Bone Marrow Cells to AMG 487 Inhibited the Expression of Co-Stimulatory Markers on DCs

We first detected the toxic effects of AMG 487 on bone marrow cells and BMDCs. We applied increasing concentrations of AMG 487 from day 0 on did not detect significant cell death at doses up to 30 µM. Instead, a dose-dependent increase in living cell count was observed. However, when the concentration was greater than 30 µM, a significant decrease in living cells was observed (Fig. 1a). The same phenomenon was observed in the number of BMDCs (Fig. 1b). To gain insight into the effects of AMG 487 on DC development from bone marrow cells, we applied increasing concentrations (3 µM, 15 µM, 30 µM) of AMG 487 from day 0 on. Although the expression of major histocompatibility complex II (I-A/I-E) was not significantly affected, the expression of co-stimulatory molecules, such as CD86,CD40 and the maturation marker CD83, on BMDCs was inhibited compared with vehicle-exposed cells (Fig. 1c). The expression of the inflammatory chemokine receptor CCR5 was not influenced. A slight increase in CXCR3 on BMDCs could indicate the binding of AMG 487 to CXCR3 leading to a feedback upregulation of CXCR3 under LPS stimulation. DC-SIGN is a pattern recognition receptor (PRR) that recognizes PAMPs (pathogen-associated molecular patterns) and mediates antigen capture and internalization (Geijtenbeek et al. 2009). Exposure to AMG 487 increased the expression of DC-SIGN on BMDCs, maintaining their capacity to capture antigen (Fig. d).

Exposure to AMG 487 Impaired LPS-Induced Activation of BMDCs

Next, we analyzed the effects of AMG 487 on LPS-induced activation of BMDCs. To exclude the effect of AMG 487 on the development of BMDCs, we cultured the cells in complete culture medium for 5 days without exposure to AMG 487. Due to the single addition of the compound for 1 day, we chose higher concentrations of AMG 487 (10 µM, 30 µM, and 60 µM). A single application of AMG 487 on day 6 and LPS-induced maturation revealed that, although I-A/I-E increased, AMG 487 dose-dependently inhibited the upregulation of CD80 and CD40 (Fig. 2a, b), and the expression of CD86 was not affected (Fig. 2c).

AMG 487 Increased the Expression of PD-L2 on BMDCS

Because AMG 487 inhibits the LPS-induced development and activation of BMDCs, we speculated that these AMG 487-treated DCs may share the characteristics of tolerogenic DC (Li and Shi 2015), which typically express a high level of PD-L1/PD-L2. We then analyzed the expression of PD-L1 and PD-L2 on AMG 487-treated DCs. The results showed that the expression of PD-L1 on the surface of AMG 487-treated DCs was all at a high level, regardless of the concentration of AMG 487. However, with an increase in AMG 487 concentration, the expression of PD-L2 up-regulated, indicating the increasing ability of BMDCs to transmit inhibitory signals (Fig. 3).

AMG 487 Reduced Murine BMDC-Induced Antigen-Specific T Cell Responses In Vitro

The most important function of DCs is to present antigens to T cells and promote T cell activation. Therefore, we analyzed the ability of AMG 487-treated DCs to induce antigen-specific $CD4^+$ mediated T cell responses. $CD4^+$ T cells from OT-II mice express a transgenic T cell receptor against the

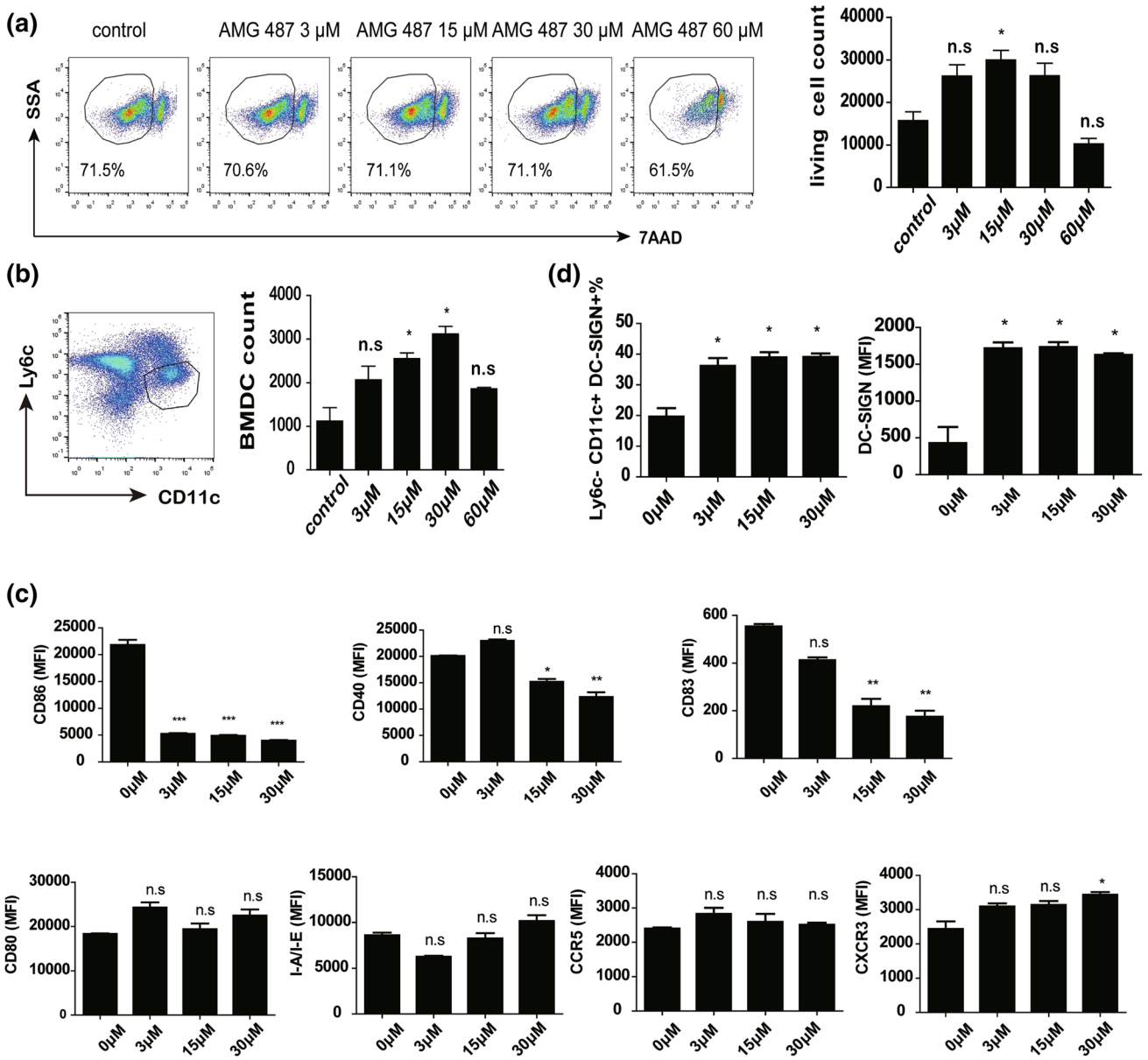

Fig. 1 AMG 487 inhibits the expression of co-stimulatory markers on DCs. **a** Proportion and absolute value of living cells (7AAD negative group) per well after exposure to different concentrations of AMG 487 (3 μM, 15 μM, 30 μM, and 60 μM) throughout the whole differentiation period. **b** The position of BMDCs ($CD11c^+$ Ly6C−) in a flow cytometry diagram and the absolute value of the BMDCs. **c** Bone marrow cells cultured under DC-driving conditions with final LPS stimulation were exposed to AMG 487(3 μM, 15 μM, 30 μM) every 3 days or vehicle and analyzed for expression of DC markers and inflammatory chemokine receptors. **d** The expression of DC-SIGN on BMDCs. The significance was calculated using the one-way ANOVA. Data are expressed as the mean ± SEM, $*p<0.05$, $**p<0.01$, $***p<0.001$ compared with vehicle (AMG 487 0 μM)

$OVA_{323–339}$ peptide presented in the context of MHC II. To exclude the direct effect of AMG 487 on T cells, BMDCs with AMG 487 were repeated washout before co-culture with T cells. The AMG 487-treated BMDCs resulted in markedly decreased activation of T cells, suggested by low expression of CD69 and CD25 expression (Fig. 4a), and reduced production of IFN-γ (Fig. 4b), retaining more T cells in an inactive state ($CD44^-CD62L^+$) (Fig. 4c).

Discussion

In recent years, more and more experimental and clinical evidence shows that the CXCR3 pathway is involved in autoimmune diseases and in graft versus host disease (GVHD) (Miao et al. 2018; Li et al. 2017; Croudace et al. 2012), especially through the production of a local inflammatory amplification cycle in the target organs, resulting in the

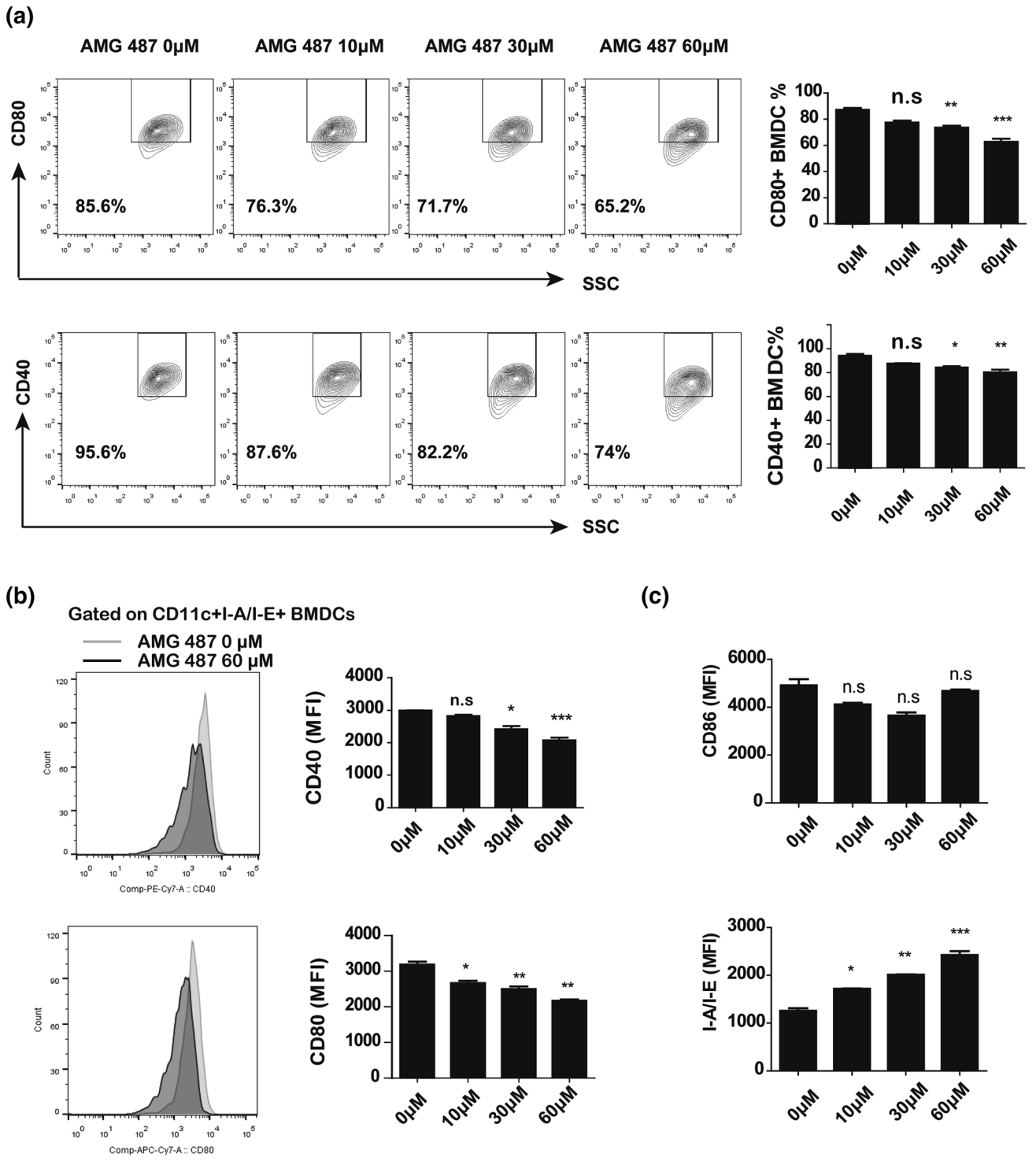

Fig. 2 AMG 487 impairs activation of BMDCs. **a** Analyses of the proportion of positive cells expressing DC activation markers. The results come from one experiment, repeated three times. **b** Expression of CD40 and CD80 on DCs and the mean fluorescence intensity (MFI) representative diagram. **c** Expression of I-A/I-E and CD86. Gray graph represent negative control with equal vehicle. The significance was calculated using a one-way ANOVA. Data are expressed as the mean ± SEM, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with vehicle (AMG 487 0 μM)

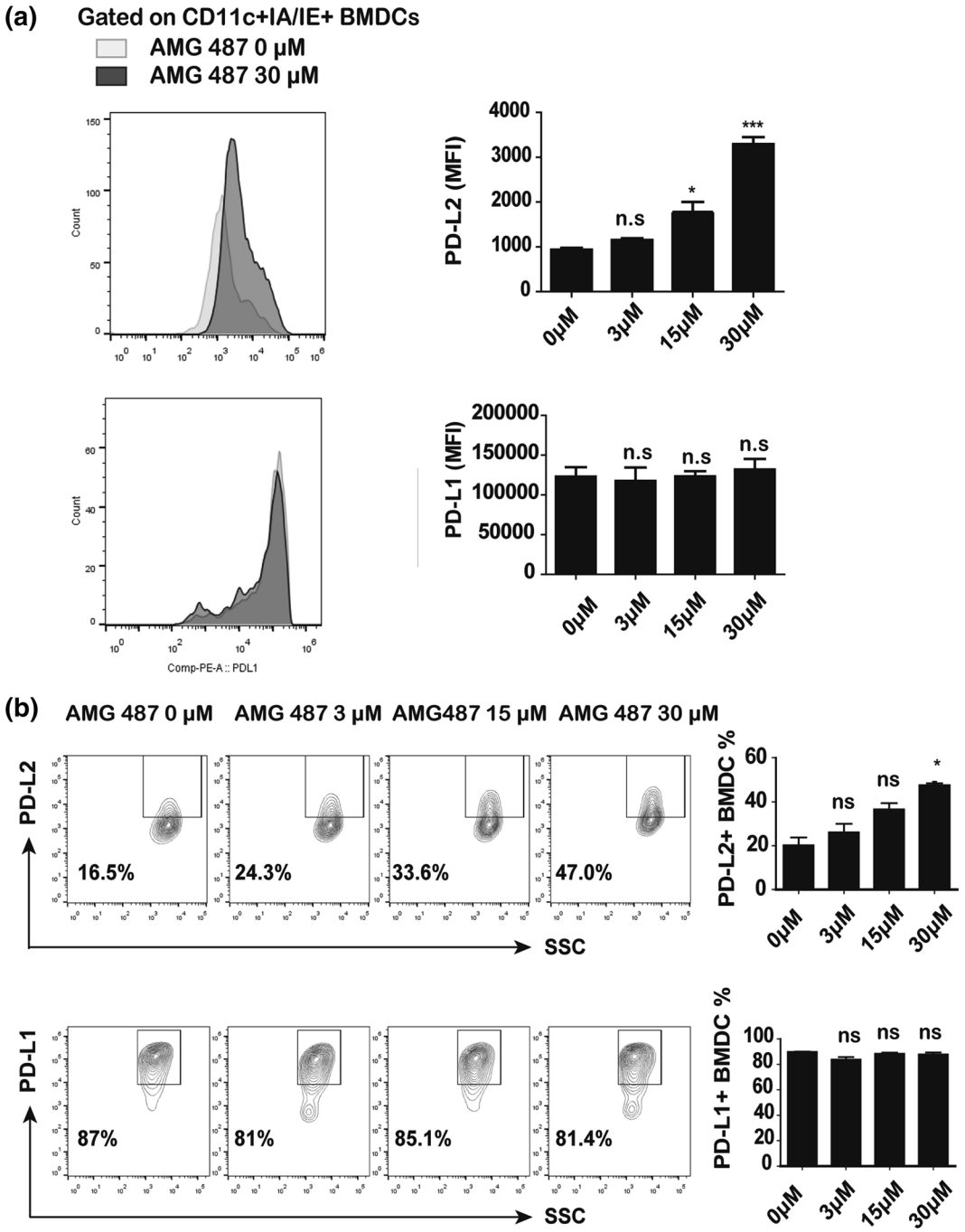

Fig. 3 AMG 487 increases the expression of PD-L2. Murine bone marrow cells cultured under DC-driving conditions with final LPS stimulation were exposed to different concentrations of AMG 487 (3 μM, 15 μM, 30 μM) or vehicle (0 μM) every three days throughout the differentiation period and tested for **a** expression of PD-L1, PD-L2, and **b** the proportion of positive cells expressing PD-L1 and PD-L2. Data are expressed as the mean ± SEM, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with vehicle (AMG 487 0 μM)

deterioration of clinical manifestations (Lacotte et al. 2009). The use of targeted blockers can alleviate clinical symptoms. However, the study of the direct immunoregulatory mechanism of AMG 487 on DCs is still very limited. Our study into the effect of AMG 487 on bone marrow-derived DCs first demonstrated that AMG 487 could inhibit BMDC maturation and LPS-induced activation, retaining the DCs in a less mature state. At the same time, the function by which DCs make specific presentations to T cells is decreased.

Immature DCs are weak T cell stimulators with few MHC and costimulatory molecules but with high antigen-capturing receptors. Once challenged with an antigen, the phenotype

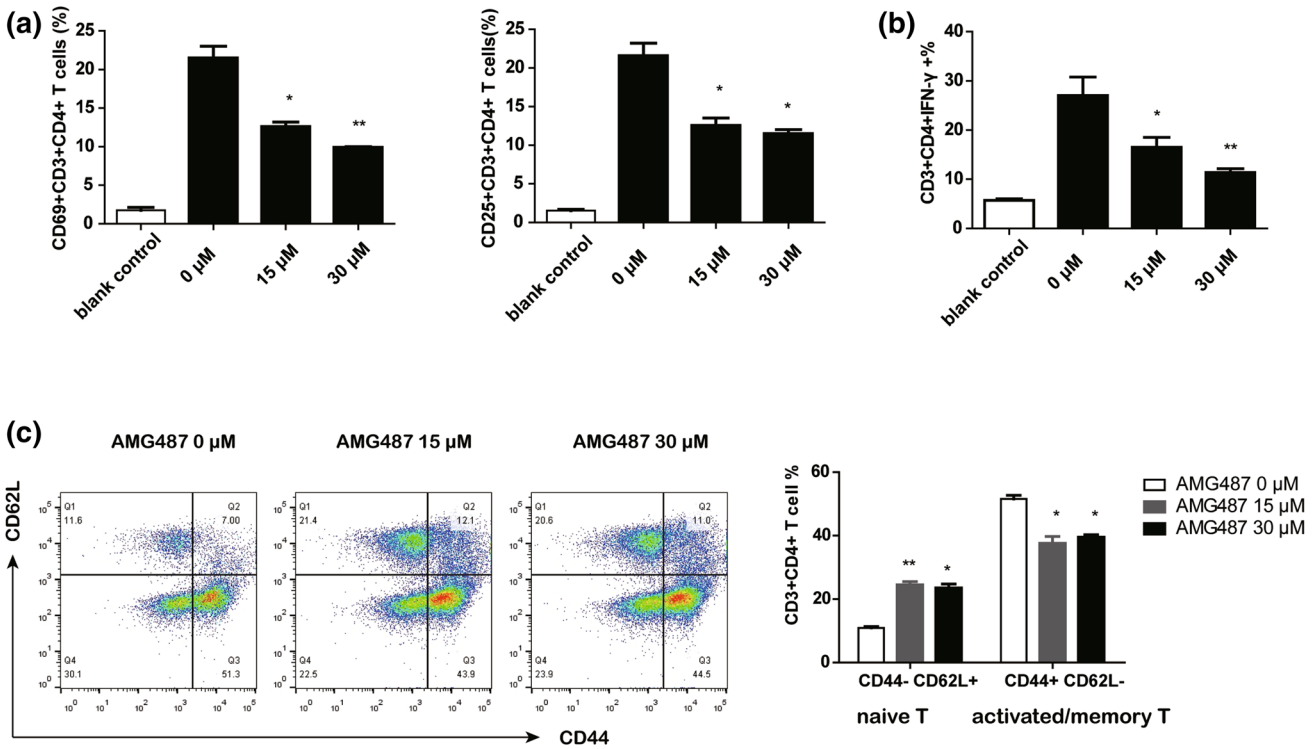

Fig. 4 AMG 487 impairs the T cell stimulatory function of BMDCs. AMG 487 (15 μM,30 μM) was added from day 0 on. Stimulation with LPS and $OVA_{323–339}$ peptide was carried out on day 6. 48–72 h later, IFN-γ production by endogenous $CD4^+$ T cells from OT II mice was analyzed by FACS after a brief re-stimulation. The AMG 487-treated BMDCs resulted in (**a**) a decreased proportion of CD69 or CD25 positive T cells and **b** low production of IFN-γ (analyzed by intracellular cytokine staining). **c** The ratio of activated T cells ($CD62L^-CD44^+$) was also decreased. Vehicle-treated BMDCs without $OVA_{323–339}$ peptide-loaded worked as a blank control. Data are expressed as the mean ± SEM, $*p<0.05$, $**p<0.01$, $***p<0.001$ compared with vehicle (AMG 487 0 μM)

changes rapidly and dramatically, down regulating the antigen-capturing devices and increasing the co-stimulatory molecules and T cell stimulatory functions (Banchereau and Steinman 1998). Our experiments showed that exposure to AMG 487 in vitro during development decreased the expression of some costimulatory molecules such as CD86, CD40 and CD83, leaving DC in a stage where it is not fully mature and that the antigen-capturing receptor DC-SIGN were still at a high level. We also demonstrated a decreased ability of DCs to induce specific T cell responses, a finding which we will discuss later, implying the AMG 487-treated BMDCs retained a semi-mature phenotype and had the capacity to capture antigens but could not stimulate T cells efficiently. To complement these data, we focus on a situation that reflected clinical situations more closely in that we also exposed already differentiated DCs to AMG 487, but only for a short period of time during LPS-induced activation. Although the expression of the MHC II molecule (I-A/I-E) was up-regulated, the expression of the costimulatory molecules, CD80 and CD40, was significantly inhibited by AMG 487, implying that the DCs activation was impaired.

The stimulating or inhibiting effect of DCs on T cells depends on the net sum of activating and inhibitory signals transmitted to T cells. Therefore, we also detected the expression of immunomodulatory molecule PD-L1/PD-L2 on the surface of AMG 487-treated DCs. The results showed that AMG 487-treated DCs upregulated the expression of PD-L2. All the PD-L1 was highly expressed and did not change with an increase in AMG 487 concentration. PD-L1 and PD-L2 are PD-1 ligands, belonging to the B7 family. PD-1 can be expressed in peripheral mature T cells, B cells, and activated myeloid cells. In the cytoplasm there is an immunoreceptor tyrosine inhibitory motif (ITIM) at the end, which generally transmits inhibitory signals and blocks the cell cycle, resulting in T cell disability and inducing immune tolerance (Sharpe and Freeman 2002). Under physiological conditions, DCs transmit inhibitory signals onto T cells through the PD-1 ligand/PD-1 axis, inducing hypo-responsiveness in auto-reactive T cells and avoiding the occurrence of autoimmune diseases (Probst et al. 2005). Therefore, our results indicate that AMG 487 enhances the ability of DCs to transmit inhibitory signals to T cells, a process that may be dominated by the PD-L2/PD-1 pathway.

With regard to the presenting function, when AMG 487-treated BMDC presented a specific polypeptide antigen ($OVA_{323–339}$) to OT-II $CD4^+$T cells, the T cells remained

more inactive and showed low reactivity than the controls, implying that the presenting function of the BMDCs was inhibited. This suggests that AMG 487 can inhibit the maturation and function of DCs, thus impairing DC-induced, antigen-specific T cell responses. Our in vivo experiment in murine aGVHD model has also found that long-term application of AMG 487 alone can inhibit the activation of T cells in the spleens of aGVHD mice after allogeneic transplantation (unpublished data). Based on our experiment results, we speculated that this phenomenon might be due to the functional inhibition of donor bone marrow-derived DC by AMG 487, which in turn inhibits T cell responses. The current in vitro data focused on a scenario that reflected more closely what happens in recipients after a hematopoietic stem cell transplantation, in that both DC and effector T cells are donor-derived after the complete chimerism of donor hematopoietic cells.

Tolerogenic DCs (tol-DCs) are a group of DCs that can induce T cell apoptosis, disability, and regulatory (Tregs) production. Recent studies have shown that tol-DCs promotes central and peripheral tolerance through a variety of mechanisms (Hill and Cuturi 2010): clearance of T cells; induction of Tregs production; incapacitation of T cells; expression of immunomodulatory molecules such as PD-L1, PD-L2, heme oxygenase-1, CD95L, and TNF-related apoptosis-inducing ligands; and production of immunosuppressive factors (Ezzelarab and Thomson 2011; Ilarregui et al. 2009; Remy et al. 2009). At present, many drugs and cytokines, such as cyclosporine A, rapamycin, vitamin A, and IL-10 (Boks et al. 2012), have been found to induce tolerogenic DCs (Hackstein and Thomson 2004; Hu and Wan 2011; Min et al. 2000). Although there are many ways to induce tol-DC, these DC subsets share common characteristics, that is a semi-mature cell state and the ability to inhibit an allogeneic T cell response. In our in vitro experiment, the maturation and activation of AMG 487-treated DCs was suppressed with high levels of PD-L2 and showed a decreased capacity to induce specific T cell responses, in accordance with this characteristic. These results suggest that AMG 487 can induce BMDCs to have characteristics similar to tolerogenic DCs, but we need more in vivo experimental data to support this conclusion. In addition, in order to further explain why AMG 487 leaves DC in a semi-mature state and exhibits characteristics similar to tolerated DC, experiments using bone marrow derived from CXCR3 knockout mice will help.

The limitation of our study is that we only studied the effect of AMG487 on mouse BMDC in vitro. However, human monocyte-derived DCs are not completely similar to mice, so our results cannot yet fully reflect the situation in humans, which is also our ongoing research.

Our results may contribute to a more detailed understanding of the effects of AMG 487 on mouse bone marrow-derived DC, making them have the characteristics and functions similar to tolerated DC. It can also provide a laboratory basis for the treatment of autoimmune diseases such as non-specific arthritis (Mohan and Issekutz 2007) or systemic lupus erythematosus (Moser et al. 2012), where DCs play a key role in the pathogenesis.

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Compliance with Ethical Standards

Conflict of Interest Statement The authors declared that they have no competing interest.

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