Continuous retinoic acid induces the differentiation of mature regulatory monocytes but fails to induce regulatory dendritic cells

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Abstract

**Background:** Myeloid cells (MCs) have potent immunoregulatory abilities that can be therapeutically useful to treat inflammatory disease. However, the factors which promote regulatory myeloid cell differentiation remain poorly understood. We have previously shown that estriol (E3) induces mature regulatory dendritic cells in vivo. To determine whether additional steroid hormones could induce mature regulatory myeloid cells, we investigated the effects of retinoic acid (RA) on MCs. Retinoic acid is a steroid hormone important in regulating mucosal immunity in the gut and promoting myeloid differentiation. We hypothesized that the presence of RA during differentiation would promote the formation of mature regulatory myeloid cells (MCregs).

**Methods:** To determine RA's ability to induce regulatory myeloid cells, we differentiated bone marrow progenitor cells with granulocytic-macrophage colony-stimulating factor (GM-CSF) under the influence of RA. We found that day 7 MCs differentiated in the presence of RA had an increase in the percent positive and relative expression levels of both maturation (CD80, CD86, and MHCII) and inhibitory (PD-L1 and PD-L2) markers compared to control cells. Functionally, these day 7 RA MCs expressed increased intracellular IL-10, induced regulatory T cells in vitro compared to controls and suppressed the proliferation of responder immune cells even after inflammatory challenge with LPS.

**Conclusion:** RA induced mature regulatory myeloid cells that were suppressive and had a CD11b+ CD11c-Ly6C low/intermediate monocyte phenotype. Surprisingly, RA CD11c+ dendritic cells were not suppressive and could contribute to enhanced proliferation. These results suggest that continuous RA has unique effects on different myeloid populations during monopoesis and dendropoesis and promotes a population of regulatory monocytes.

**Keywords:** Regulatory myeloid cells, Dendritic cells, Retinoic acid, Monocyte

Background

Myeloid cells (MCs) are a diverse population of cells that form during hematopoiesis and play a critical role in host defense. Comprised of granulocytes, mononuclear phagocytes and their precursors, MCs are innate immune cells that have an important role in promoting inflammation and the induction of adaptive immune responses. Inflammatory MCs are induced and increased in numbers following exposure to exogenous (e.g. pathogens) or endogenous “danger” (e.g. post-necrotic release of high-mobility group box 1 or HMGB1) signals by [1]. These and other environmental factors present within peripheral tissue and bone marrow impact granulopoiesis, monopoiesis and dendropoiesis to influence the ultimate fate of inflammatory granulocytes, monocytes/macrophages and DCs, respectively. While inflammatory MCs have been well characterized, within the last several years the potent regulatory abilities of these cells has increasingly been recognized. Such regulatory MCs (MCregs) are a diverse population of cells with the ability to control inflammation and, thus, are a promising target to treat a wide array of inflammatory diseases. To date, however, factors involved in the differentiation of MCregs populations remain poorly understood.
MC\textsubscript{reg} subsets are particularly diverse, both in terminology and in function. Regulatory, tolerogenic, type II or steady-state are terms applied to regulatory populations of DCs, macrophages, monocytes, and their precursors [1-3]. By and large, the regulatory abilities of macrophages, and more recently, DCs have been most thoroughly studied. First described over 30 years ago, alternatively activated macrophages are able to promote wound healing and resolve inflammation [4,5]. Over the last 10 years the regulatory abilities of DCs and their therapeutic potential have been the focus of many studies [2,6]. Monocytes are circulating myeloid cells that give rise to tissue macrophages and DCs. Monocytes have been recognized as a contributor to the inflammatory responses, and are now known to contribute to immune regulation [7]. MC\textsubscript{regs} can regulate immune responses through the production of soluble regulatory factors (e.g. IL-10, TGF-beta, indoleamine 2,3 deoxygenase (IDO), arginase, nitric oxide (NO), etc.), expression of inhibitory or regulatory cell surface molecules (e.g. PD-L1, PD-L2) and induction other regulatory cells (e.g. regulatory T cells; T\textsubscript{regs}) or enhance regulatory feed-back loops [8,9]. At present, MC\textsubscript{regs} are identified based on combination of phenotype and function, with no equivalent to T\textsubscript{regs} FoxP3 marker being as yet identified [10-13]. Through cell-cell interactions and the production of soluble immunoregulatory molecules, MC\textsubscript{regs} have very potent and diverse means of inducing immune regulation. However, much remains to be characterized about factors controlling MC\textsubscript{reg} induction and how different MC\textsubscript{reg} subsets regulate immune responses. Given that MC\textsubscript{reg} therapy has the potential to diminish disease in the 100+ millions of individuals impacted by immune-mediated, chronic inflammatory and autoimmune diseases worldwide, it is critical to determine the factors which govern the induction and function of these cells [14-16]. The therapeutic potential of MC\textsubscript{regs}, has been described in several experimental models of inflammatory and autoimmune disease. Specifically, MC\textsubscript{regs}, including MDSC, conventional DCs, lung-resident tissue macrophages, monocytes, and plasmacytoid DCs have all been shown to impact disease course in animal models of diabetes [17], colitis [18], allergic asthma [19], experimental autoimmune disease [20], and rheumatoid arthritis [21] respectively.

For many MC\textsubscript{regs} an arrest in immature and/or altered functionality contributes to their regulatory abilities [22,23]. Glucocorticoids, vitamin D and IL-10 are the most common means to induce these immature MC\textsubscript{regs}. These altered MC\textsubscript{regs} cells have decreased expression levels of maturation/activation markers CD80, CD86 and MHC class II [2,24-28]. Additionally, these immature MC\textsubscript{regs} can have reduced inflammatory cytokine expression [29,30], overall blunted function, induce T\textsubscript{regs} and suppress the action of other immune cells. However, a primary concern with using immature MC\textsubscript{regs} for therapy is that they may mature into inflammatory MCs under inflammatory disease conditions. Such inflammatory MCs could then actually exacerbate the very inflammatory disease they were used to treat [2,22,23,31]. Thus, mature (and stable) MC\textsubscript{regs} may avoid such concerns but, to date only a handful of studies have significantly explored the induction of such mature MC\textsubscript{regs} [18,22,32]. Typically, mature MC\textsubscript{regs} have been induced by combining traditional immature MC\textsubscript{reg} induction protocols with the addition of inflammatory stimuli such as LPS or TNF-alpha [33,34]. Our laboratory has focused on identifying non-inflammatory systems to induce mature MC\textsubscript{regs} and we have previously found that estriol (E3), a steroid hormone of pregnancy, produce mature activated DC\textsubscript{regs} [35]. These E3 DC\textsubscript{regs} maintained their regulatory abilities within an inflammatory environment and protected mice against the inflammatory autoimmune disease, experimental autoimmune encephalomyelitis (EAE) [35]. Although E3 shows promise, the fact that there are limitations on using estrogens broadly in the human patient population necessitated investigating alternative means of inducing mature stable MC\textsubscript{reg} populations.

All-trans retinoic acid (RA) is a steroid hormone metabolite of vitamin A that plays both an important role during embryonic development and has recently been identified as the key metabolite regulating immune responses at mucosal sites [36-38]. RA is a logical candidate for inducing mature MC\textsubscript{regs} given its defined role in both mucosal immunoregulation and its ability to promote myeloid cell differentiation and maturation. Within the gut, RA influences the balance between T\textsubscript{reg} and Th17 cells, B cell iso-type switching, antibody production and mucosal homing of numerous immune cells [6,37,39-43]. Mucosal myeloid cells are largely responsible for producing local RA which acts in a paracrine and autocrine manner to regulate mucosal immune responses [6,37]. Although mucosal DCs produce much of the RA required for immune regulation at mucosal sites, much less is known about RA's direct impact on MC populations at both mucosal and non-mucosal sites [9,19,39,40].

RA regulates myeloid cell survival and promotes the differentiation of immature myeloid cells into mature populations of DCs, macrophages and granulocytes [18,44-46]. Additionally, RA appears to be required for the production of mature phagocytes in the bone marrow through its effects on MHC class II and co-stimulatory molecule expression [47]. Therapeutically, RA has long been used to treat myeloid leukemia given that it promotes myeloid cell differentiation and maturation [48,49]. More recently, it has been used to promote the differentiation of immature myeloid cells (i.e. myeloid derived suppressor cells; MDSCs) in cancer patients to diminish immunosuppressive MDSC effects.
[36,44,50-53]. Given RA’s important roles in both mucosal immunoregulation and myeloid cell differentiation we hypothesized that RA would induce mature MCregs.

Using an in vitro model to induce differentiation of MC populations (i.e. DCS, macrophages and monocytes), we evaluated the ability of RA to generate mature MCregs [42,54]. We demonstrated that bone marrow cells differentiated with GM-CSF for 7 days in the presence of RA had an activated regulatory phenotype (i.e. increased CD80, CD86, MHC class II, PD-L1 and PD-L2), produced increased IL-10, increased the induction of Treg and suppressed the proliferation of responder immune cells. We found that the suppressive population was a small but potent CD11b+ CD11c+ Ly6Clow/intermediate population whose phenotype is consistent with a regulatory monocyte. Surprisingly the CD11c+ DCs were not suppressive. Taken together these results demonstrate a differential effect of RA during monopoiesis and dendropoiesis which results in the induction of regulatory monocytes but not regulatory DCs.

**Results**

**Differentiation with retinoic acid induced mature activated regulatory myeloid cells**

Given that RA is a regulator of mucosal immunity and influences myelopoiesis, we hypothesized that RA would induce a population of mature MCregs. Day 6–7 BM cells differentiated with GM-CSF in the presence of RA were able to suppress the proliferation of responder immune cells and this suppression was markedly greater than either control or E3 treated cells (Figure 1A). The ability of RA differentiated cells to suppress proliferation was apparent regardless of whether responder immune cells were stimulated with either peptide or anti-CD3. Interestingly, cells treated with E3 suppressed proliferation after stimulation with peptide but not anti-CD3 (Figure 1A).

We next determined whether the RA differentiated cells remained regulatory when exposed to the inflammatory stimulus LPS. Figure 1B shows that RA differentiated cells maintained their ability to suppress proliferation even after exposure to LPS challenge and that this was present following stimulation of co-cultures with either peptide or anti-CD3. This effect was entirely lost in E3 treated cells. These results suggest that RA differentiated cells are more potent and stable than E3 differentiated cells and that RA differentiated cells maintain their regulatory ability following exposure to an inflammatory stimulus.

Given that increased IL-10 is seen in E3 DCregs [35] and other MCregs populations [50,55] we next evaluated whether RA induced an increase number of IL-10+ cells. Figure 1C shows that RA differentiated cells had an increased percentage of IL-10-producing cells compared to either media or E3 control cells. We next evaluated whether RA differentiated cells could increase Treg numbers. We found that RA differentiated cells were able to induce a significant increased percentage of FoxP3+ cells following a 5 day culture with naive immune cells (Figure 1D). Cells differentiated in vitro in the presence of E3 failed to significantly increase either IL-10+ cells or induce Treg cells (Figures 1C, D). These results show that RA differentiated cells suppressed the proliferative abilities of responder immune cells and induced FoxP3+ (Treg) cells.

To determine whether these RA differentiated cells were mature, we evaluated the cell surface expression of maturation markers CD80, CD86 and MHC class II and inhibitory markers PD-L1 and PD-L2. RA differentiated cells demonstrated an increased percentage of CD80+, CD86+ and MHC class II (Figure 2A), indicating that an increased proportion of the cells were mature and/or activated in comparison to E3 or control cells. Additionally, there were increases in the mean fluorescence intensity (MFI) of CD80, CD86 and MHC class II in RA differentiated cells as depicted in Figures 2C and D, indicating that the relative expression levels on a per cell basis were increased in RA differentiated cells. Although E3 differentiated cells had mildly increased expression levels of CD80, CD86 and MHC class II, RA differentiated cells had consistently higher levels than either E3 differentiated or control cells. To confirm that RA differentiated cells demonstrated an “activated regulatory” phenotype as previously described for E3, we evaluated the expression of inhibitory co-stimulatory molecules PD-L1 and PD-L2 [35]. RA increased the percentage of PD-L1+ cells (but not PD-L2+) (Figure 2B) and the MFI of both PD-L1 and PD-L2 (Figure 2C, D) compared to E3 or media controls. These results demonstrate that during differentiation RA induces a population of mature activated MCregs that suppress the proliferation of responder immune cells even in the face of inflammatory challenge. Additionally, our data shows that although both RA and E3 may induce MCregs which suppress proliferation (Figure 1A) RA MCregs appear to have superior regulatory abilities compared to E3 MCregs.

**CD11b+ but not CD11c+ cells were the suppressive population**

The in vitro differentiation of bone marrow cells with GM-CSF is a commonly used protocol to produce large numbers (>80%) of highly enriched CD11c+ DCs [38,56] that, as a population, are considered immature DCs. However, our data demonstrated that while approximately 80-90% of the cells were CD11c+, the remaining 10-20% were CD11c- but still CD11b+ (Additional file 1: Figure S1A). To determine whether the MCregs induced by RA were DCs, we purified CD11c+ cells from day 7 differentiated cells and cultured them with responder
immune cells. Although RA induction of mucosal "DCregs" have been described [9,36,57], we found that RA-treated CD11c+ cells were not the suppressive cell population (Figure 3A). In all experiments, RA-treated CD11c+ cells failed to suppress proliferation and had variable to no effect on proliferation with some experiments actually demonstrating enhanced proliferation (data not shown). Phenotypic evaluation of these CD11c+ cells showed no difference in percentage (Figure 3B) or expression levels of CD80, CD86, MHC class II, PD-L1 and PD-L2 compared to media controls. To determine the source of the suppressive MCregs, we evaluated the CD11c- population and found that the RA CD11c- cells suppressed proliferation of responder cells (Figure 3C). These CD11c- cells had a marked (>30%) increase in the percentage of CD80+, CD86+, MHC class II+ and PD-L1+ cells (with no differences in PD-L2+ cells) (Figure 3D) when differentiated with RA, consistent with an activated regulatory phenotype in these cells described previously [35]. In contrast, levels of CD80, MHC class II and PD-L1 did not change, remaining consistently high (>80%) in RA versus control MCs. These data suggest that RA present during GM-CSF differentiation increased an activated regulatory phenotype in the CD11c- (non-DC) populations.

Both differentiated and precursor populations within the bone marrow are predominantly but not completely CD11b+ (>90%) (Additional file 1: Figure S1A). To definitively isolate the effects of CD11b+ CD11c- cells, we serially purified CD11b+ cells from the CD11c- fraction and evaluated their phenotype and function. As expected, the increases in the percentage of CD80+, CD86+, MHC class II+ and PD-L1+ cells seen in Figure 3D was also seen in the CD11b+ CD11c- serially purified population.
We then went on to evaluate the ability of these cells to influence CD4 and CD8 responses. We found that the CD11b+ CD11c- population was able to suppress the proliferation of responder immune cells (Figure 4B) and could modify the cytokine profile of T cells. The proliferating CD4+ responder immune cells cultured with RA CD11b+ CD11c- cells were also shown to have reduced expression of IL-17 IFN-gamma (Figure 4C) and IL-10 (Additional file 2: Figure S2) but no change in IL-4 production as determined by intracellular cytokine staining (Figure 4C). Intracellular IL-10 and FoxP3+ cells were also increased as expected (Additional file 2: Figure S2A and S2B, respectively). We also evaluated the ability of RA CD11b+ CD11c- cells to influence CD8+ T cell responses. Figure 4D demonstrates reduced cytotoxicity in CD8+ T cells cultured with RA CD11b+ CD11c- cells. Taken together, these results suggest that RA induced an activated regulatory population of CD11b+ CD11c- cells that were able to suppress both CD4+ and CD8+ adaptive immune responses.

CD11b+ CD11c-Ly6Clo/intermediate were the primary population responsible for suppression

Although used primarily to induce large numbers of DCs, differentiation with GM-CSF can potentially promote the differentiation of a mixture of granulocytes, monocytes, macrophages and DCs [42,54,56,58-60] In our GM-CSF cultures, we found that Ly-6G+ granulocytes were no longer present in CD11b+ cells at day 7 of differentiation (Additional file 1: Figure S1B), indicating that granulocytes were not responsible for the suppression seen [61,62]. To determine whether monocytes were present and may be responsible for the suppressive effects, we evaluated day 7 non-adherent cells sorted based on their relative expression of the monocyte marker Ly-6C. Ly-6C expression levels have been shown to correlate with cellular function and maturation level where Ly-6C<sub>high</sub> monocytes are inflammatory and Ly-6C<sub>low</sub> monocytes are steady-state or regulatory [7,63]. Figure 5A shows that the presence of RA during differentiation increased the percentage of cells expressing...
low to intermediate levels of Ly6C. To determine whether the increase in these cells was responsible for the suppression seen in the CD11b+ CD11c+ population, we sorted cells based on Ly-6Clow, Ly-6C intermediate, and Ly-6C high expression patterns. Figure 5B demonstrates that both Ly-6Clow and Ly-6C intermediate cells were able to suppress up to a 6-fold decrease in proliferation of responder cells following antigenic stimulation while Ly-6C high cells failed to influence peptide-specific proliferation. Similarly, Ly-6C low and Ly-6C intermediate cells maintained their ability to suppress proliferation (Figure 5C) even when co-cultures were stimulated with LPS. In contrast, Ly-6C high actually significantly increased the proliferation of responder immune cells (Figure 5C) following stimulation with LPS. These results demonstrate that RA Ly-6C low and Ly-6C intermediate cells are suppressive populations and are able to maintain suppressive abilities even in the presence of inflammatory (LPS) challenge. Phenotypically, RA Ly-6C low cells showed the most marked increase in the percentage of PD-L1+ compared to CD86+ and MHC class II+ cells, with over 90% of the Ly-6C low cells expressing PD-L1 (Figure 6). A similar but less dramatic phenotype was seen in the Ly-6C intermediate cells (data not shown). Taken together, these data show that RA induces a small but potent population of CD11b+ CD11c+ Ly-6C low/intermediate MCregs consistent with an activated regulatory monocyte phenotype that are able to suppress immune cell proliferation.

Discussion

The principal objective of this study was to determine whether RA, a steroid hormone known to play important roles in regulating both mucosal immune responses and differentiation of myeloid cells could generate an activated (or mature) MCreg population. We demonstrate that RA influences myelopoiesis to a regulatory MCreg (monocyte) with the phenotype of CD11b+ CD11c+ Ly-6C low/intermediate MCregs, consistent with an activated regulatory monocyte phenotype that are able to suppress immune cell proliferation.

MCregs are a diverse population of cells and much attention has focused on the in vitro generation and clinical application of MCregs. While the in vitro generation of such MCreg populations has great therapeutic potential, understanding the mechanisms of their development under different conditions is crucial.

Figure 3 RA mediated suppression of T cell proliferation is not mediated by CD11c+ BM-MCs. BM-MCs were magnetically separated with CD11c+ beads. Purity of CD11c+ and CD11c- cells was confirmed and cells were analyzed on the BD Accuri C6 Flow cytometer. Purified CD11c+ (A) and CD11c- (C) were co-cultured with responder immune cells for 96 hours with media or anti-CD3 stimulation and then pulsed with H3 thymidine in the final 18 hours of culture; and the relative percentages of CD11c+ (B) and CD11c- (D) cells expressing maturation markers CD80, CD86, MHCII, PD-L1 and PD-L2 were determined. Data are representative of three separate experiments.* = p < 0.05.
potential, much remains to be learned regarding the factors which contribute to MCreg induction. The majority of in vitro generated MCregs are arrested in an immature or hypo-functional state. An emerging concern is that these immature MCregs populations may mature to become inflammatory DCs or macrophages and, thus, contribute to...
inflammatory disease pathology [2,22,23,29-31,64]. A more recent approach is to induce mature MCregs which would be stable and maintain regulatory potential in an inflammatory environment [22,32,65,66]. Anderson and colleagues have demonstrated that human DCregs (generated with dexamethasone, vitamin D and LPS) maintain tolerogenic activity and actually induce significantly higher levels of IL-10 production by resultant T cells [33]. However, the relative stability and ability of MCregs (such as DCregs) to maintain regulatory abilities during inflammation may still be in question. For example, a study by Voigtlander et al. suggests that DCregs induced by TNF-alpha do not maintain their regulatory abilities upon a secondary stimulation with TNF-alpha in vivo [34]. Obviously, this is of considerable concern given that TNF-alpha is present in a large array of inflammatory conditions where such DCregs (or other MCreg populations) may be applied therapeutically. Much work remains to determine critical factors important in generating mature MCregs for anti-inflammatory therapies but we have focused on non-inflammatory pathways to induce mature MCregs.

We have shown that mature MCregs can be generated with the use of steroid hormones alone [35]. Our previous work has shown that the sex steroid hormone estriol (E3) induces a mature activated MCreg population of CD11c+ DCregs that protects against inflammatory challenge in vitro and in an in vivo disease model [35]. In the present study, we have extended our research of pathways involved in normal homeostatic induction of mature MCregs by investigating the ability of the steroid hormone RA to induce mature MCregs that are resistant to inflammatory challenge. Our results show that RA is more effective than E3 in vitro in generating MCregs and that these MCregs are resistant to LPS inflammatory challenge.

Figure 5 RA CD11b+ CD11c− Ly6Clow/intermediate are the suppressive population. BM cells were differentiated in the presence of GM-CSF with or without 100 nM RA for 7 days, stained with fluorescently-labeled antibodies against CD11c, CD11b+, and Ly-6C to determine relative expression of Ly6C on CD11b+ CD11c cells (A). Cells were then sorted based on relative Ly6C expression and purified populations co-cultured with antigen-specific T cell receptor transgenic T cells for 96 hours with media, peptide or LPS and fold change in proliferation shown for peptide-stimulated (B) or LPS-stimulated (C) co-cultures. Data are representative of at least three separate experiments. * = p < 0.05.

RA is known for its ability to promote the differentiation, and maturation, of myeloid cell populations. This ability, along with its known immunoregulatory role at mucosal sites, made it a logical candidate for these studies [44,52]. RA is present in relatively large concentrations within mucosal sites and is largely produced by local antigen presenting cells (APCs) residing within these mucosal sites. Specifically, mucosal CD103+ DCs are the primary immunoregulatory myeloid cells within the gut. These DCs have up-regulated raldh2 gene expression, constitutively produce RA, and produce increased TGF-beta. They also have a significant ability to induce Foxp3+ Tregs, mucosal homing receptors CCR9 and α4β7 expression on lymphocytes and enhance antibody production and Ig isotype switching [6,9,36,57]. These mucosal DCs are the most common MCs investigated regarding RA biology and induced mucosal DCs have been generated from monocytes...
or splenic DCs with GM-CSF with IL-4 [8,43] or bone marrow precursors with RA [18,43,57,67,68]. Increasingly, the non-mucosal and therapeutic applications of RA (i.e. in cancer) are being investigated [9,19,43,44,53] and this study focused on RA’s ability to induce mature activated MCregs that are able to suppress responder immune cell proliferation [8,35,41,43,57,69].

Given RA’s critical role in DC-mediated immunoregulation within the gut, it was quite surprising that RA CD11c+ cells were not suppressive. One possibility is that DC’s differentiated with RA could generate mucosal DCs but wouldn’t generate mature activated DCregs that could suppress proliferation as seen with E3 DCregs. While induction of mucosal DCs can be accomplished with RA [18,43], the immunomodulatory abilities of these DCs as described in these studies was not the focus of this study. Alternatively, timing of RA administration may have resulted in the lack of DCreg induction as described by Feng and colleagues [41]. Specifically, their studies showed that the presence of 1 μM RA from day 0 throughout differentiation failed to induce mucosal DCs. Although different dosages and criteria were used to generate and identify DCs as mucosal (versus DCreg in our study), the continuous presence of RA during differentiation may have resulted in the inability to induce DCreg in our study. Similarly, Wada’s group showed that the use of a synthetic RARx and β agonist (AM-80) could differentiate human peripheral blood monocytes into dendritic cells that have a tolerogenic phenotype and function [18]. The use of AM-80 versus ATRA in our study or the differentiation of human monocytes versus murine myeloid progenitors could explain the differences in DCreg versus MCreg in our study.

It could be argued that CD11c DC precursors existed within the population of CD11b+ CD11c+ cells. Given the described effects of RA in promoting differentiation and maturation, in conjunction with our data demonstrating an activated phenotype, we believe this to be unlikely [57,70-72]. Rather, our data on Ly6C expression strongly support that the suppressive cells were regulatory monocytes with an activated regulatory phenotype (increased CD80, CD86, MHC class II and PD-L1) consistent with previous work within our lab. Given that the CD11b+ CD11c population is comprised less than 20% of the entire population, the ability of these cells to suppress both CD4+ and CD8+ responses is noteworthy. The specific contributions of cell contact-dependent (i.e. PD-L1) versus cell contact-independent (i.e. IL-10, TGF-beta, etc.) mechanisms responsible for the regulatory abilities of these cells was beyond the scope of this study. However, we did see increases in regulatory markers including PD-L1, IL-10 and the percentage of FoxP3+ cells with RA MCregs.

Monocytes are circulating myeloid cells which give rise to tissue DCs and macrophages, and their regulatory abilities have recently been recognized [7]. Although numerous markers can be present on mouse monocytes (e.g.
CD11b, CD115, CCR2, CX3CR1 and Ly-6C), we chose to investigate Ly6C expression levels given that they have been correlated with monocyte function [7,63,71,73]. Specifically, Ly-6C<sup>high</sup> represents an inflammatory monocytes while, Ly-6C<sup>low/intermediate</sup> monocytes have been shown to play important roles in patrolling the vasculature and potentially resolving inflammation and tissue repair [7,63,74-76]. Ly6C is also down regulated following differentiation which is consistent with our findings where RA, a molecule known to promote differentiation and maturation, increases the percentage of cells that are Ly-6C<sup>low/intermediate</sup> (Figure 5A) [3,44]. Our data suggest that Ly-6C levels correlate with suppressive abilities with the lowest Ly-6C expression associated with the most suppressive ability. Given that Ly-6C<sup>high</sup> monocytes are typically inflammatory monocytes, it is not surprising that proliferation is actually enhanced following LPS stimulation in this cell population (Figure 5C). Taken together, these data showed a progression from Ly-6C<sup>high</sup> to Ly-6C<sup>low</sup> associated with increasing regulatory abilities. These results are consistent with the association seen between Ly-6C expression and blood monocyte function described by others [7,63,71,77]. Currently, the mechanisms and pathways by which RA maturation of monocytes imparts them with increased regulatory abilities remain undefined. Whether a specific signal during differentiation drives monocytes to become regulatory in an active process or whether differentiation under homeostatic or regulatory (i.e. RA) conditions in the absence of inflammatory stimuli is a default mechanism for regulatory monocyte induction is unknown. Additionally, whether these RA Ly-6C<sup>low/intermediate</sup> monocytes have the potential to further differentiate into DC<sub>reg</sub> or regulatory macrophage populations remains to be determined and is the subject of ongoing studies within the laboratory [7].

**Conclusion**

Our findings show that continuous RA exposure during myelopoiesis promotes the induction of MC<sub>reg</sub>. Specifically, RA induced CD11b<sup>+</sup> CD11c<sup>-</sup> Ly-6C<sub>low</sub> regulatory monocytes which suppressed the proliferation of immune cells but RA failed to induce DC<sub>reg</sub>. Our data suggests that RA has unique effects on different myeloid populations during differentiation that may influence the regulatory abilities of monocytes and DCs. A more thorough understanding of how RA mediates these differential effects has important implications in our understanding of MC<sub>reg</sub> biology and the potential application of these cells to treat a wide variety of inflammatory diseases.

**Methods**

**Mice**

C57BL/6 (H-2<sup>b</sup>) mice (4–8 wk old), C57BL/6-Tg (TcraTcrb) 425Cbn/J, C57BL/6-Tg(Tcra2D2,Tcrb2D2)1Kuch/J and reporter Foxp3EGFP (B6.Cg-Foxp3<sup>tm2Tch</sup>) were purchased from Jackson Laboratories (Bar Harbor, ME, USA) or bred in-house. Mice were housed five per cage and maintained on a 12 hr. light/dark cycle, maintained under specific pathogen-free conditions and were housed and cared for according to the institutional guidelines of the Ohio State University’s Institute for Animal Care and Use Committee.

**Cell lines**

EG7 and EL7 (kindly provided by P. Boyaka, Ohio State University) were used to study the MHC class I-restricted response of CTLs in mice. The EG7 cells have been transfected with plasma to synthesize and constitutively secrete OVA 257–264 peptide and should be cultured in 10% RPMI. The EL4 cells are the non-OVA secreting duplicate of the EG7. Both are commonly found at ATCC but were acquired through Dr. Boyaka. The DC2.4 cell line was kindly provided by K. Rock, University of Massachusetts and as a DC antigen-presenting cell.

**BM-MC differentiation and development of regulatory MC differentiation model**

Bone marrow (BM) cells were collected from C57Bl/6 mice femurs and tibias. After erythrocyte lysis (AKC or in-house lysis buffer), cells were cultured with RPMI 1640 (Invitrogen) supplemented with 10% FBS, 25 mM HEPES, 2 mM L-glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin, 5 × 10<sup>-5</sup> M 2-mercaptoethanol and 200U/ml recombinant murine GM-CSF (R&D Systems) ± 100 nM of either estradiol (E3) or all-trans retinoic acid (RA) (Sigma-Aldrich) for 6–7 days at a density of 2 × 10<sup>6</sup> cells/ml. Day 6–7 cells were considered differentiated BM-MCs (media control) and BM-MC<sub>reg</sub> (RA and E3). Cells were challenged with inflammatory stimulus LPS (1 μg/ml, 055:B5, Sigma-Aldrich) during culture as indicated at day 6 or later for BM-DCs.

**Functional immunosuppressive assays: T cell proliferation assay**

Myeloid cells (BM-MCs or BM-MC<sub>reg</sub>) were cultured with responder spleen cells from antigen-specific T cell receptor transgenic (TCR Tg; where antigen was either OVA323–339 or MOG35-55) or Foxp3EGFP mice as indicated. To assess T cell proliferation co-cultures were stimulated with anti-CD3 (BD Bioscience), T cell-receptor specific antigen MOG35-55 (Bio Matic) or T cell-receptor specific antigen OVA 323–339 (Anaspec). To assess the effects of myeloid cell activation, co-cultures were stimulated with LPS from Escherichia coli, 055:B5 (Sigma-Aldrich) for 96 hours, pulsed with (H<sup>3</sup> thymidine) (Perkin Elmer Life Sciences or MP Biomedicals) in the last 18 hours, harvested and counted, data is expressed as counts per million (cpm) ± SEM [35].
Functional immunosuppressive assay: CD8\(^+\) cytotoxic assay

To generate CTLs, spleen and lymph nodes (LN) were removed from OT-1 mice and co-cultured with OVA (257–264) pulsed DC2.4 cells (kindly provided by Kenneth Rock, University of Massachusetts) for 4 days, removed and cultured with mIL-2 (R&D Systems) for 2 days. OVA-expressing (EG7) and non-transfected control cells (EL4) were seeded at \(2 \times 10^4\) cells per well and co-cultured with CTLs (\(1 \times 10^5\)) and control or RA treated monocytes (\(2 \times 10^5\)) for 6–8 hours [73,78]. The MTT assay (Sigma-Aldrich) was used to determine the quantity of live cells. Briefly, after incubation, cells were centrifuged (1500 RPM for 5 min) media was decanted and 100 ul of fresh media was added. 10ul of 5 mg/ml thiazolyl blue tetrazolium bromide (MTT) was added to each well for 2 hours at 37°C. After incubation cells were centrifuged (1500 RPM for 7 min) and media was decanted, cells were allowed to dry for 15–30 min before 100 ul of DMSO was added, mixed well and read at 570 nm on a Spectra Max 2. The absorbance levels were calculated by averaging the non-specific and specific absorbance levels of five separate data sets. Media control is compared to RA treated cells.

In vitro T\(_{reg}\) induction

Bone marrow (BM) cells were collected from C57Bl/6 mice femurs and tibias. After erythrocyte lysis, BM cells were differentiated for 6–7 days at a density of \(2 \times 10^6\) cells/ml +/- RA. Spleens from mice with reporter Foxp3EGFP (B6. Cg-Foxp3\(^{tm2Tch}\)) were harvested, passed through cell strainers (70 μm, BD Falcon), collected by centrifugation (1500 RPM for 7 Min at 4°C) and subjected to erythrocyte lysis. Responder cells and MCs or CD11c- MCs were cultured for 4–6 days and aliquots from cultures assessed for Foxp3 expression by flow cytometry.

Flow cytometry

In vivo and in vitro derived DCs and MCs were labeled and evaluated by three-color flow cytometry using combination of the following conjugated directly antibodies (clone): CD11c (HL3), CD11b (M1/70), Gr-1 (RB6-8C5), Ly-6G (1A8), Ly-6C (AL-21), MHC class II (AF6-120.1), CD80 (16-10A1), CD86 (IT 2.2), PD-L1 (MIH5), and PD-L2 (YT25) with appropriate isotype controls. (BD Bioscience, eBiosciences or Miltenyi Biotec). Cells were stained with fluorochrome-labeled antibodies or isotype controls for 20 min in the dark at 4°C, washed twice with FACS buffer (2x with 1 ml) and then washed with FACS buffer (2x with 1 ml) and then fixed and permeabilized using FIX/PERM solution (BD Bioscience), briefly vortexed and incubated in the dark at 4°C for 20 min. Cells were then washed twice with 1 ml of PERM/WASH buffer (BD Bioscience), re-suspended in PERM/WASH buffer and stained with 0.2 mg/ml anti-IL-10 (BD Bioscience) for 30 min. in the dark at 4°C. All flow samples were processed on an Accuri C6 flow cytometer and results analyzed using the Accuri C6 Flow software (BD Biosciences).

Myeloid cell purification

Day 6–7 differentiated BM cells were incubated with manufacturer suggested amounts of CD11c/CD11b microbeads (Miltenyi Biotec) for 15 minutes in the dark at 4°C. Cells were washed with running buffer (10% FBS in PBS with 900 mg of NaN\(_2\) per 1 L of PBS), and centrifuged (1500 RPM, 7Min). Cell separation was performed using either the Auto Macs (Miltenyi Biotec) magnetic separation instrument or the FACS Aria III 12 color, 4 laser cell sorter. The Auto Macs was used according to the manufacturer’s instructions. Cell sorting with the FACS Aria III was performed at the OSU Flow Cytometry Core and isotype control antibodies were included to detect determination levels. CD11b\(^+\) CD11c\(^-\) Ly-6C\(^{low}\) monocyte populations were serially gated on CD11c\(^+\) cells, followed by CD11b\(^+\) with gates set around distinct populations of Ly-6C low, intermediate and high. The purity of the cell populations was ≥95%.

Statistical analysis

Data are represented as mean +/- SEM or fold change. Statistical significance was determined using a Student’s t-test or 1 way ANOVA with a significance level (p-value) < 0.05 and the Wilcoxon signed-rank test. All analyses were performed using Excel and/or GraphPad Prism software (La Jolla, CA).

Additional files

Additional file 1: Figure S1. GM-CSF induced myeloid cells. BM-MCs were differentiated for 6–7 days and characterized phenotypically using the Accuri C6 Flow cytometer to identify the relative percentage of the cell population expressing (A) of CD11c\(^+\) and CD11b\(^+\) media and RA differentiated cells. Expression of Ly-6G was evaluated by the Accuri C6 cytometer of the (B) CD11b\(^+\) cells. Data are representative of at least three separate experiments.
Additional file 2: Figure S2. CD11c+ IL-10 and T_{reg} cell induction. Bone marrow cells were differentiated in the presence of GM-CSF with or without 100 nM of or retinoic acid over 7 days to generate BM-MCs. Following differentiation MCs were magnetically labeled with CD11c+ beads and separated with the AutoMacs. Purity was confirmed by routine staining of positive and negative cells with FITC-conjugated anti-CD11c antibody and cells were run on the Accuri C6 Flow cytometer. (A) The relative percentage of IL-10+ cells was determined in control MCs and RA-MCs. Data are representative of at least three separate experiments. (B) Day 7 media CD11c+ CD11c+ MCs or RA CD11c+ MCs were co-cultured in the presence of Foxp3EGFP reporter cells and expression of Foxp3+ cells was evaluated in the lymphocyte population over time in the cultures by flow cytometry. Data shown is a representation of 3 experiments.

Abbreviations

APC: Antigen presenting cell; GM-CSF: Granulocyte-macrophage colony-stimulating factor; PD-L1: PD-L2: Program death ligand 1 and 2; E3: Estrol; DC: Dendritic cell; idC/idC_{reg} Tolerogenic/Regulatory Dendritic cell.

Competing interests

The authors declare that they have no competing of interest.

Authors’ contributions

ZCV, JDB, DCM, HRS, MG-d-A and TLP performed research and analyzed data. ZCV and JDB, DCM, HRS, MG-d-A and TLP designed the research. SO-M provided statistical data analysis. ZCV, JDB, DCM, HRS, MG-d-A and TLP wrote the manuscript. TLP and HRS revised and edited the manuscript. All authors read and approved the final manuscript.

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References

1. Geissmann F, Manz MG, Jung S, Siveke MH, Merad M, Ley K: Development of monocytes, macrophages, and dendritic cells. Science (New York, NY) 2010, 327:656–661.
2. Steinman RM, Hawiger D, Nussenzweig MC: Tolerogenic dendritic cells. Annu Rev Immunol 2003, 21:685–711.
3. Gabrilovich DJ, Velders MP, Sotomayer EM, Kast WM: Mechanism of immune dysfunction in cancer mediated by immature Gr-1+ myeloid cells. J Immunol 2001, 166:5398–5406.
4. Martinez FO, Helming L, Gordon S: Alternative activation of macrophages: an immunologic functional perspective. Annu Rev Immunol 2009, 27:451–483.
5. Gordon S, Taylor PR: Monocyte and macrophage heterogeneity. Nat Rev Immunol 2005, 5:953–964.
6. Manicassamy S, Pulendran B: Dendritic cell control of tolerogenic responses. Immunological reviews 2011, 241:206–227.
7. Geissmann F, Auffray C, Palfaner R, Wirig C, Cocca A, Campoli L, Nam-Mancinelli E, Lauvau G: Blood monocytes: distinct subsets, how they relate to dendritic cells, and their possible roles in the regulation of T-cell responses. Immunology and cell biology 2008, 86:398–408.
8. Yokota A, Takeuchi H, Maeda N, Ohara K, Yato C, Song SY, Ivata M: GM-CSF and IL-4 synergistically trigger dendritic cells to acquire retinoic acid-producing capacity. International immunology 2009, 21:361–377.
9. Guilliams M, Crozet K, Henri S, Tamoutounour S, Grenot P, Devillard E, de Bois B, Alexopoulou L, Dalod M, Malissen B: Skin-draining lymph nodes contain dermis-derived CD103+ dendritic cells that constitutively produce retinoic acid and induce Foxp3+ regulatory T cells. Blood, 2010, 115:958–968.
10. Fontenot JD, Gavin MA, Rudensky AY: Foxp3 programs the development and function of CD4+ CD25+ regulatory T cells. Nat immunol 2003, 4:330–336.
11. Gavin MA, Rasmussen JP, Fontenot JD, Vasta Y, Manganello VC, Beavo JA, Rudensky AY: Foxp3-dependent programme of regulatory T-cell differentiation. Nature 2007, 445:771–775.
12. Hori S, Nomura T, Sakaguchi S: Control of regulatory T cell development by the transcription factor Foxp3. Science (New York, NY) 2003, 299:1057–1061.
13. Genscher RK, Cohen P, Henhic R, Liebhaber SA: Suppressor T cells. J Immunol 1972, 108:586–590.
14. Haney RA, Anderson AE, Isaacs JD, Hilkens CM: Generation and characterisation of therapeutic tolerogenic dendritic cells for rheumatoid arthritis. Ann Rheum Dis 2010, 69:2042–2050.
15. Giannoukakis N, Phillips B, Finegold D, Hampshire J, Trucco M: Phase I (safety) study of autologous tolerogenic dendritic cells in type 1 diabetic patients. Diabetes care 2011, 34:2026–2032.
16. Geissler EK: The ONE Study compares cell therapy products in organ transplantation: introduction to a review series on suppressive monocyte-derived cells. Transplantation research 2012, 1:11.
17. Yin B, Ma G, Yen CY, Zhou Z, Wang GX, Divino CM, Casares S, Chen SH, Yang WC, Pan PY: Myeloid-suppressor cells prevent type 1 diabetes in murine models. J Immunol 2010, 185:5528–5534.
18. Wada Y, Hisamatsu T, Kamada N, Okamoto S, Hibi T: Retinoic acid contributes to the induction of IL-12-hypoproducing dendritic cells. Inflammation 2009, 15:1548–1556.
19. Sorosoo P, Doherty TA, Duane W, Mehta AK, Choi H, Adams YF, Mikulski Z, Khonan N, Rosenthal P, Bodea DH, Croft M: Lung-resident tissue macrophages generate Foxp3+ regulatory T cells and promote airway tolerance. J Exp Med 2013, 210:775–788.
20. Weber MS, Prod’homme T, Youssef S, Dunn SE, Rundell CD, Lee L, Patarroyo JC, Stuve O, Sobel RA, Steinman L, Zamvil SS: Type II monocytes modulate T-cell mediated central nervous system autoimmune disease. Nature medicine 2007, 13:935–943.
21. Kavadasaki M, Makrigiannakis A, Bouroumas D, Verginis P: Novel role of plasmacytoid dendritic cells in humans: induction of interleukin-10-producing Treg cells by plasmacytoid dendritic cells in patients with rheumatoid arthritis responding to therapy. Arthritis Rheum 2010, 62:53–63.
22. Anderson AE, Sayers BL, Haniffa MA, Swan DJ, Diboll J, Wang XN, Isaacs JD, Hilkens CM: Differential regulation of naive and memory CD4+ T cells by alternatively activated dendritic cells. Journal of leukocyte biology 2008, 84:14–133.
23. Thomson AW, Robbins PD: Tolerogenic dendritic cells for autoimmune disease and transplantation. Annals of the rheumatic diseases 2008, 67(Suppl 3):iii90–96.
24. Dong X, Bachman LA, Kumar R, Griffin MD: Generation of antigen-specific, interleukin-10-producing T-cells using dendritic cell stimulation and steroid hormone conditioning. Transpl Immunol 2003, 11:323–333.
25. Steinbrink K, Wolff M, Junelich H, Knop J, Erik AH: Induction of tolerance by IL-10-treated dendritic cells. J Immunol 1997, 159:4772–4780.
26. Verma PL, Kallinikos P, Vieregg EA, Kapsenberg ML, de Jong EC: Glucocorticoids inhibit bioactive IL-12p70 production by in vitro-generated human dendritic cells without affecting their T cell stimulatory potential. J Immunol 1998, 161:5245–5251.
27. Adorini L, Giarratana N, Penna G: Pharmacological induction of tolerogenic dendritic cells and regulatory T cells. Semin Immunol 2004, 16:271–274.
28. Hackstein H, Thomson AW: Dendritic cells: emerging pharmacological targets of immunosuppressive drugs. Nat Rev Immunol 2004, 4:24–34.
39. Kodaira Y, Nar SK, Wrenshall LE, Gilboa E, Platt JL: Phenotypic and functional maturation of dendritic cells mediated by heparan sulfate. J Immunol 2000, 165:1599–1604.

40. Bros M, Jahling F, Renzing A, Wieschmann N, Dang NA, Sutter A, Ross R, Knoop J, Sudowe S, Reske-Kunz AB: A newly established murine immature dendritic cell line can be differentiated into a mature state, but exerts tolerogenic function upon maturation in the presence of glucocorticoid. Blood 2007, 109:3820–3829.

41. Steinman RM, Banchereau J: Dendritic cells in medicine. Nature 2007, 449:419–426.

42. Lan YY, Wang Z, Raimondi G, Wu W, Colvin BL, de Creus A, Thomson AW: "Alternatively activated" dendritic cells preferentially secrete IL-10, expand Foxp3+ CD4+ T cells, and induce long-term organ allograft survival in combination with CTLA-4 Ig. J Immunol 2006, 177:5868–5877.

43. Anderson AE, Swan DJ, Sayens BL, Harry RA, Patterson AM, von Delwig A, Robinson JH, Issac JD, Hilkens CM: LPS activation is required for migratory activity and antigen presentation by tolerogenic dendritic cells. J Leukoc Biol 2009, 85:243–250.

44. Veldhuizen G, Rossner S, Cierpka E, Theiner G, Wiethe C, Menges M, Schuler G, Lutz MB: Dendritic cells matured with TNF can be further activated in vitro and after subcutaneous injection in vivo which converts their tolerogenicity into immunogenicity. Journal of Immunotherapy (Hagerstown, Md: 1997) 2006, 29:407–415.

45. Papenfuss TL, Powell ND, McClain MA, Bedarf A, Singh A, Gienapp IE, Austyn JM: Generation of large numbers of dendritic cells from mouse bone marrow cells supplemented with granulocyte/macrophage colony-stimulating factor. J Exp Med 1992, 176:1693–1702.

46. van de Laar L, Coffer PJ, Wolpert AM: Regulation of dendritic cell development by GM-CSF: molecular control and implications for immune homeostasis and therapy. Blood 2012, 119:3383–3393.

47. Hengelsbach LM, Hoag KA: Physiological concentrations of retinoic acid favor myeloid dendritic cell development over granulocyte development in cultures of bone marrow cells from mice. J Nutr 2004, 134:2653–2659.

48. Athens JW, Haab OP, Raab SO, Mauer AM, Ashenbrucker H, Cartwright GE, Wintrobe MM: Leukokinetik studies. IV. The total blood, circulating and marginal granulocyte pools and the granulocyte turnover rate in normal subjects. J Clin Invest 1961, 40:899–905.

49. Suratt BT, Young SK, Lieber J, Nick JA, Henson PM, Worthen GS: Neutrophil maturation and activation determine anatomic site of clearance from circulation. Am J Physiol Lung Cell Mol Physiol 2001, 281:R913–921.

50. Sanderkotter C, Nikolic T, Dillon MJ, Van Rooijen N, Stehling M, Drevets DA, Leenen PJ: Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. J Immunol 2004, 172:4410–4417.

51. Panna G, Adorini L: 1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. J Immunol 2000, 164:2405–2411.

52. Emmer PM, van der Vlag J, Adema GJ, Hilbrands LB: Dendritic cells activated by lipopolysaccharide after dexamethasone treatment induce donor-specific allograft hypersensitiveness. Transplantation 2006, 81:1451–1459.

53. Fujihara M, Murai M, Tanamoto K, Suzuki T, Azuma H, Ikeda H: Cellular mechanisms of macrophage activation and deactivation by lipopolysaccharide: roles of the receptor complex. Pharmacology & Therapeutics 2003, 100:171–194.

54. Suratt BT, Young SK, Lieber J, Nick JA, Henson PM, Worthen GS: Neutrophil maturation and activation determine anatomic site of clearance from circulation. Am J Physiol Lung Cell Mol Physiol 2001, 281:R913–921.

55. Sanderkotter C, Nikolic T, Dillon MJ, Van Rooijen N, Stehling M, Drevets DA, Leenen PJ: Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. J Immunol 2004, 172:4410–4417.

56. Panna G, Adorini L: 1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. J Immunol 2000, 164:2405–2411.

57. Emmer PM, van der Vlag J, Adema GJ, Hilbrands LB: Dendritic cells activated by lipopolysaccharide after dexamethasone treatment induce donor-specific allograft hypersensitiveness. Transplantation 2006, 81:1451–1459.

58. Fujihara M, Murai M, Tanamoto K, Suzuki T, Azuma H, Ikeda H: Cellular mechanisms of macrophage activation and deactivation by lipopolysaccharide: roles of the receptor complex. Pharmacology & Therapeutics 2003, 100:171–194.

59. Suratt BT, Young SK, Lieber J, Nick JA, Henson PM, Worthen GS: Neutrophil maturation and activation determine anatomic site of clearance from circulation. Am J Physiol Lung Cell Mol Physiol 2001, 281:R913–921.

60. Sanderkotter C, Nikolic T, Dillon MJ, Van Rooijen N, Stehling M, Drevets DA, Leenen PJ: Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. J Immunol 2004, 172:4410–4417.

61. Panna G, Adorini L: 1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. J Immunol 2000, 164:2405–2411.

62. Emmer PM, van der Vlag J, Adema GJ, Hilbrands LB: Dendritic cells activated by lipopolysaccharide after dexamethasone treatment induce donor-specific allograft hypersensitiveness. Transplantation 2006, 81:1451–1459.
72. del Hoyo GM, Martin P, Vargas HH, Ruiz S, Arias CF, Ardavin C: Characterization of a common precursor population for dendritic cells. *Nature* 2002, 415:1043–1047.

73. Sutherland AP, Joller N, Michaud M, Liu SM, Kuchroo VK, Grusby MJ: IL-21 promotes CD8+ CTL activity via the transcription factor T-bet. *J Immunol* 2013, 190:3977–3984.

74. Auffray C, Siegelman SH, Geissmann F: Blood monocytes: development, heterogeneity, and relationship with dendritic cells. *Annu Rev Immunol* 2009, 27:669–692.

75. Nahrendorf M, Swirski FK, Aikawa E, Stangenberg L, Wurdinger T, Figueiredo JL, Libby P, Weissleder R, Pittet M: The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *J Exp Med* 2007, 204:3037–3047.

76. Schiopu A, Nadig SN, Cotoi OS, Hester J, van Rooijen N, Wood KJ: Inflammatory Ly-6C(hi) monocytes play an important role in the development of severe transplant arteriosclerosis in hyperlipidemic recipients. *Atherosclerosis* 2012, 223:291–298.

77. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, Leenen PJ, Liu Yi, MacPherson G, Randolph GJ, Scheberich J, Schmitz J, Shortman K, Sozzani S, Strobl H, Zembala M, Austyn JM, Lutz MB: Nomenclature of monocytes and dendritic cells in blood. *Blood* 2010, 116:74–80.

78. Robinson MJ, Ronchese F, Miller JH, La Flamme AC: Paclitaxel inhibits killing by murine cytotoxic T lymphocytes in vivo but not in vitro. *Immunology and cell biology* 2010, 88:291–296.

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