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OUTSTANDING OBSERVATION

Naïve blood monocytes suppress T-cell function. A possible mechanism for protection from autoimmunity

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In certain disease context, cells of the monocyte/macrophage lineage are known to exhibit T-cell suppressor function. However, whether naïve monocytes are also able to suppress T-cell responses has not been previously investigated. In this study, we have discovered that CD11b$^+$Ly6G$^-$ mononuclear cells in the blood of naïve mice are potent suppressors of T-cell proliferation *in vitro*. The suppression of T-cell proliferation requires cell-cell contact and is partially dependent on nitric oxide production. Following the induction of experimental autoimmune encephalomyelitis in mice, the suppressor function of this blood CD11b$^+$Ly6G$^-$ cell population is impaired. Therefore, blood CD11b$^+$Ly6G$^-$ cells appear to be intrinsically suppressive and may have a key role in maintaining immune homoeostasis. Loss of this suppressive function may contribute to development of autoimmunity.
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Cells of the monocyte/macrophage lineage are highly plastic in nature. A characteristic feature of this plasticity is the ability to alter their functional phenotype in response to their immune microenvironment.[1]

Monocyte-macrophages are well known for raising pro-inflammatory responses following activation with microbial stimuli and cytokines such as interferon-γ (IFN-γ).[2] However, it is now widely understood that this cell lineage can also be polarised towards anti-inflammatory or modulating functions including T-cell suppression.[3] In general, this T-cell suppressor function is observed in monocyte/macrophage populations present in a disease context. For example, myeloid-derived suppressor cells (MDSCs) that exhibit T-cell suppressive activities linked to tumour progression in tumour-bearing mice and humans.[4–11]

Surprisingly, little work has been carried out to determine whether a change in the microenvironment is necessary for the development of T-cell suppressor function by monocytes or whether T-cell suppressor function is an integral feature of the naïve monocyte phenotype. In this study, we investigated the ability of blood monocytes from naïve mice to suppress T-cell responses and whether T-cell suppression by blood monocytes may have a role in protecting against development of autoimmunity.

RESULTS

Characterisation of the CD11b$^+$Ly6G$^-$ blood monocytes

MDSCs are often identified by the co-expression of CD11b$^+$Gr-1$^+$.[9] However, CD11b is expressed on both monocytes and neutrophils, and Gr-1 antibody binds both Ly6G and Ly6C; therefore, CD11b$^+$ Gr-1$^+$ staining does not differentiate between monocyte and neutrophil populations. To specifically identify blood monocytes in naïve mice, cells from lymph nodes, spleens and blood were analysed by flow cytometry for surface expression of CD11b, Ly6C and neutrophil-specific Ly6G. As shown in Figure 1a, the CD11b$^+$ cell population was comprised of a distinct Ly6G$^+$ and Ly6G$^-$ cell population. The CD11b$^+$Ly6G$^+$ cells were also Ly6C^{hi} and presented a typical neutrophilic morphology (data not shown).[5,12,13] The CD11b$^+$Ly6G$^-$ cells made up 5–20% of all the leucocytes in the blood and ~1–2% of the splenocytes, but were absent in lymph nodes (Figure 1a and data not shown). Isolated CD11b$^+$Ly6G$^-$cells were heterogeneous in their Ly6C expression (Figure 1b) and exhibited a monocyte morphology possessing a large, ovoid or kidney-shaped nucleus (Figure 1c). Further surface marker analysis of the CD11b$^+$Ly6G$^-$ cells showed that they expressed high levels of the surface markers CD11a, CD45, CD49d and intermediate levels of Gr-1, F4/80 and CD80, but lacked classic dendritic, B cell or natural killer cell markers (Table 1). This pattern of surface marker expression was consistent with previously described naïve blood monocyte populations[14] and provided further confirmation that the blood CD11b$^+$Ly6G$^-$ cells were of the monocyte lineage.

The blood CD11b$^+$Ly6G$^-$ cells are potent suppressors of T-cell proliferation

To determine whether blood CD11b$^+$Ly6G$^-$ cells had suppressor function, the CD11b$^+$Ly6G$^-$ cells were purified from mouse blood

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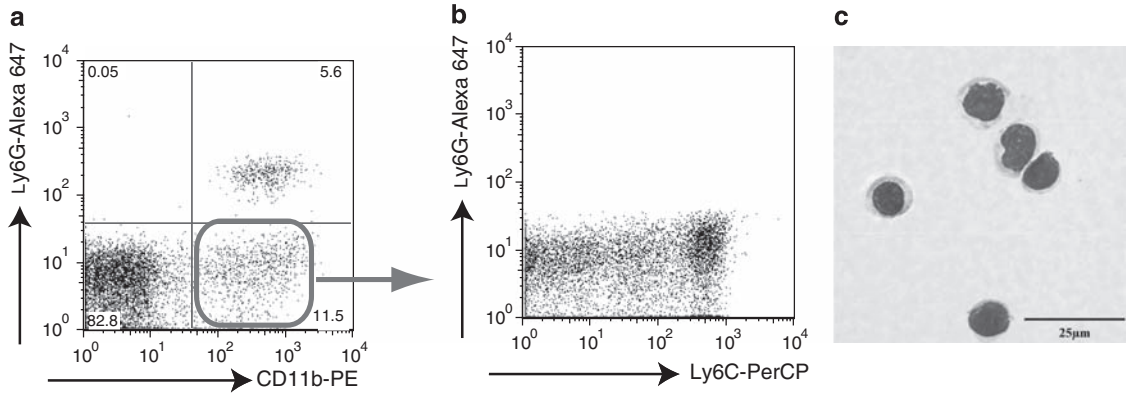

Figure 1 Morphology and cell surface markers of blood CD11b$^+$Ly6G$^-$ cells. Blood cells from C57BL/6J mice were analysed by flow cytometry for the expression of (**a**) Ly6G and CD11b and (**b**) expression of Ly6G and Ly6C. (**c**) FACS-sorted blood CD11b$^+$Ly6G$^-$ cells were stained by using Diff-Quik stain set (×400).

Table 1 Surface staining of the blood CD11b$^+$Ly6G$^-$ cells

Myeloid lineage markers	
F4/80	+
CD11b (MAC-1)	++
Monocyte subset markers	
Ly6C	−, +, ++
Adhesion molecules	
CD11a (LFA-1)	++
CD44	++
CD54 (ICAM-1)	−
CD49d (VLA-4)	++
Antigen presentation/co-stimulatory markers	
MHCII	−
CD80	−, +
CD86	−
CD40	−, +
T-cell markers	
CD90.2 (Thy1.2)	−
CD4	−
CD8	−
Dendritic cell markers	
CD11c	−
PDCA-1	−
Neutrophil marker	
Ly6G	−
B-cell marker	
CD45R (B220)	−
Miscellaneous	
CD62L	−,+
CD69	−
Fcγ receptors II or III (24G2)	++
CD45	++
CD1d	−

Data have been assigned arbitrary symbols that represent '−' no staining and increasing amount of staining '+, ++'.

and tested for their ability to suppress T-cell proliferation *in vitro* using a combination of [^{3}H]-thymidine uptake and Carboxyfluorescein succinimidyl ester (CFSE) dilution assays. Purified blood CD11b$^+$Ly6G$^-$ cells suppressed proliferation of both 2D2 and C57BL/6J splenocytes, induced by either anti-CD3/CD28 coated expander beads or plate-bound anti-CD3/CD28 (Figure 2a). These data also confirmed that the CD11b$^+$Ly6G$^-$ cells were suppressing T-cell proliferation and were not blocking T-cell expansion because of phagocytosis of expander beads. It was possible that the observed T-cell suppression was the result of direct metabolic or space filling competition from the CD11b$^+$Ly6G$^-$ cells. T-cell expansion was not affected when CD11b$^+$Ly6G$^-$ cells were replaced by bone marrow-derived dendritic cells (Supplementary Figure 1), indicating that decreased T-cell proliferation by CD11b$^+$Ly6G$^-$ cells was not an artefact of competition. Flow cytometric analysis of T-cell expansion by CFSE dilution showed that both CD4$^+$ and CD8$^+$ T-cell proliferation was suppressed by the presence of blood CD11b$^+$Ly6G$^-$ cells (Figure 2b). The blood CD11b$^+$Ly6G$^-$ cells also suppressed 2D2 transgenic lymph node cell and purified CD4$^+$ T-cell proliferation induced by MOG_{35-55} peptide (Figures 2c and d). Together, these data showed that naïve blood CD11b$^+$Ly6G$^-$ cells were capable of suppressing both antigen non-specific and antigen-specific T-cell responses.

The suppression of T-cell proliferation by blood CD11b$^+$Ly6G$^-$ cells requires cell contact and is partially dependent on NOS

Excessive activation and proliferation of T cells is known to lead to T-cell apoptosis.[15] To determine whether the observed blood CD11b$^+$Ly6G$^-$ cell-mediated T-cell suppression was because of enhanced T-cell activation-induced apoptosis, blood CD11b$^+$Ly6G$^-$ cells were cultured with CFSE-labelled splenocytes, and Annexin V expression was monitored over 48 h. In fact, the presence of blood CD11b$^+$Ly6G$^-$ cells lowered the percentage of Annexin V$^+$ cells in culture (Figure 3a), confirming that blood CD11b$^+$Ly6G$^-$ cells possess a true suppressor phenotype as opposed to other MDSC populations that simply induce T-cell apoptosis.[16]

To determine whether cell contact was important for the suppression of T-cell proliferation by blood CD11b$^+$Ly6G$^-$ cells, a transwell experiment was performed. The suppression of T-cell proliferation by blood CD11b$^+$Ly6G$^-$ cells was abolished when the blood CD11b$^+$Ly6G$^-$ cells and responder cells were in different chambers (Figure 3b). This result indicated that T-cell suppression by blood CD11b$^+$Ly6G$^-$ cells required cell-cell contact. To identify possible molecules involved in the cell contact-dependent suppression,

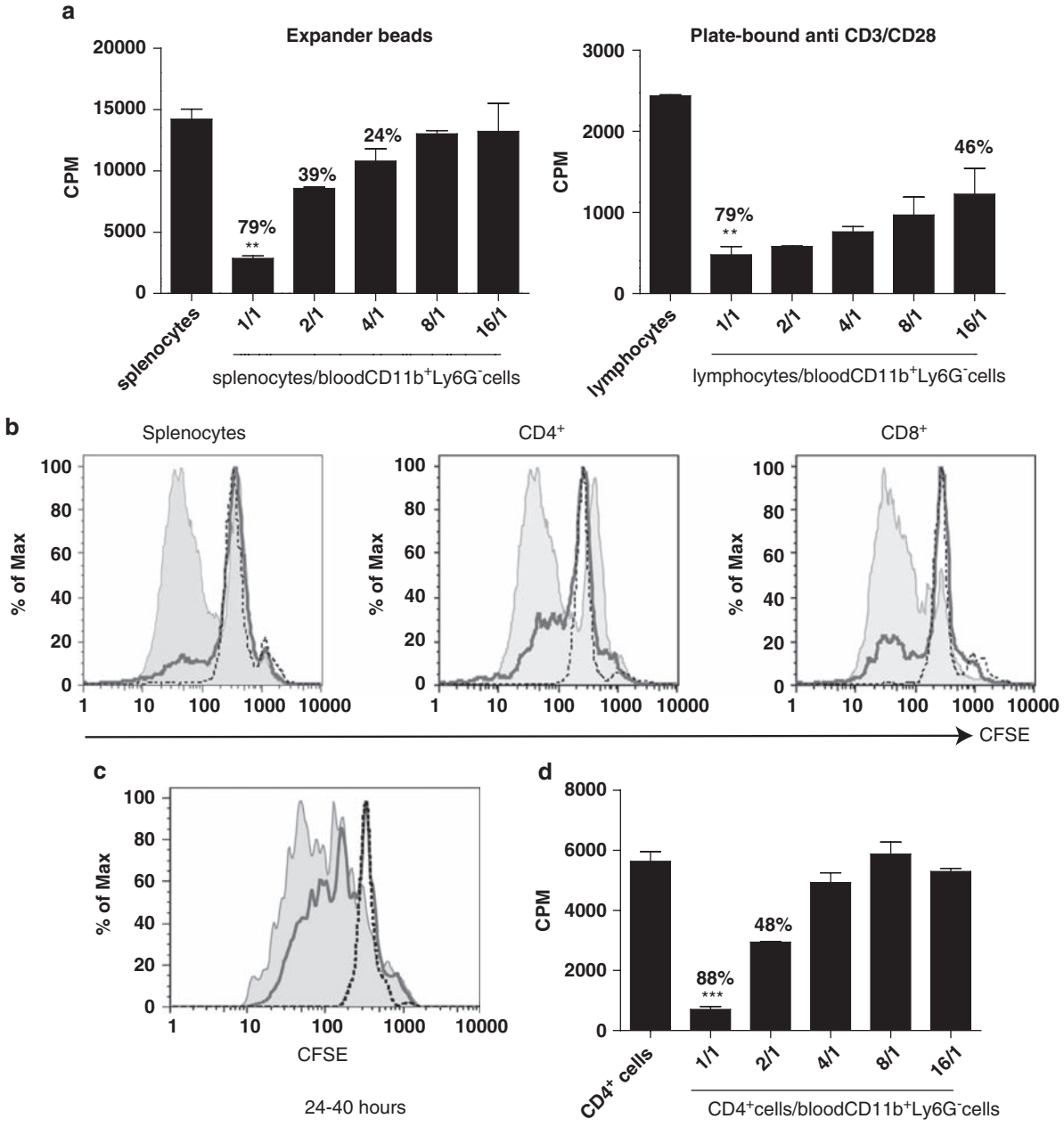

Figure 2 Blood CD11b$^+$Ly6G$^-$ cells suppress T-cell proliferation. (**a**) 2D2 mouse splenocytes were activated with anti-CD3/CD28 coated expander beads, or plate-bound anti-CD3/CD28, and co-cultured with naïve CD11b$^+$Ly6G$^-$ cells from C57BL/6J mice. Total cell proliferation was measured by [^{3}H]-thymidine uptake. (**b**) CFSE-labelled splenocytes were incubated with CD11b$^+$Ly6G$^-$ cells (ratio 1:1) and total, CD4$^+$ and CD8$^+$ T-cell proliferations at 72 h was measured by flow cytometry. Splenocytes alone (dashed line), splenocytes + expander beads (grey fill), splenocytes + expander beads + CD11b$^+$Ly6G$^-$ cells (solid line). (**c**) CFSE-labelled 2D2 transgenic LN cells were activated with 3 μg ml^{-1} MOG_{35-55} peptide. CD11b$^+$Ly6G$^-$ cells from naïve 2D2 mice were added to the culture at T=24 h. Cell proliferation was measured by flow cytometry at T=48 h. LN cells alone (dashed line), LN cells+MOG_{35-55} peptide (grey fill), LN cells + MOG_{35-55} peptide + CD11b$^+$Ly6G$^-$ cells (solid line). (**d**) Purified CD4$^+$ cells from 2D2 transgenic mice were activated with anti-CD3/CD28 expander beads in the presence of CD11b$^+$Ly6G$^-$ cells. Total cell expansion was measured by [^{3}H]-thymidine uptake. Statistical analysis was carried out using one-way ANOVA followed by Bonferroni tests $^{**}P<0.01$, $^{***}P<0.001$. Results are representative of at least three separate experiments.

blocking antibodies targeting the cell surface co-stimulatory molecules CD40 (Clone 1C10), CD80 (Clone 16-10A1) and CD86 (Clone PO3.1), and the Fcγ receptors II and III (Clone 24G2) were added to the cell co-cultures. None of these antibodies, either alone or in combination, blocked the ability of blood CD11b$^+$Ly6G$^-$ cells to suppress T-cell proliferation (Figure 3c). These data indicated that the above molecules do not have a role in the blood CD11b$^+$Ly6G$^-$ cell-mediated T-cell suppression.

Published evidence points to a fundamental role of nitric oxide synthase (NOS) and/or arginase-1 in the suppression of T-cell proliferation by MDSCs in tumours.[5,7] In this study, the arginase-1 inhibitor nor-NOHA failed to reduce the blood CD11b$^+$Ly6G$^-$ cells' suppressive activity (Figure 3d). However, the NOS inhibitor L-NMMA partially blocked the suppression of T-cell proliferation, indicating that the mechanism of suppression involves nitric oxide. Co-addition of nor-NOHA and L-NMMA to the cell co-culture

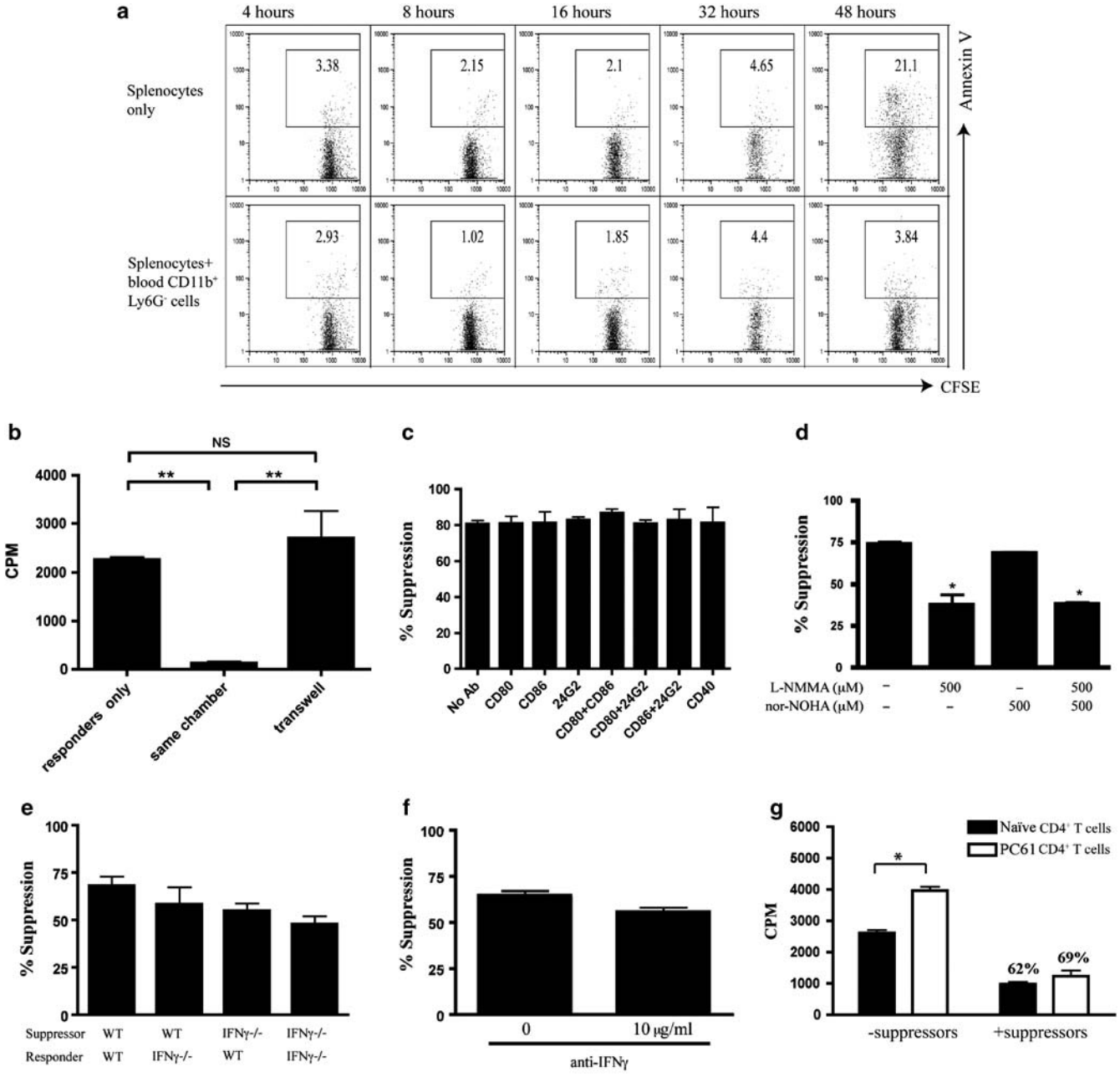

Figure 3 *In vitro* T-cell suppression by blood CD11b$^+$Ly6G$^-$ cells requires cell contact and NOS. (**a**) CFSE-labelled splenocytes from C57BL/6J mice (50 000 cells per well) were activated with expander beads in the presence of purified blood CD11b$^+$Ly6G$^-$ cells (1:1 ratio). At different time points, the cells were stained with Annexin V and analysed by flow cytometry. Percentages of Annexin V$^+$ cells in the CFSE$^+$ population are shown. (**b**) Splenocytes from C57BL/6J mice were co-cultured with purified blood CD11b$^+$Ly6G$^-$ cells (1:1 ratio) or in separate chambers in a transwell plate (3 μm pore size). Splenocytes from C57BL/6J mice (50 000 cells per well) were activated with anti-CD3/CD28 coated expander beads in the presence of purified blood CD11b$^+$Ly6G$^-$ cells (1:1 ratio) and (**c**) different antibodies, or (**d**) 500 μg ml^{-1} L-NMMA or 500 μg ml^{-1} nor-NOHA. (**e**) Splenocytes from C57BL/6J or IFN-$\gamma^{-/-}$ mice were activated with expander beads in the presence of purified blood CD11b$^+$Ly6G$^-$ cells from either C57BL/6J or IFN-$\gamma^{-/-}$ mice (1:1 ratio). (**f**) C57BL/6J splenocytes were activated with expander beads in the presence of purified blood CD11b$^+$Ly6G$^-$ cells from C57BL/6J mice (1:1 ratio) and neutralising IFN-γ antibody (10 μg ml^{-1}). (**g**) CD4$^+$ cells from Foxp3-GFP KI mice were purified from the lymph nodes following depletion of CD25$^+$ Tregs (Clone PC61, i.p. 200 μg). CD4$^+$ T cells were activated with expander beads in the presence of blood CD11b$^+$Ly6G$^-$ cells (1:1 ratio). (**b**–**f**) Cell expansion was measured by [^{3}H]-thymidine uptake. Statistical analysis was carried out using one-way ANOVA followed by Bonferroni tests (**b, c**) and non-parametric two-tailed student *t*-test (**g**) $*P<0.05$, $**P<0.01$; NS, not significant. Results are representative of more than two separate experiments.

did not enhance the blocking effect of L-NMMA. Together, these data confirmed that CD11b$^+$Ly6G$^-$ cell-mediated suppression of T-cell proliferation was partially dependent on NOS but not arginase-1.

As CD11b$^+$Ly6G$^-$ cell-mediated T-cell suppression was not fully dependent on NOS, we investigated alternative mechanisms that may contribute to suppressive activity. A number of studies have shown that IFN-γ has an important role in the MDSC-mediated suppression of

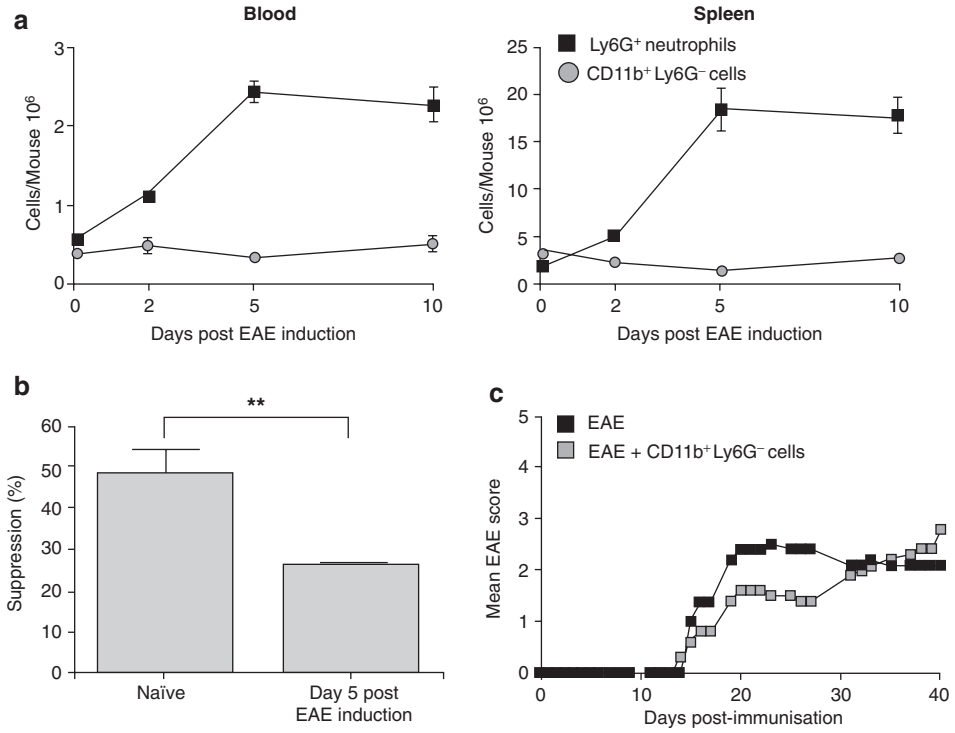

Figure 4 Blood CD11b$^+$Ly6G$^-$ cells from EAE-immunised mice exhibit impaired T-cell suppressor function. EAE was induced in mice by s.c. injection of 50 μg MOG_{35-55} peptide and 500 μg heat-killed *Mycobacterium tuberculosis* strain H37RA in 0.2 ml complete Freud's adjuvant followed by 250 ng pertussis toxin on day 1. (**a**) Blood and spleen cells were collected and analysed by flow cytometry (n=3). (**b**) At day 5, blood CD11b$^+$Ly6G$^-$ cells were purified from naïve mice and EAE mice and assayed for T-cell suppressor function measured as [^{3}H]-thymidine uptake by bead-activated lymph node cells. Cell ratio1:1. (**c**) At day 5, purified blood CD11b$^+$Ly6G$^-$ cells from naïve mice were adoptively transferred (2×10^5 cells per mouse) into EAE-immunised mice. Statistical analysis was performed using a one-tailed Mann–Whitney U-test. $^{**}P<0.01$.

T-cell responses.[5,16,17] As shown in Figure 3e, there was no significant difference in the suppression of wild-type or IFN-$\gamma^{-/-}$ responder cells by blood CD11b$^+$Ly6G$^-$ cells derived from wild-type or IFN-$\gamma^{-/-}$ mice. However, there was a trend towards reduced suppression of IFN-$\gamma^{-/-}$ responder cells by the IFN-$\gamma^{-/-}$ CD11b$^+$Ly6G$^-$ cells, possibly because of the anti-proliferative effects of IFN-γ.[18–22] To determine whether this marginal difference was dependent on the presence of IFN-γ, IFN-γ neutralising antibody was added to wild-type cultures to block any IFN-γ-mediated responses. The addition of the IFN-γ antibody did not reverse the suppression by the blood CD11b$^+$Ly6G$^-$ cells (Figure 3f), indicating that naïve blood CD11b$^+$Ly6G$^-$ cells suppressed T cells in a predominantly IFN-γ independent manner.

It has also been reported that the MDSCs derived from tumour-bearing mice can suppress T-cell proliferation by inducing the expansion of Foxp3$^+$ regulatory T cells (Tregs).[7] To assess whether the presence of Tregs in the T-cell population was important for the suppressive activity of blood CD11b$^+$Ly6G$^-$ cells, Foxp3-GFP knock-in male mice were injected with anti-CD25 antibody (Clone PC61) to neutralise Tregs *in vivo*[23] (Supplementary Figure 2). As shown in Figure 3g, blood CD11b$^+$Ly6G$^-$ cells suppressed the proliferation of purified CD4$^+$ T cells from both CD25$^+$ Treg-depleted and naïve mice equally (Figure 3g). This finding indicates that the mechanism of suppression of CD4$^+$ T-cell proliferation *in vitro* by blood CD11b$^+$Ly6G$^-$ cells was not dependent on CD25$^+$ Treg expansion.

Induction of experimental autoimmune encephalomyelitis impairs blood CD11b$^+$Ly6G$^-$ cells' T-cell suppressor function

To determine whether blood CD11b$^+$Ly6G$^-$ cells lose their suppressive function in a model of autoimmunity, we compared the T-cell suppressor function of CD11b$^+$Ly6G$^-$ blood cells from naïve mice with cells from mice with EAE. As shown in Figure 4a, 5 days after experimental autoimmune encephalomyelitis (EAE) induction, Ly6G$^+$ neutrophils accumulated in both the spleen and blood of mice, confirming that the immune system was activated. The blood CD11b$^+$Ly6G$^-$ cells from mice immunised with EAE showed a reduced suppression of T-cell proliferation compared with blood CD11b$^+$Ly6G$^-$ cells from naïve mice. Adoptive transfer of a single dose of naïve CD11b$^+$Ly6G$^-$ cells into EAE mice did not confer significant protection from EAE (Figure 4c), indicating that the suppressive capacity of the naïve cells, as in the recipient EAE mice, was turned off by the immune environment following adoptive transfer.

DISCUSSION

In this study, we report that a mononuclear CD11b$^+$Ly6G$^-$ monocytic cell population in naïve mouse blood exhibits the ability to suppress T-cell proliferation *in vitro* without inducing apoptosis. This T-cell suppressor function is dependent on both cell contact and nitric oxide production but is independent of CD25$^+$ Tregs and IFN-γ.

Previous studies have reported the presence of Gr-1$^+$CD11b$^+$ cells in the spleens of naïve mice.[5,16] The majority of studies investigating *in vitro* T-cell suppression by Gr-1$^+$CD11b$^+$ splenocytes in naïve mice indicate that they are not suppressive.[5,8,24–26] One paper has reported that naïve Gr-1$^+$CD11b$^+$ splenocytes are able to suppress CD4$^+$ T-cell responses.[27] However, results from earlier studies are confounded by the characterisation of the cells based on the expression of Gr-l, which does not differentiate between monocyte and granulocytes. In contrast, our study specifically characterised suppressor function in the CD11b$^+$ Ly6G$^-$ monocytes from the blood of naïve mice. There

has been no prior evidence to indicate that freshly isolated $Ly6G^{-}CD11b^{+}$ monocytes from naïve mouse blood are able to suppress T-cell proliferation. The existence of this $CD11b^{+}Ly6G^{-}$ suppressor monocyte population in a naïve environment could well represent a previously unknown suppressive mechanism that has evolved to regulate immune homoeostasis and limit immune responses.

It is interesting to note that the naïve blood monocyte population investigated in this study expresses a similar pattern of surface marker expression to that of MDSC identified in the spleens of tumour-bearing mice.[9–11] Like the naïve blood $CD11b^{+}Ly6G^{-}$ suppressor monocytes reported here, some mononuclear MDSCs in the spleen of tumour-bearing mice also suppress T-cell responses in a NOS-dependent manner.[5] It is therefore possible that, rather than being driven by the tumour environment, the NOS-dependent MDSC population represents infiltration of naïve blood monocytes[14] into the tumour. It is possible that the blood suppressor monocyte is a precursor for other MDSCs phenotypes that utilise alternative suppression pathways (arginase-1, Tregs). Indeed, the induction of different suppressor phenotypes has been linked to the immune microenvironment.[5,28,29] In addition, bone marrow $Gr\text{-}1^{+}CD11b^{+}$ mononuclear cells exposed to IFN-γ, granulocyte-macrophage colony stimulating factor and interleukin-3[30–32] also exhibit T-cell suppressor function *in vitro*. Together, this illustrates the potential for modification of the naive blood monocyte suppressor phenotype in response to a change in the immune environment.

The accumulation of a $CD11b^{+}Ly6C^{+}$ suppressor cell population has been reported in the spleens of EAE mice,[16] indicating that autoimmunity may also induce development of cells with an MDSC-like phenotype. However, our data indicate that the previously reported accumulating cell population was likely comprised of $Ly6G^{high}$ neutrophils rather than the $CD11b^{+}Ly6G^{-}$ mononuclear cells investigated in this study. Other studies have proposed that some autoimmune diseases are caused by a loss-of-function of the suppressive mechanisms involved in immunoregulation.[33,34] Consistent with this pattern of immune disregulation, our data showed that the induction of EAE in mice impaired the suppressive function of blood $CD11b^{+}Ly6G^{-}$ monocytes. This finding implies that the immune environment induced by EAE switches off suppressor function in blood monocytes, an event that may contribute to the development of unregulated self-reactive T-cell responses *in vivo*. Therefore, it is not surprising that adoptive transfer of naïve cells provided no significant delay in EAE progression as it is likely that suppressor function in the naïve donor cells would also be switched off on exposure to the EAE environment of the host. At this stage, the mechanism(s) involved in the loss of T-cell suppressor function by $CD11b^{+}Ly6C^{+}Ly6G^{-}$ monocytes, and whether this phenomenon also occurs in human patients with autoimmune diseases is unknown. Nevertheless, the loss of suppressive function by blood $CD11b^{+}Ly6G^{-}$ cells following induction of EAE provides evidence to suggest that these cells may have a part in maintaining immune tolerance and protection from autoimmunity. Monocyte suppressor function therefore appears to be a valid target for the development of new autoimmune therapies.

In summary, the findings from this study uncover a previously unknown T-cell suppressor function in naïve blood $CD11b^{+}Ly6G^{-}$ monocytes that may be a precursor for other mononuclear suppressor phenotypes. Importantly, the identification of the naïve suppressor phenotype provides new insight into a possible regulatory role for naïve blood $CD11b^{+}Ly6G^{-}$ cells in the maintenance of immunological tolerance and in the development of autoimmune disease.

METHODS

Mice and reagents

All mice were bred and maintained on standard laboratory food and water *ad libitum* in the Biomedical Research Unit of the Malaghan Institute of Medical Research. All animal studies were approved by the Victoria University of Wellington Animal Ethics Committee and performed in accordance with the guidelines of the Victoria University of Wellington Animal Ethics Committee, New Zealand. All mice utilised in this study were maintained by inbreeding.

Foxp3-GFP breeding mice harbouring a GFP-Foxp3 fusion protein reporter knock-in allele (B6×129, Foxp3-GFP-g2) were a kind gift from Dr A Rudensky and imported from the University of Washington (Seattle, WA, USA), and have been described by Fontenot *et al*.[35] 2D2 mice expressing transgenic T cell receptors specific for the MOG_{35-55} peptide (MEVGWYRSPFSRVVH-LYRNGK) presented by IA^{b} were obtained from Harvard Medical School (Boston, MA, USA) and derived as described.[36] IFN-γ deficient ($IFN\text{-}\gamma^{-/-}$) C57BL/6J mice were generated by targeted disruption of the *IFN-γ* gene as described[18] and breeding pairs were imported from the Walter and Eliza Hall Institute of Medical Research (Melbourne, Victoria, Australia).

The MOG_{35-55C} peptide was synthesised by Mimotopes (Chiron, Clayton, Victoria, Australia) with a purity of >97%. Anti-CD3/CD28 expander beads and Dynabeads Comp Mouse $CD4^{+}$ kit were purchased from Invitrogen, Carlsbad, CA, USA. Lympholyte-Mammal cell separation medium and Lympholyte-M desity gradient was purchased from Cedarlane Laboratories Limited (Burlington, Ontario, Canada). N^{G}-monomethyl-L-arginine (L-NMMA) and lipopolysaccharide were purchased from Sigma-Aldrich (Auckland, New Zealand) and the inhibitor of arginase-1, N^{ω}-hydroxy-nor-L-arginine (nor-NOHA) was purchased from Calbiochem (Darmstadt, Germany). Anti-PE Automacs beads and Automacs Running Buffer were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). [^{3}H]-thymidine was purchased from GE Healthcare (Piscataway, NJ, USA) and filter mats for harvesting plates of suppression assay were purchased from Perkin-Elmer Life Sciences (Turku, Finland). All other reagents were purchased from Invitrogen (Auckland, New Zealand).

Purification of blood $CD11b^{+}Ly6G^{-}$ cells

Whole blood was collected from mice through cardiac puncture and spun on a Lympholyte-M density gradient as per manufacturer's instructions to separate the peripheral blood mononuclear cells from the neutrophils. The peripheral blood mononuclear cells band containing the blood $CD11b^{+}Ly6G^{-}$ cells was carefully removed, washed in complete Iscoves modified Dulbecco's medium and resuspended at 1×10^{6} cells ml^{-1} in AutoMACS running buffer. The $CD11b^{+}Ly6G^{-}$ cells were passed through an AutoMACS column to remove non-specific binding and dead cells. The cells were resuspended in AutoMACS buffer (5×10^{8} cells ml^{-1}) and incubated on ice with CD11b-PE antibody (1:800 dilution, 15 min). The cells were washed, resuspended in AutoMACS buffer (5×10^{8} cells ml^{-1}) and incubated under rotation with anti-PE beads (10 µl per 5×10^{7} cells, 30 min, 4 °C). Cells were washed twice in AutoMACS buffer and passed through a MACS column to positively select for $CD11b^{+}$ cells.

Purification of $CD4^{+}$ T cells

$CD4^{+}$ T cells were purified from inguinal and mesenteric lymph nodes using a Dynabeads Comp Mouse $CD4^{+}$ kit as per manufacturer's instructions.

Suppression assay

Splenocytes, inguinal and mesenteric lymph node cells or purified $CD4^{+}$ T cells (50 000 cells per well) from different mice strains were activated using either anti-CD3/CD28 expander beads (2.5×10^{5} or 5×10^{5} beads per well) or a combination of plate-bound anti-CD3 (2 µg ml^{-1}) and anti-CD28 (3 µg ml^{-1}), and incubated in the presence of different ratios of purified blood $CD11b^{+}Ly6G^{-}$ cells or DC. 2D2 cells from 2D2 transgenic mice were also activated with 3 µg ml^{-1} MOG_{35-55} peptide. [^{3}H]-thymidine (0.25 µCi) was added to the cell culture at 24 h, and the cells were incubated for an additional 16 h. The cells were harvested to filter mats using an automated cell harvester (Tomtec Inc., Hamden, CT, USA) and read on a Topcount Microplate scintillation counter (Perkin Elmer, Melbourne, Australia).

In some experiments, the responder cells (T cells/splenocytes, 50 000 cells per well) were labelled with CFSE and cells were incubated at 37 °C for 72 h. T-cell

proliferation (total CD3, CD8 and/or CD4) was measured as CFSE dilution using flow cytometry.

%Suppression was calculated using the following formula:[37]

$$[1 - \text{c.p.m.(target cells with suppressor cells)}/\text{c.p.m.(target cells alone)}] \times 100\%$$

EAE induction

EAE was induced in 8–12-week-old C57BL/6J mice by subcutaneous immunisation into hind limb flanks with 0.2 ml of an emulsion containing 50 μg MOG_{35-55} peptide in complete Freud's adjuvant, supplemented with 500 μg heat-killed *Mycobacterium tuberculosis* strain H37RA. Mice also received 250 ng pertussis toxin on day 1.

In other experiments, mice were immunised as described above, and on day 5 after immunisation, purified blood $CD11b^+Ly6G^-$ cells (2×10^5 cells per mouse) were adoptively transferred intravenously.

Statistics

Unless otherwise stated, statistical analysis was performed by one-way analysis of variance followed by Bonferroni tests and the statistical program Prism (GraphPad Software Inc., La Jolla, CA, USA). $P \leqslant 0.05$ was considered significant.

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