Reciprocal relationship of T regulatory cells and monocytic myeloid-derived suppressor cells in LP-BM5 murine retrovirus-induced immunodeficiency

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Immunomodulatory cellular subsets, including myeloid-derived suppressor cells (MDSCs) and T regulatory cells (Tregs), contribute to the immunosuppressive tumour microenvironment and are targets of immunotherapy, but their role in retroviral-associated immunosuppression is less well understood. Due to known crosstalk between Tregs and MDSCs in the tumour microenvironment, and also their hypothesized involvement during human immunodeficiency virus/simian immunodeficiency virus infection, studying the interplay between these immune cells during LP-BM5 retrovirus-induced murine AIDS is of interest. IL-10-producing FoxP3+ Tregs expanded after LP-BM5 infection. Following in vivo adoptive transfer of natural Treg (nTreg)-depleted CD4+ T-cells, and subsequent LP-BM5 retroviral infection, enriched monocytic MDSCs (M-MDSCs) from these nTreg-depleted mice displayed altered phenotypic subsets. In addition, M-MDSCs from LP-BM5-infected nTreg-depleted mice exhibited increased suppression of T-cell, but not B-cell, responses, compared with M-MDSCs derived from non-depleted LP-BM5-infected controls. Additionally, LP-BM5-infused M-MDSCs modulated the production of IL-10 by FoxP3+ Tregs in vitro. These collective data highlight in vitro reciprocal modulation between retroviral-induced M-MDSCs and Tregs, and may provide insight into the immunotherapeutic targeting of such regulatory cells during retroviral infection.

INTRODUCTION

Due to their immunosuppressive properties, T regulatory (Treg) cells are immunotherapeutic targets for the treatment of cancer (Dannull et al., 2005; Duraiswamy et al., 2013; Rech et al., 2012; Taieb et al., 2006; Tan et al., 2013). Alterations in the Treg compartment are observed during retroviral infections, including human immunodeficiency virus (HIV) (Angin et al., 2012; Apoil et al., 2005; Bandera et al., 2010; Bi et al., 2009; Kinter et al., 2007; Moreno-Fernandez et al., 2012; Schulze Zur Wiesch et al., 2011; Simonetta et al., 2012; Suchard et al., 2010), simian immunodeficiency virus (SIV) (Allers et al., 2010; Moreno-Fernandez et al., 2012), and LP-BM5 (Beilharz et al., 2004; Li & Green, 2006) and Friend (Dietze et al., 2011; Dittmer et al., 2004; Robertson et al., 2006; Zelinsky et al., 2006, 2009) murine retroviral infections. HIV and SIV studies have reported increased (Bandera et al., 2010; Bi et al., 2009; Kinter et al., 2007; Moreno-Fernandez et al., 2012; Schulze Zur Wiesch et al., 2011; Suchard et al., 2010), decreased (Angin et al., 2012; Apoil et al., 2005; Simonetta et al., 2012) or no change (Chevalier et al., 2015) in the Treg compartment. In the aforementioned studies, variability in cell surface markers used to define Tregs, such as identification of human Tregs using only CD25 (Baecher-Allan et al., 2005), has led to difficulty in comparatively interpreting these results. The role of Tregs during HIV infection remains unclear: do Tregs (i) suppress protective HIV-immune responses; (ii) decrease immune activation associated with HIV-pathogenesis; and/or (iii) act as a reservoir for HIV infection (Chase et al., 2008; Chevalier & Weiss, 2013; Favre et al., 2009; Kinter et al., 2007; Moreno-Fernandez et al., 2012). Therefore, understanding the role of retrovirus-induced Tregs is critical to determine their potential as immunotherapeutic targets.

Tumour-induced myeloid-derived suppressor cells (MDSCs) are well described (reviewed by Gabrilovich & Nagaraj, 2009), but the role of MDSCs during infections is less understood. MDSC expansion occurs in several microbial infections including bacterial (Delano et al., 2007; Goñi et al., 2002; Sunderkötter et al., 2004), fungal (Mencacci et al., 2002), parasitic (Giordanengo et al., 2002; Terrazas et al., 2001; Voisin et al., 2004) and viral infections, including, but not limited to, hepatitis B virus (Chen et al., 2011), cytomegalovirus (Daley-Bauer et al., 2012), vaccinia virus (Fortin et al., 2012) and very recently...
retroviruses: SIV (Sui et al., 2014), HIV (Bowers et al., 2014; Garg & Spector, 2014; Qin et al., 2013; Vollbrecht et al., 2012) and LP-BM5 murine retrovirus (Green et al., 2013). In murine models, MDSCs are identified as CD11b^+GR^+1^+, with two major subsets: granulocytic (G-MDSCs; Ly6C^hiLy6G^hi) and monocytic (M-MDSCs; Ly6C^loLy6G^hi) (reviewed, for example, by Talmadge & Gabrilovich, 2013). Human MDSCs express CD11b^+CD33^+ and can be further subdivided into CD14^+CD15^G-MDSCs and CD14^+CD15^-M-MDSCs, although a phenotypic consensus on these subsets has not been achieved (reviewed, for example, by Talmadge & Gabrilovich, 2013). In the few available studies, retrovirus-induced MDSCs frequently display a monocytic phenotype (Garg & Spector, 2014; Green et al., 2013; Qin et al., 2013), in contrast to the granulocytic phenotype more prominently observed in cancer models, and are hypothesized to contribute to in vivo immunosuppression displayed in these retroviral models. Regardless of the disparate MDSC markers used in these studies, retrovirus-induced MDSCs consistently suppress in vitro T-cell responses, utilizing suppressive mechanisms including, but not limited to, nitric oxide (NO) and reactive oxygen species production (Garg & Spector, 2014; Green et al., 2013; Qin et al., 2013; Sui et al., 2014; Vollbrecht et al., 2012). MDSCs contribute to in vitro and ex vivo immunosuppression, but how MDSCs influence retroviral infection in vivo remains unclear. Understanding MDSC biology is necessary to define efficient immunotherapeutic approaches to potentially target these cells.

LP-BM5 murine retroviral infection induces a profound and progressive immunodeficiency disease known as murine AIDS (MAIDS). MAIDS results in severe immunodeficiency of T- and B-cell responses, an increased incidence of B-cell lymphomas and increased susceptibility to opportunistic infections at later stages of disease, and thus demonstrates similarities to HIV/AIDS (Cerny et al., 1990; Klinman & Morse, 1989; Morse et al., 1989; Mosier et al., 1985). Pathogenic CD4^+ T-cells are required for disease, as mice lacking CD4^+ T-cells, either by use of depleting antibodies or by genetic mutation, although susceptible to infection by LP-BM5, are resistant to MAIDS pathogenesis (Li & Green, 2006; Mosier et al., 1987; Simard et al., 1997). As in murine Friend retroviral infection (Dietze et al., 2011), Tregs expand within the first few weeks of LP-BM5 infection (Beilharz et al., 2004; Li & Green, 2011). Our laboratory also identified an increase in a CD11b^Ly6G^LoLy6C^hi M-MDSC population as LP-BM5-associated M-MDSCs. We demonstrated for the first time, to the best of our knowledge, in any retroviral or viral model, functional modulation of MDSCs by Tregs in vivo. In addition, M-MDSCs were able to alter Treg function in vitro. Collectively, these studies report a reciprocal relationship between Tregs and M-MDSCs during murine LP-BM5 retrovirus infection.

RESULTS

Induction of IL-10-producing FoxP3^+ Tregs following LP-BM5 infection

Following LP-BM5 retroviral infection, FoxP3^+CD4^+ Tregs expand as early as 1 week post-infection (p.i.), with peak expansion occurring at 2–3 weeks p.i. (Beilharz et al., 2004; Li & Green, 2011). To further characterize these LP-BM5-induced Tregs, FoxP3–GFP mice were infected and analysed for Treg markers at 14 days p.i. As reported previously (Beilharz et al., 2004; Li & Green, 2011), the frequency of GFP^+ (FoxP3^+) CD4^+ T-cells increased significantly at 14 days p.i. (Fig. 1a). No change in CD39 or glucocorticoid-induced TNF receptor (GITR) expression was observed between uninfected and 14 days p.i. FoxP3^+ Tregs (Fig. 1b). In contrast, expression of inducible T-cell costimulator (ICOS), a key mediator in the production of IL-10 (Redpath et al., 2013; Witsch et al., 2002), was significantly upregulated on CD4^+FoxP3^+ Tregs 14 days p.i. (Fig. 1b). Because it has been reported that early peak IL-10 levels occur in the first 3 weeks of LP-BM5 infection (Beilharz et al., 2004; Gazzinelli et al., 1992; Uehara et al., 1994) and it was relevant to the studies here, it was determined whether FoxP3^+ Tregs produced IL-10 during this time. To test this, we used 10BiT mice, which contain a Thy1.1 reporter transgene for IL-10 production such that once the IL-10 promoter is activated, Thy1.1 is expressed continuously on the cell surface (Maynard et al., 2007). LP-BM5-induced Treg production of IL-10 was demonstrated: both the percentage and numbers of IL-10-producing FoxP3^+ Tregs increased significantly by 14 days p.i. (Fig. 1c, d). Additionally, the density of Thy1.1 expression increased significantly on IL-10^+FoxP3^+CD4^+ T-cells from LP-BM5-infected mice compared with those from uninfected mice, indicating increased IL-10 production on a per cell basis (Fig. 1e).

IL-10 production at the protein level was further confirmed in that, at 14 days p.i., LP-BM5-infected mice exhibited: (i) increased IL-10 secretion by splenocytes stimulated with T-cell mitogens (anti-CD3 and CD28); and (ii) increased frequency of IL-10-producing splenocytes, stimulated with T-cell mitogens in vitro and
Adoptive transfer of nTreg-depleted CD4+ T-cells (Li & Green, 2011) was shown). The frequency of Tregs remained parallel with control WT B6 mice. As reported previously infected with LP-BM5 retrovirus at 48 h post-transfer, in CD4+FoxP3+ T-cells (purity 90%) were isolated from FoxP3–GFP mice and sorted to deplete nTregs (CD4+ AT) or sorted CD4+ cells lacking nTregs (nTreg-depl. CD4+ AT) underwent AT into TCRα−/− mice (Fig. 2a). Mice were infected with LP-BM5 retrovirus at 48 h post-transfer, in parallel with control WT B6 mice. As reported previously (Li & Green, 2011), the frequency of Tregs remained absent or very low in nTreg-depleted CD4+ AT mice throughout infection, and the relative percentage of FoxP3+(GFP+) of CD4+ cells was less than one-third of the percentage found in CD4+ AT mice by 5 weeks p.i. (data not shown). As an established MAIDS parameter (Green et al., 2013; O’Connor & Green, 2014) and to identify murine MDSCs by their canonical marker GR-1 (Gabrilovich & Nagaraj, 2009), we first evaluated the frequency of CD11b+GR-1+ cells in LP-BM5-infected mice at 5 weeks p.i. The percentage of CD11b+GR-1+ cells increased in all infected experimental mice, with a trend towards even higher levels in infected mice receiving the nTreg-depleted CD4+ AT (Fig. 2b). In the LP-BM5 infection model, isolated monocytic (Ly6C+Ly6G−), MDSCs, are suppressive (Green et al., 2013). Because anti-GR-1 binds both Ly6C and Ly6G, we wanted to determine if either of these MDSC compartments was altered. The frequency of granulocytic CD11b+Ly6G+ cells increased in all infected mice compared with uninfected controls but with no significant differences between the different infected experimental groups (Fig. 2c). The frequency of CD11b+Ly6C+ M-MDSCs also increased significantly in all infected mice (Fig. 2d). Of greater interest, the frequency of CD11b+Ly6C+ cells was significantly higher in infected nTreg-depleted CD4+ AT mice compared with the infected CD4+ AT controls (Fig. 2d), suggesting that nTregs

Fig. 1. Induction of IL-10-producing FoxP3+ Tregs following LP-BM5 infection. FoxP3–GFP mice (a, b) or 10BiT mice (c–e) were infected with LP-BM5 and splenocytes were stained from uninfected (D0) and 14 days p.i. (D14) mice. All flow cytometry plots (a–c) are representative with accompanying histogram means ± SD (a–e) of three to four mice from a single experiment. All data are representative of at least three independent experiments (a–e). (a) Flow cytometry plots and frequency of GFP+ (FoxP3+) cells of CD4+ cells. (b) GITR, CD39 and ICOS expression in GFP+ (FoxP3+)CD4+ Tregs from D14 mice (shaded), D0 mice (dashed line) or fluorescence minus one (FMO) control (solid line). The accompanying histogram indicates the mean fluorescence intensities (MFI) of GFP+ (FoxP3+)CD4+ Tregs. (c) Flow cytometry dot plots and histogram frequencies of Thy1.1+(IL-10+) of FoxP3+ CD4+ cells. (d) Absolute numbers per spleen of CD4+FoxP3+Thy1.1+ cells. (e) Thy1.1 (IL-10) expression by CD4+FoxP3+Thy1.1+ cells. *P<0.05; **P<0.01; NS, not significant.

Adoptive transfer of nTreg-depleted CD4+ T-cells during LP-BM5 infection enhances the myeloid compartment

Substantial expansion of the myeloid cell compartment occurs in LP-BM5-infected mice by approximately 5 weeks p.i. and is exaggerated in the absence of IL-10 (Green et al., 2008). As Tregs expand earlier during LP-BM5 infection and are capable of producing IL-10 (Green et al., 2008), we determined whether nTregs modulate the myeloid compartment in vivo and utilized an established adoptive transfer (AT) model to test this (Li & Green, 2006, 2007, 2011). First, enriched CD4+ T-cells (purity ≥90%) were isolated from FoxP3–GFP mice and sorted to deplete FoxP3+ (GFP+) Tregs (depletion ≥99%). Unsorted CD4+ T-cells containing FoxP3+ Tregs (CD4+ AT) or sorted CD4+ cells lacking nTregs (nTreg-depl. CD4+ AT) underwent AT into TCRα−/− mice (Fig. 2a). Mice were infected with LP-BM5 retrovirus at 48 h post-transfer, in parallel with control WT B6 mice. As reported previously (Li & Green, 2011), the frequency of Tregs remained absent or very low in nTreg-depleted CD4+ AT mice throughout infection, and the relative percentage of FoxP3+(GFP+) of CD4+ cells was less than one-third of the percentage found in CD4+ AT mice by 5 weeks p.i. (data not shown). As an established MAIDS parameter (Green et al., 2013; O’Connor & Green, 2014) and to identify murine MDSCs by their canonical marker GR-1 (Gabrilovich & Nagaraj, 2009), we first evaluated the frequency of CD11b+GR-1+ cells in LP-BM5-infected mice at 5 weeks p.i. The percentage of CD11b+GR-1+ cells increased in all infected experimental mice, with a trend towards even higher levels in infected mice receiving the nTreg-depleted CD4+ AT (Fig. 2b). In the LP-BM5 infection model, isolated monocytic (Ly6C+Ly6G−), but not granulocytic (Ly6G+Ly6C+), MDSCs, are suppressive (Green et al., 2013). Because anti-GR-1 binds both Ly6C and Ly6G, we wanted to determine if either of these MDSC compartments was altered. The frequency of granulocytic CD11b+Ly6G+ cells increased in all infected mice compared with uninfected controls but with no significant differences between the different infected experimental groups (Fig. 2c). The frequency of CD11b+Ly6C+ M-MDSCs also increased significantly in all infected mice (Fig. 2d). Of greater interest, the frequency of CD11b+Ly6C+ cells was significantly higher in infected nTreg-depleted CD4+ AT mice compared with the infected CD4+ AT controls (Fig. 2d), suggesting that nTregs

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Fig. 2. AT of nTreg-depleted CD4+ T cells during LP-BM5 infection enhances the myeloid compartment. (a) AT protocol. Splenocytes were isolated from naïve FoxP3–GFP mice, positively selected for CD4+ T-cells, and remained unsorted or were sorted to deplete GFP+(FoxP3+) CD4+ Tregs. Purified CD4 T-cells (1 x 10^7), non-depleted (CD4+ AT) or nTreg-depleted (nTreg-depl. CD4+ AT), underwent AT into naïve TCRα−/− mice. Recipient (Recip.) mice were infected, in parallel with B6 mice, 48 h after transfer with LP-BM5 retrovirus or left as uninfected controls. All mice were assessed at 5 weeks p.i. (b–d) All flow cytometry dot plots are representative, and the graphs are cumulative, with points indicating individual mice from all the experiments. Solid black horizontal lines indicate means. Flow cytometry plots and summary of CD11b+GR-1+ cells (b), CD11b+Ly6G+ cells (c), CD11b+Ly6C+ cells (d): uninfected B6 (■) and TCRα−/− (●) mice; infected B6 (□), CD4+ AT (○), or nTreg-depleted CD4+ AT (shaded circle) mice. *P < 0.05; **P < 0.01; NS, not significant.
antagonistically modulated the M-MDSC compartment during LP-BM5 infection.

**Depletion of nTregs during LP-BM5 infection alters the M-MDSC phenotype**

We considered whether the CD11b⁺Ly6C⁺ cells from nTreg-depleted CD4⁺ AT mice, observed in Fig. 2, displayed an altered phenotype compared with control M-MDSCs. Miltenyi bead-enriched M-MDSCs (Ly6G⁺/loLy6C⁺) (Green et al., 2013) from infected B6 and TCRα⁻/⁻ recipient mice of CD4⁺ AT and nTreg-depleted CD4⁺ AT, were assessed at 5 weeks p.i. M-MDSCs from B6 and CD4⁺ AT mice demonstrated similar levels of enrichment. For the interest of these studies, enriched M-MDSCs contained ~5.5 % CD4⁺T-cells and <0.5 % FoxP3⁺CD4⁺ Tregs.

Fig. 3. Depletion of nTregs during LP-BM5 infection alters the M-MDSC phenotype. M-MDSCs were enriched by Ly6G bead depletion, followed by CD11b bead enrichment, from individual mice at 5 weeks p.i. All flow cytometry dot plots (a, b) are representative and the accompanying graphs (a–c) sum up a total of six independent experiments, with points indicating individual mice. Solid black horizontal lines indicate means. (a) Flow cytometry dot plot and graphic summary of CD11b and Ly6C expression of M-MDSCs. (b) Ly6C expression and MFI of M-MDSCs: infected B6 (dotted line), CD4⁺ AT (dashed line), and nTreg-depleted CD4⁺ AT (shaded) mice, and isotype control (solid line). (c) Frequency of Ly6C⁻/Lo, Ly6C⁺/Mid and Ly6C⁺/Hi M-MDSC subpopulations. The means ± SD of individual mice from six aggregated experiments are given. **P<0.01; #P<0.05 in comparison with B6; †P<0.05 in comparison with CD4⁺ AT; NS, not significant.
and were inconsistent with any substantial functional contribution. In contrast, M-MDSCs from nTreg-depleted CD4+ AT mice had a significant increase in the proportion of CD11b+Ly6C+ cells (Fig. 3a). Similarly, the mean fluorescence intensity (MFI) of Ly6C was significantly greater on M-MDSCs from nTreg-depleted CD4+ AT mice (mean ± SD, 2588 ± 862), compared with M-MDSCs from B6 (1013 ± 511) or CD4+ AT mice (880 ± 465) (Fig. 3b).

M-MDSCs from infected mice display varied levels of Ly6C expression (Green et al., 2013) and these different subpopulations (i.e. Ly6C+/Lo, Ly6C+/Mid and Ly6C+/Hi) have been associated with differential suppression of T- and B-cell responses (O’Connor et al., 2015). Here, the Ly6C expression profiles were similar between enriched M-MDSCs from B6 and CD4+ AT mice (Fig. 3c). In contrast, M-MDSCs from nTreg-depleted CD4+ AT recipient mice demonstrated a significant increase in the proportion of Ly6C+/Mid cells, and a correspondingly significant decrease in Ly6C+/Lo cells, with no statistical change but an increasing trend, in Ly6C+/Hi cells – all as compared with M-MDSCs from B6 and CD4+ AT mice (Fig. 3c). These alterations in Ly6C expression were consistent with the possibility that M-MDSCs from nTreg-depleted CD4+ AT mice might display differential suppressive capabilities.

![Diagram](image-url)

**Fig. 4.** Suppression of T-cell, but not B-cell, responses is enhanced by M-MDSCs from nTreg-depleted mice. M-MDSCs were co-cultured with naïve responder cells in a suppression assay and stimulated with lipopolysaccharide (LPS) (B-cell) (a–d) or anti-CD3/CD28 (T-cell) (e–h), as described previously (Green et al., 2013). (a–d) Responder : suppressor cell (R : S) ratio of 1 : 0.33; (e–h) R : S of 1 : 0.166. All histograms represent the means ± SD of triplicate samples and are representative of at least three independent experiments. The percent suppression against B-cell (a) or T-cell (e) proliferative responses by M-MDSCs, as measured by incorporation of [3H]thymidine, is shown. The percent suppression (b, f) and effect on suppression (blockade) (c, g) by addition of the iNOS inhibitor L-NIL on B-cell (b, c) or T-cell (f, g) responsiveness is depicted. ND, No detectable suppression. Suppression assay-derived supernatants, from co-cultures of responder cells with B-cell (d) or T-cell (h) stimuli, per above, with or without M-MDSCs, were collected to measure the amount of NO production using Griess reagent for nitrite. ND, not detectable NO. *P<0.05; **P<0.01; NS, not significant.
Suppression of T-cell responses, but not B-cell responses, is enhanced by M-MDSCs from nTreg-depleted mice

We next evaluated the suppressive capacity of enriched M-MDSCs from nTreg-depleted CD4^+ AT mice against B- and T-cell responsiveness, as described previously (Green et al., 2013). Consistent with the data from Figs 2 and 3, and our previous work with the CD4^+ AT TCRζ^-/- model (Li & Green, 2011), we found no significant difference between the infected B6 and CD4^+ AT
groups; therefore, we focused solely on the two AT groups for the subsequent functional studies.

The percent suppression by M-MDSCs was determined as published previously (Green et al., 2013). Briefly, naïve splenic responder cells stimulated with polyclonal activators were cultured with or without enriched M-MDSCs at multiple responder:suppressor (R:S) cell ratios to titrate the M-MDSC suppressive capability. Suppression of B-cell responsiveness by enriched M-MDSCs from CD4\(^+\) AT and nTreg-depleted CD4\(^+\) AT mice was comparable (Fig. 4a). In contrast, M-MDSCs from nTreg-depleted CD4\(^+\) AT mice were more suppressive of T-cell responsiveness than M-MDSCs from CD4\(^+\) AT mice (Fig. 4g), suggesting that Tregs played a critical role in regulating the extent of M-MDSC suppression of T-cell responses.

For M-MDSCs from WT B6 mice, the dominant mechanism of suppression of T-cell, and about half of the suppression of B-cell responsiveness, is mediated by iNOS/NO (Green et al., 2013). Therefore, additional suppression assays were set up as described above with or without the addition of the iNOS inhibitor L-N6-(1-iminoethyl)lysine (L-NIL). In keeping with our previous results (Green et al., 2013), L-NIL blocked almost half (up to 45%) of the suppression of B-cell responses by M-MDSCs derived from CD4\(^+\) AT or nTreg-depleted CD4\(^+\) AT mice (Fig. 4b, c). Furthermore, the amounts of NO in supernatants from these suppression assays, as assessed by the Griess reagent, were essentially equivalent (Fig. 4d). In parallel suppression assays, also comparing M-MDSCs derived from CD4\(^+\) AT or nTreg-depleted mice, addition of L-NIL blocked almost all M-MDSC-mediated suppression (80–100%) of T-cell responsiveness (Fig. 4f, g). Although a significantly greater L-NIL blockade for the M-MDSCs from CD4\(^+\) AT was observed in this particular experiment, this difference was not a consistent finding in repeat experiments. This near-complete dependence of M-MDSC suppression on iNOS/NO was expected for the CD4\(^+\) AT mice but also occurred in the nTreg-depleted CD4\(^+\) AT mice, despite their greater suppressive activity (Fig. 4e).

In this context, we wondered whether increased NO production could explain the increased suppression of T-cell responsiveness by M-MDSCs from nTreg-depleted CD4\(^+\) AT mice; however, M-MDSCs from either set of mice produced roughly equivalent amounts of NO (Fig. 4h).

**LP-BM5-induced M-MDSCs modulate Treg function**

In Figs 2–4 we demonstrated that Tregs selectively modulated the M-MDSC phenotype and function in the context of the LP-BM5 retrovirus infection system. We next questioned whether LP-BM5-induced M-MDSCs could reciprocally modulate CD4\(^+\) Tregs. To address this possibility, responder cells from FoxP3\(^{+}\)/GFP or 10B11 (Thy1.1 under the IL-10 promoter) mice were stimulated in vitro by the same T-cell polyclonal activation as above and co-cultured with enriched M-MDSCs from LP-BM5-infected WT mice. The percentage of GFP\(^{+}\) (FoxP3\(^{+}\)) of CD4\(^+\) T-cells increased significantly in the presence of M-MDSCs compared with control cultures lacking LP-BM5-induced M-MDSCs (Fig. 5a), with no overall change in the proportion of CD4\(^+\) T-cells within the culture (data not shown). FoxP3\(^{+}\) CD4\(^+\) Tregs did not increase in cultures containing M-MDSCs and the iNOS inhibitor L-NIL (Fig. 5a), indicating that the M-MDSC-dependent increase in Treg percentage was iNOS/NO dependent. These experiments were repeated using direct intranuclear FoxP3 staining with similar results (data not shown).

FoxP3\(^{+}\) CD4\(^+\) Tregs derived from LP-BM5-infected mice readily produced IL-10, as assessed ex vivo via surrogate Thy1.1 induction (Fig. 1c), and here also in control in vitro cultures of stimulated responder cells (Fig. 5b). The addition of M-MDSCs partially inhibited (59±16%) the percentage of IL-10-producing FoxP3\(^{+}\) CD4\(^+\) Tregs (Fig. 5b) and IL-10 produced per Treg cell, based on Thy1.1 MFI (Fig. 5c). Somewhat surprisingly, however, total IL-10 production by FoxP3\(^{+}\) CD4\(^+\) Tregs (Fig. 5b) and the amount of IL-10 produced per cell (Fig. 5c), were decreased significantly by the M-MDSCs in spite of their treatment with L-NIL, indicating that M-MDSC inhibition of IL-10 production by FoxP3\(^{+}\) CD4\(^+\) Tregs was iNOS independent. Furthermore, the presence of M-MDSCs did not alter ICOS levels on IL-10\(^+\) Tregs, a key mediator of IL-10 production, even though IL-10\(^+\) Tregs consistently expressed higher ICOS levels than IL-10\(^+\) Tregs (data not shown). These data collectively indicated that M-MDSCs from LP-BM5-infected mice, themselves affected by nTregs, can reciprocally downregulate Treg function, even in the face of a moderate increase in Treg numbers.

**DISCUSSION**

In these studies we have described, for the first time, to the best of our knowledge, in a retroviral model, reciprocal regulation of Tregs and M-MDSCs in vitro and/or in vivo. LP-BM5 infection caused expansion of the CD4\(^+\) FoxP3\(^{+}\) Treg population, which displayed increased ICOS expression and IL-10 production (Fig. 1). Following in vivo AT of nTreg-depleted CD4\(^+\) T-cells into TCR\(^{−}\)/− recipients, a significant increase in, and an alteration of the Ly6C phenotype of, the CD11b\(^+\) Ly6C\(^{+}\)/lo Ly6C\(^{−}\) M-MDSC population was observed (Figs 2 and 3). M-MDSCs from nTreg-depleted CD4\(^+\) AT mice exhibited significantly more suppressive activity towards T-cell, but not B-cell, responsiveness, compared with M-MDSCs derived from nTreg-intact CD4\(^+\) AT mice (Fig. 4a, e). We identified suppression of T-cell, but not B-cell, responses by the Ly6C\(^{+}\)/Mtd M-MDSCs (O’Connor et al., 2015) and the observed increased suppression of T-cell responses (Fig. 4e) may be attributed to an increased proportion of these Ly6C\(^{+}\)/Mtd cells (Fig. 3c) in the nTreg-depleted CD4\(^+\) AT mice. Using an iNOS-specific inhibitor, we confirmed that almost all of the suppression of T-cell (Fig. 4f, g),
and a substantial proportion, up to 40%, of the suppression of B-cell (Fig. 4b, c), responses by M-MDSCs from nTreg-depleted CD4+ AT mice were mediated by an iNOS/NO-dependent mechanism. Furthermore, we identified a reciprocal relationship between these two cell types and demonstrated that LP-BM5-induced M-MDSCs suppressed IL-10 production by FoxP3+ Tregs in vitro (Fig. 5).

Many HIV/AIDS patients exhibit increased serum IL-10 levels, which may be related to increased ICOS expression observed on, and increased IL-10 production by, CD4+ Tregs from the PBMCs (Chevalier et al., 2015; Liovat et al., 2012). In this report, we also described increased ICOS expression and IL-10 production by FoxP3+ CD4+ Tregs (Fig. 1), in keeping with the utility of the murine AIDS model for studying Tregs during retrovirus infection. Previously, using IL-10−/− mice, our results suggested that IL-10 may normally act to diminish MAIDS, in part by negatively regulating the pathogenic CD4+ T-cell compartment (Green et al., 2008), and we have further observed, in additional studies, increased MDSC activity in these LP-BM5 infected IL-10−/− mice (Green et al., 2013). The work highlighted here suggests that IL-10 derived from CD4+ Tregs may also limit MDSC immunosuppressive functions.

Although our studies suggest that FoxP3+ Tregs may modulate M-MDSCs via IL-10 production, several other mechanisms may also be involved. A recent report by Mascanfroni et al. (2015) indicated that the metabolic programming of murine FoxP3+ Tregs versus FoxP3− type 1 regulatory T (Tr1) cells is closely regulated by hypoxia inducible factor-1α (HIF-1α) and aryl hydrocarbon receptor (Mascanfroni et al., 2015). Additionally, HIF-1α regulates murine MDSC differentiation and function (Corzo et al., 2010; Liu et al., 2014; Noman et al., 2014). Therefore, metabolic regulation of glycolysis versus oxidative phosphorylation may also influence the relationship between MDSCs and Tregs. Future studies are needed to determine whether induced Tr1 cells (Workman et al., 2009), in addition to nTregs, contribute to M-MDSC modulation in the LP-BM5 viral model, as the subjects of Treg plasticity (Hamann, 2012) and FoxP3 instability (Zhou et al., 2009) remain controversial.

FoxP3+ Tregs from HIV-infected patients also co-express CD39 and are correlates of HIV disease (Schulze zur Wiesch et al., 2011). CD39 is an ectoenzyme involved in immunosuppression via release of adenosine (Chevalier et al., 2015) and suppression of IL-2 production by activated T-cells (Jenabian et al., 2013). Although increased CD39 expression was not observed on FoxP3+ Tregs from LP-BM5-infected mice (Fig. 1b), the role of adenosine metabolism in regulating immune responses, including MDSC function, to LP-BM5 retroviral infection remains a possibility.

MDSCs isolated from HIV patients, or expanded in vitro with HIV gp120 envelope glycoprotein, induce Treg expansion in vitro (Garg & Spector, 2014; Vollbrecht et al., 2012). Our experiments herein are consistent with, and expand upon, these findings, as LP-BM5-induced M-MDSCs elicited FoxP3+ Treg expansion in vitro, in an iNOS-dependent manner (Fig. 5). NO itself is capable of generating FoxP3+ Tregs both in vitro and in vivo (Niedbala et al., 2007), supporting the data presented here. However, whereas in vitro HIV gp120-expanded human MDSCs induce IL-10 production by CD4+ T-cells (Garg & Spector, 2014), M-MDSCs from LP-BM5-infected mice inhibited IL-10 production by FoxP3+ Tregs (Fig. 5) but did not alter IL-10 production by CD4+ non-Tregs (data not shown). MDSCs induced in vitro, versus in vivo as in our studies, may exhibit differential functional effects on Tregs. If this is the case, the in vivo LP-BM5-associated MDSCs may represent a physiological model of the Treg–MDSC relationships occurring in vivo in other retroviral models. Phenotypic and/or functional differences between murine and human MDSC subsets may also influence their relationship with Tregs. In a humanized mouse model for HIV disease, it was observed that HIV infects FoxP3+ Tregs. Furthermore, denileukin diftitox depletion of FoxP3+ Tregs impairs HIV infection, in part due to decreased cell targets for virus infection (Jiang et al., 2008), but it is also possible that Treg depletion modulates other immune responses, including MDSC suppressive function. Further characterization in this humanized mouse model may help to bridge the gap between our results in the murine LP-BM5 retroviral model and observations in HIV-infected patients.

Tumour-induced MDSCs can cause Treg expansion in vivo (Huang et al., 2006; Pan et al., 2010; Serafini et al., 2008; Yang et al., 2006), and depletion, physically or functionally, of one immunosuppressive cell type (MDSCs or Tregs) in vivo can result in diminished numbers and/or functions of the other immunosuppressive cell type (Tregs or MDSCs) (Fujimura et al., 2012; Ko et al., 2009; Tseng et al., 2008; Wesołowski et al., 2013). However, this in vivo relationship is not well characterized in retroviral models. Therefore, it was of special interest to find, in our LP-BM5 system, that M-MDSCs were significantly expanded and exhibited increased suppression of T-cell responses, but not of B-cell responses, in the absence of nTregs. This functional dichotomy of MDSCs is contrary to most reports in tumour models and further suggests the heterogeneity of these M-MDSCs (Fig. 2–4). HIV-infected patients as well as LP-BM5-infected mice have several B-cell defects, including polyclonal activation and hypergammaglobulinaemia (Klinman & Morse, 1989; Klinken et al., 1988; Moir & Fauci, 2008), and B-cell abnormalities may be attributed to direct or indirect immunosuppression by M-MDSCs and/or Tregs (Lim et al., 2004; Phetsouphanh et al., 2014). Increased plasma levels of sCD40L are observed in HIV-infected patients and correlate with in vivo Treg frequency (Jenabian et al., 2014). In vitro, sCD40L promotes expansion of Tregs (Jenabian et al., 2014) and MDSCs (Huang et al., 2012).
In the LP-BM5 retroviral system, interactions between CD40 on B-cells and CD40L on T-cells are required for pathogenesis (Green et al., 1996, 1998, 2001, 2002), suggesting that CD40/CD40L signalling may also play a role in shaping the Treg and MDSC immune response to MAIDS. During HIV infection, MDSCs are thought to contribute to immunosuppression by Treg induction (Garg & Spector, 2014; Vollbrecht et al., 2012), but the data presented here suggest that the in vivo relationship between MDSCs and Tregs during retrovirus infections may be more complex.

In conclusion, these studies demonstrate a largely reciprocal regulatory relationship between M-MDSCs and Tregs in a murine model of retroviral immunodeficiency. From the standpoint of the retroviral life cycle and retroviral genome persistence, we hypothesize that Tregs and M-MDSCs constitute functionally overlapping, but distinguishable, immunosuppressive mechanisms, collectively allowing the retrovirus to evade immune clearance, while keeping its host alive, thus resulting in no overt differences in viral load (Li & Green, 2011). During retrovirus infection, cross-talk between M-MDSCs and Tregs may regulate differential suppression of T- versus B-cell targets by these two cell types. The data presented here begin to address the complex relationship between M-MDSCs and Tregs, and how they may contribute to the global immunodeficiency observed during retroviral infections; however, a full in vivo approach is needed to confirm our ex vivo and in vitro observations. Additional studies are needed to understand the precise molecular mechanisms by which M-MDSCs and Tregs functionally regulate each other, and also how these cells influence other arms of the immune response and contribute to LP-BM5, SIV and HIV retrovirus-induced immunodeficiency.

METHODS

Mice. C57BL/6 (B6; WT) mice were purchased from the National Cancer Institute (Bethesda, MD, USA) or Charles River Laboratories (Wilmington, MA, USA), and B6.TCRβ–/– (TCRβ–/–) mice from The Jackson Laboratory (Bar Harbor, ME, USA). B6, FoxP3–GFP (FoxP3–GFP) and 10B11 (Tly1.1 gene under the control of IL-10 promoter) mice (Maynard et al., 2007) were generous gifts from the laboratories of Steve Fiering and Lloyd Kasper (both Geisel School of Medicine at Dartmouth, Lebanon, NH, USA), respectively. All mice were housed and/or bred in house at the Center for Comparative Medicine and Research (CCMR) at the Geisel School of Medicine at Dartmouth. All animal experiments were done with the approval of the Institutional Animal Care and Use Committee of Dartmouth College, and in conjunction with the Dartmouth CCMR, an AALAC-approved animal facility.

nTreg depletion and AT. Splenocytes from naïve FoxP3–GFP mice were positively selected for CD4+ T-cells (purity >90%) using MACS purification beads (Miltenyi Biotec) and negatively sorted using a FACSAria (BD Biosciences) to deplete GFP+ (FoxP3+) CD4+ nTregs (depletion >99%). A suspension of 1 x 10^7 purified CD4+ T-cells [non-Treg depleted (CD4+) or nTreg-depleted CD4+ (nTreg-depl. CD4+) cells] underwent AT via the tail vein into naïve TCRβ–/– mice, as described previously (Li & Green, 2006, 2007, 2011).

LP-BM5 virus inoculation. LP-BM5 retrovirus was prepared as described previously (Green et al., 1998; Klinken et al., 1988). Mice were given an intraperitoneal injection of 5 x 10^4 p.f.u. LP-BM5 at between 6 and 8 weeks of age. Mice receiving AT were infected, in parallel, at 48 h post-transfer.

Flow cytometry. Surface staining was performed as described previously (O’Connor & Green, 2014). Cells were stained with FITC-, peridinin chlorophyll (PerCP)-, phycoerythrin (PE)-, PerCP-eFluor 710, allophycocyanin (APC)- or APC-Cy7-conjugated antibodies to detect the expression of murine CD4 (clone RM4-5), CD11b (clone M1/70), Ly6C (clone HK1.4) or GR-1 (clone RB6-8C5) (all from BioLegend); Thy1.1 (clone HIS51), ICOS (clones 7E.17G9 and 15F9) or CD39 (clone 24DMS1) (all from ebioscience), or GITR (clone DTA-1) (BD Biosciences). Cells were fixed and permeabilized as per the manufacturer’s protocol (BioLegend) and stained with PE-conjugated FoxP3 (clone 150D) (BioLegend) or FoxP3 (clone FJK-16s) (ebioscience). Samples were analysed using a MACSQuant (Miltenyi Biotec) and analysed with Flowjo software (Tree Star). Positive gates were based on isotype or fluorescence minus one (FMO) controls.

M-MDSC cell enrichment and suppression assays. Suppressor cells (S) from pooled or individual LP-BM5-infected (5 weeks p.i.) mice, were negatively selected for Ly6G and then positively selected for CD11b using paramagnetic beads and subsequent MACS column purification (Miltenyi Biotec), as described previously (Green et al., 2013). Enriched M-MDSCs contain ~5.5% MACS*, <0.05% FoxP3+ CD4+, ~4.5% CD8+ and ~20% CD19+ cells, but extensive analysis of suppression by sorting of M-MDSC subpopulations suggests that these cells do not significantly contribute to the suppression of T- and/or B-cell responses (O’Connor et al., 2015). Responders cells (R) were isolated from pooled splenocytes of naïve WT mice. Suppression assays were set up as described previously (Green et al., 2013) at different R:S cell ratios. Samples were plated in triplicate with supplemented medium, and stimulated by either a final concentration of 10 μg LPS ml⁻¹ (B-cell activation) or 2.5 μg anti-CD3 mAb-1 and 1 μg CD28 ml⁻¹ (T-cell activation), in the presence/absence of 100 μM L-NIL (Enzo Life Sciences). The percent suppression was calculated from the change in proliferation when responder cells were cultured alone versus with MDSCs, as described previously (Green et al., 2013).

NO production. Fifty microlitres of cell supernatants from suppression assays (described above) were collected after 65–72 h of culture and combined with 50 μl Griess reagent for nitrite (Sigma-Aldrich). Absorbance was measured at 570 nm, after 10 min of incubation. The concentration of nitrite (NO production) was determined in reference to a NaNO₂ standard curve.

In vitro co-culture assays. Co-culture assays using responder cells from WT, 10B11 or FoxP3–GFP mice were set up similar to the above-described suppression assay. Wells were combined after 72 h to allow for triplicate samples and stained for flow cytometric markers as described above.

IL-10 ELISA and ELISPOT. Plasma or cell supernatants from suppression assays (described above) were collected after 65–72 h of culture and combined with 50 μl Griess reagent for nitrite (Sigma-Aldrich). Absorbance was measured at 570 nm, after 10 min of incubation. The concentration of nitrite (NO production) was determined in reference to a NaNO₂ standard curve.
(eBioscience) and aminoethyl carbazole (Sigma-Aldrich) substrate solution.

Statistical analysis. Statistical analyses between groups were tested using Student’s t-test, and the Holm–Bonferroni method was used to correct for multiple testing.

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