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Macrophage polarization to a unique phenotype driven by B cells

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Regulation of adaptive immunity by innate immune cells is widely accepted. Conversely, adaptive immune cells can also regulate cells of the innate immune system. Here, we report for the first time the essential role of B cells in regulating macrophage (Mϕ) phenotype. *In vitro* B cell/Mϕ co-culture experiments together with experiments in transgenic mice models for B-cell deficiency or overexpression showed B1 cells to polarize Mϕ to a distinct phenotype. This was characterized by downregulated TNF-α, IL-1β and CCL3, but upregulated IL-10 upon LPS stimulation; constitutive expression of M2 Mϕ markers (*e.g.* *Ym1*, *Fizz1*) and overexpression of TRIF-dependent cytokines (IFN-β, CCL5). Mechanistically, this phenotype was linked to a defective NF-κB activation, but a functional TRIF/STAT1 pathway. B1-cell-derived IL-10 was found to be instrumental in the polarization of these Mϕ. Finally, *in vivo* relevance of B1-cell-induced Mϕ polarization was confirmed using the B16 melanoma tumor model where adoptive transfer of B1 cells induced an M2 polarization of tumor-associated Mϕ. Collectively, our results define a new mechanism of Mϕ polarization wherein B1 cells play a key role in driving Mϕ to a unique, but M2-biased phenotype. Future studies along these lines may lead to targeting of B1 cells to regulate Mϕ response in inflammation and cancer.

Key words: B1 cells · Inflammation · Mϕ · M2 polarization · Tumor

See accompanying Commentary by Sica *et al.*

Supporting Information available online

Introduction

Macrophages (Mϕ) are a heterogeneous cell population involved in diverse physiological processes including anti-microbial defence, wound resolution, inflammation, tissue remodeling, plaque formation in atherosclerosis and promotion of tumor growth [1–7]. Despite their heterogeneity, Mϕ can be broadly divided into M1 (classically activated) or M2 (alternatively activated) phenotypes [1, 8–12]. The M1 phenotype is induced in response to microbial products such as LPS or proinflammatory cytokines including IFN-γ, IL-1β or TNF-α. M1 Mϕ produce further proinflammatory cytokines (TNF-α, IL-12 and CCL3) with reactive oxygen and nitrogen intermediates, which combine to give M1 Mϕ potent anti-microbial, tumoricidal and inflammatory properties [12]. In contrast, the M2 phenotype results from exposure to anti-inflammatory molecules such as glucocorticoid hormones, IL-4,

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IL-13, IL-10 or immune complexes [10–12]. M2 Mϕ are anti-inflammatory and immunosuppressive in nature. They are highly phagocytic, preferentially activate the arginase pathway, promote angiogenesis, tissue remodeling and have pro-tumoral activity [8, 12]. However, *in vivo*, the division between M1 and M2 cells may be blurred, with the above phenotypes likely representing two extremes in a continuum of Mϕ functional states [9, 13–15].

Transcriptome data demonstrate the existence of distinct polarization phenotypes for Mϕ associated with specific pathological conditions [4, 7, 16–18]. Based on this, it is believed that the phenotype of an Mϕ is a reflection of its immediate microenvironment. Indeed, several studies have shown that the tumor microenvironment influences the infiltrating Mϕ to promote tumor progression [4, 13]. This necessitates a detailed understanding of these cells, their microenvironmental stimuli and the signaling pathways which shape their pro-tumoral phenotype. Interestingly, de Visser *et al.* [19] demonstrated for the first time, the requirement of B cells in mediating the recruitment of inflammatory cells into pre-malignant skin and in neoplastic progression, using a HPV16-induced mouse skin carcinoma model. The study marks a seminal observation suggesting an interaction between B cells and inflammatory immune cells *in vivo*. This posed certain questions such as whether B cells interact with Mϕ, do they influence Mϕ polarization and if so, what is the molecular basis for such an interaction [20].

B cells also exist in two subsets with different tissue distributions, phenotypes and functional properties [21]. B1 cells inhabit the gut lamina propria, peritoneal and pleural cavities, whereas the "conventional" B2-cell subset mainly occurs in the spleen and lymph nodes [22]. Phenotypically, B1 cells are characterized by a $B220^{lo}IgM^{hi}CD11b^{+}$ staining pattern, whereas B2 cells are characterized by a $B220^{hi}IgM^{lo}CD11b^{-}$ surface expression profile. B1 cells in fact appear to constitute a class of "tolerant" B cells. In contrast to B2 cells, they fail to proliferate following engagement of their B-cell antigen receptor [23, 24], exhibiting atypical downstream signaling characterized by defective PLCγ2, Akt, p38 MAPK and NF-κB activation [25]. Functionally, B1 cells contribute to protection against microbes through release of natural polyvalent Ab in response infection as well as those directed toward self-antigens [26].

In this study, we asked whether interactions between B cells and Mϕ could contribute to Mϕ polarization. We employed *in vitro* B cell/Mϕ co-cultures as well as transgenic mouse models that either lacked B cells or overexpressed B1 cells. We uncovered a crucial role for B1 cells in polarizing Mϕ to a unique, M2-like phenotype, both *in vitro* and *in vivo*. We also revealed the signaling mechanisms behind this polarization and its relevance in a pathological setting.

Results

Absence of B cells induces an inflammatory phenotype in Mϕ

To determine the role of B cells in Mϕ polarization, we compared peritoneal Mϕ from B-cell-deficient μMT mice (μMT-Mϕ) [27] and WT mice (WT-Mϕ). Following LPS stimulation *in vitro*, μMT-Mϕ showed significantly higher expression of inflammatory genes *Tnfa*, *Ccl3* and *Il1b* than the WT-Mϕ (Fig. 1A). In contrast, LPS-induced expression of the anti-inflammatory cytokine, *Il10* was significantly lower in the μMT-Mϕ than the WT-Mϕ. These results were confirmed at the protein level by ELISA (Fig. 1B). Unstimulated Mϕ from WT or μMT mice showed no significant difference in base-line cytokine expression. Thus, Mϕ from a B-cell-deficient background exhibited a predominantly pro-inflammatory phenotype upon activation. This prompted us to investigate further whether B-cell populations had a role in regulating Mϕ phenotype and polarization.

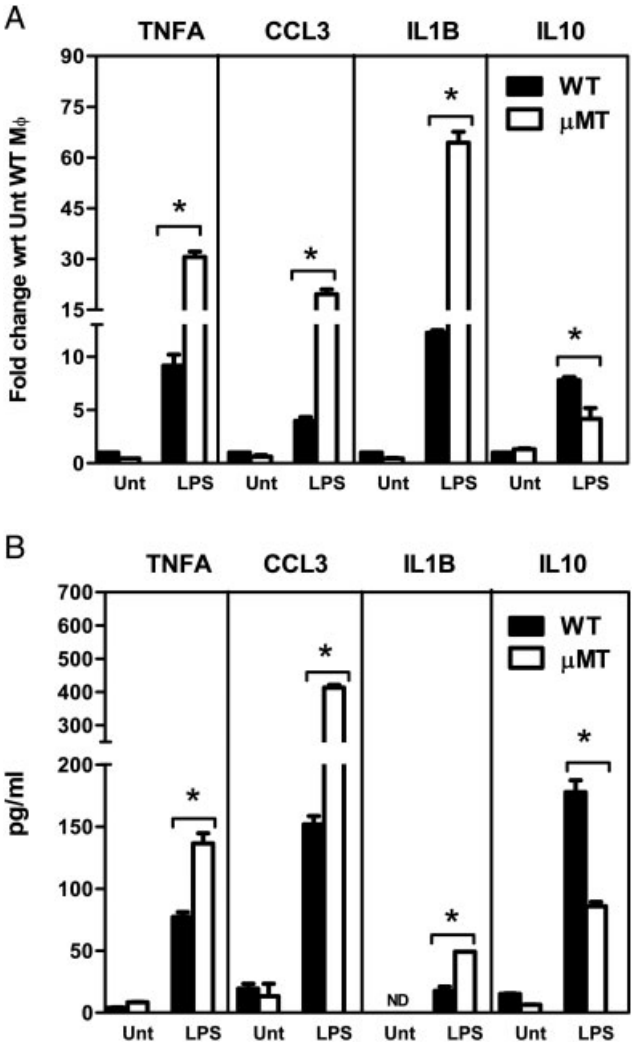

Figure 1. Mϕ from μMT mice show an inflammatory phenotype. Mϕ from WT and μMT mice were left untreated or treated with LPS (100 ng/mL) for 4–24 h for the following assays. (A) Gene expression at 4 h after LPS treatment was assessed by qPCR. Gene expression is represented as fold change with respect to untreated WT Mϕ ("Unt WT Mϕ"). (B) Cytokines in the Mϕ culture supernatants at 24 h after LPS treatment were detected by ELISA. Names on the top of each panel indicate the particular gene or cytokine being assayed. Data show mean ± SEM ($n = 3$) and are representative of three independent *$p<0.05$, WT *versus* μMT.

B1 cells are responsible for Mϕ polarization *in vitro*

Next, we determined whether specific subsets of B cells, *i.e*. B1 or B2 cells could influence the activation and polarization of Mϕ *in vitro*. WT-Mϕ were either cultured alone or in the presence of B1 or B2 cells for 36 h. The B cells were then washed off and Mϕ were cultured for an additional 4 h in the presence or absence of LPS. Quantitative PCR (qPCR) analysis revealed that LPS-treated Mϕ co-cultured with B1 cells expressed lower levels of proinflammatory cytokine genes including *Tnfa, Il1b* and *Ccl3*, but higher levels of the anti-inflammatory cytokine gene, *Il10*, when compared with the LPS-treated Mϕ cultured alone (Fig. 2A). In contrast, Mϕ co-cultured with B2 cells upon LPS treatment did not show any appreciable difference in the induction of the above cytokine/chemokine genes as compared with LPS-treated Mϕ that were cultured alone. Mϕ co-cultured with either B1 or B2 cells in the absence of LPS stimulation did not exhibit any significant modulation in the basal expression of cytokine/chemokine genes.

In all the above experiments, B1 cells were derived from V_H12f (VH12) transgenic mice which preferentially develop B1 cells ([28] and Supporting Information Fig. 1). LPS response of Mϕ co-cultured with B1 cells isolated from WT mice (Fig. 2B and C) showed a similar cytokine profile to those co-cultured with B1 cells from the VH12 mice (Fig. 2A). These results reiterate that the distinct cytokine expression profile of Mϕ co-cultured with B1 cells remained the same, irrespective of whether B1 cells were derived from the WT or VH12 mice. Based on our data, it is clear that B1 cells can induce a distinct polarization of Mϕ with respect to its cytokine/chemokine profile.

B1 cells in VH12 mice influence Mϕ phenotype *in vivo*

To investigate whether B1 cells could also induce polarization of Mϕ *in vivo*, we studied resident peritoneal Mϕ isolated from the VH12 transgenic mice which overexpress B1 cells. Peritoneal VH12-Mϕ and WT-Mϕ were stimulated with LPS and assessed for the expression of cytokines and chemokines. Supporting our *in vitro* findings, LPS-stimulated VH12-Mϕ showed significantly lower expression of *Tnfa*, *Il1b* and *Ccl3* but significantly higher *Il10*, compared with WT-Mϕ (Fig. 3A, left panel). This trend was also confirmed at the protein level by ELISA for IL-1β, CCL3 and IL-10 (Fig. 3A, right panel). Furthermore, we observed that expression of *Ifnb* and the type I IFN-inducible chemokines *Ccl5* and *Cxcl10* was significantly upregulated in LPS-treated VH12-Mϕ compared with WT-Mϕ (Fig. 3B, left panel), also confirmed by ELISA (Fig. 3B right panel).

LPS-induced transcription of genes such as *Ifnb*, *Ccl5* and *Cxcl10* is mediated *via* the TLR4/TRIF-dependent pathway [29]. Based on the above data, we hypothesized that VH12-Mϕ might be predisposed to more efficiently activate this pathway. Indeed, stimulating these VH12-Mϕ with the TRIF pathway-specific activator, polyI:C did result in significantly higher *Ccl5* and *Cxcl10* expression compared with WT-Mϕ (Fig. 3C). In contrast, expression of the MyD88-dependent gene, *Tnfa* was almost twofolds lower in poly I:C-treated VH-Mϕ than WT cells.

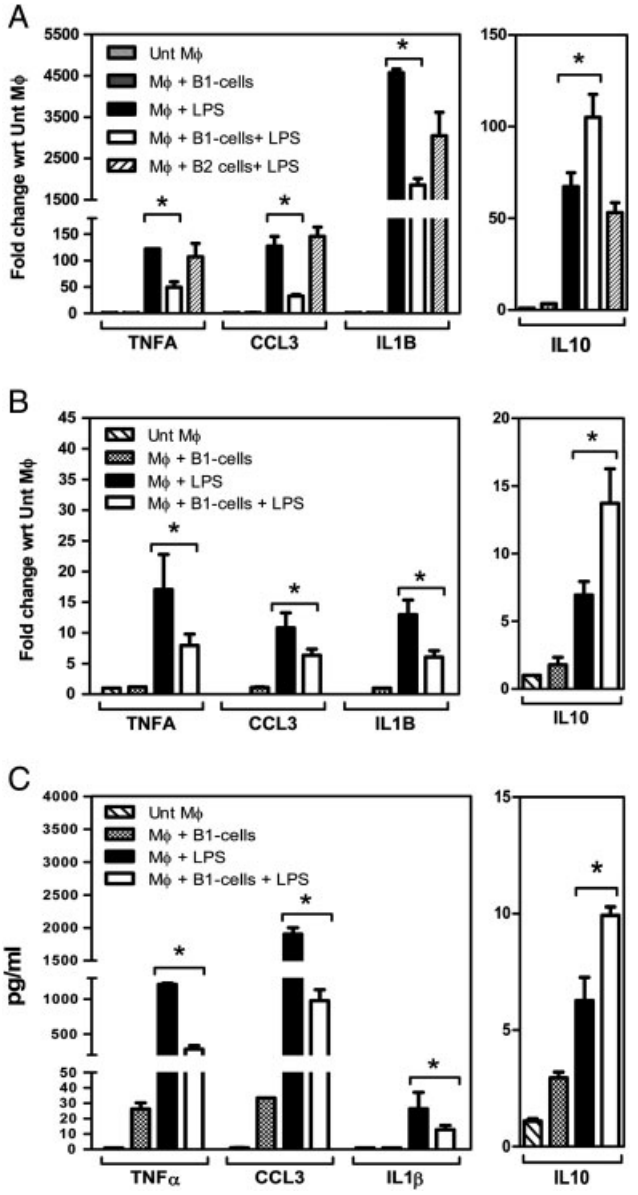

Figure 2. B1 cells induce Mϕ polarization *in vitro*. (A) Mϕ were cultured alone or with B1 or B2 cells (Mϕ:B cells at 1:2 ratio) for 36 h. B cells were then washed off and Mϕ cultured for an additional 4 h either in the presence or absence of LPS (100 ng/mL). RNA was extracted and gene expression detected by qPCR. Mϕ cultured alone for 4 h in the presence of LPS (100 ng/mL) was taken as the positive control for gene expression. B1 cells were derived from VH12 mice whereas B2 cells from WT mice. (B) Mϕ and B-cell co-culture experiment was performed as described above, but with B1 cells derived from WT mice. RNA was extracted and gene expression was detected by qPCR. For (A) and (B), gene expression is represented as fold change with respect to untreated Mϕ ("Unt Mϕ"). (C) Cytokine levels in the supernatants of Mϕ and WT B1-cell co-cultures after 24 h LPS stimulation, determined by ELISA. In all panels, names on the X-axis indicate the gene or cytokine being studied. Data show mean ± SEM ($n = 3$) and are representative of three independent experiments. $^*p<0.05$, Mϕ+B1+LPS *versus* Mϕ+LPS.

Stimulation of Mϕ with the TLR2 ligand, Pam_3Cys_4 which signals through MyD88 but not the TRIF pathway, showed no *Ccl5* and *Cxc10* expression in WT- or VH12-Mϕ. However, Pam_3Cys_4-

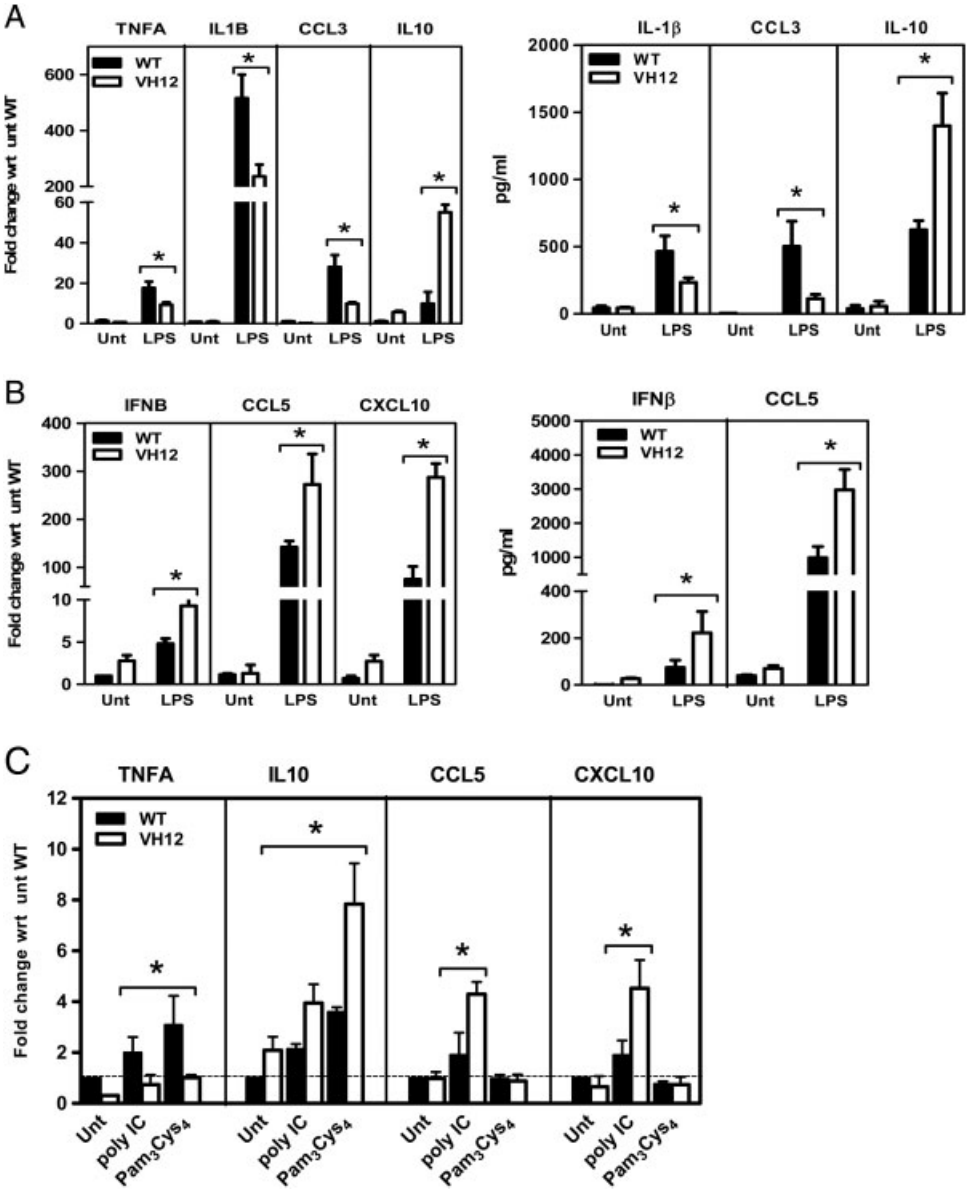

Figure 3. Production of cytokines/chemokines by *in vivo*-polarized VH12 Mϕ. Mϕ from WT or VH12 mice were untreated or treated with LPS (100 ng/mL) for either 4 or 24 h for the following assays. Expression of (A) MyD88-dependent cytokines and (B) TRIF-dependent cytokines was determined either by qPCR at 4 h after LPS stimulation or by ELISA in the Mϕ culture supernatants at 24 h after LPS treatment. $^{*}p<0.05$, WT *versus* VH12 (C) Mϕ from WT and VH12 mice were untreated or treated with TLR3 ligand, polyI:C (10 μg/mL) or TLR2 ligand, Pam 3Cys4 (100 ng/mL) for 4 h and expression of the indicated genes was assessed by qPCR. Gene expression in all cases is represented as fold change with respect to untreated WT Mϕ ("Unt WT"). Names on the top of each panel indicate the particular gene or cytokine being assayed. Data show mean ± SEM of three independent experiments, $^{*}p<0.05$, WT *versus* VH12.

stimulated VH12-Mϕ showed almost twofold lower *Tnfa*, but higher *Il10* expression as compared with the WT-Mϕ (Fig. 3C). This was similar to our observations with LPS-treated VH12-Mϕ, shown earlier in Fig. 2.

Taken together, the above data suggest that VH12-Mϕ upon LPS stimulation show a phenotype characterized by impaired expression of several proinflammatory cytokine genes such as *Tnfa*, *Il1b* and *Ccl3*, but upregulated expression of anti-inflammatory cytokine, *Il10*. This profile exactly coincides and corresponds to those observed in the *in vitro* B1-cell co-cultured Mϕ and thereby provides an *in vivo* validation of this phenotype. However, a unique feature of the VH12-Mϕ was the overexpression of TRIF-dependent genes such as *Ifnb*, *Ccl5* and *Cxcl10* upon LPS as well as polyI:C stimulation.

B1-cell-polarized Mϕ from VH12 mice express several M2 markers

M2 polarized Mϕ are reported to exhibit reduced expression of proinflammatory cytokines (*e.g.* *Tnfa*, *Il1b*) but enhanced

expression of anti-inflammatory cytokines (*e.g. Il10*) [8, 12]. In this respect, VH12-Mϕ (Fig. 3A) and WT-Mϕ co-cultured with B1 cells (Fig. 2) display an M2-like phenotype.

To understand the extent of M2 polarization in VH12-Mϕ, we studied the expression of several well-known M2 markers. VH12-Mϕ constitutively expressed elevated levels of some M2 genes including *Fizz1*, *Ym1*, *Msr2* (or *Cd36*) and *Il1ra*, in contrast to WT-Mϕ (Fig. 4A). However, other M2 genes such as *ArgII* and *Ccl22* were not detected in either Mϕ population under basal conditions. Immunofluorescence images confirmed the presence of YM1 and FIZZ1 protein in VH12-Mϕ (Fig. 4B). Therefore, alongside our cytokine expression results, these data confirm the polarization of VH12-Mϕ toward a distinct M2-like phenotype.

B1-cell-polarized Mϕ exhibit defective NF-κB but functional STAT1 signaling

To determine the molecular basis of the altered phenotype of VH12-Mϕ, we studied the kinetics of some key TLR4 signaling events, namely MyD88-dependent activation of NF-κB and TRIF-dependent activation of STAT1 [29, 30]. We measured the degradation of IκBα as an indicator of NF-κB activation and the expression of phospho-STAT1 (Tyr 701) as a readout for TRIF pathway activation. Figure 5A shows significant IκBα degradation in WT-Mϕ following 30–60 min of LPS stimulation, but not in the LPS-treated VH12-Mϕ. In contrast, VH12-Mϕ showed higher phospho-STAT1 (Tyr 701) expression following 15–30 min of LPS treatment, compared with their WT counterparts (Fig. 5A). These observations indicate defective NF-κB activation, but a functional STAT1 pathway in the VH12-Mϕ, which correlates with our earlier gene expression data. To exclude the possibility that the differential signaling pattern was due to differences in the surface expression of TLR4, we also measured TLR4 expression by flow cytometry. WT and VH12-Mϕ showed comparable TLR4 surface expression (Fig. 5B).

IL-10 is responsible for B-cell-induced polarization of Mϕ

IL-10 polarizes Mϕ to an M2 phenotype [8]. Since B1 cells can constitutively release this cytokine [31], we asked whether IL-10 was involved in our observed B1-cell-induced Mϕ polarization. WT-Mϕ co-cultured with B1 cells *in vitro* expressed higher levels of the IL-10-inducible *Bcl3* and *Socs3* under both basal- and LPS-treated conditions (Fig. 5C, left panel). VH12-Mϕ also displayed higher *Bcl3* and *Socs3* expression compared with WT-Mϕ under LPS stimulated and unstimulated conditions (Fig. 5C, right panel). Thus the interaction of Mϕ with B1 cells induced upregulation of IL-10 inducible genes in the Mϕ.

IL-10-induced gene expression is primarily mediated by the transcription factor STAT3 [32]. Therefore, we compared STAT3 activation in LPS-treated WT- and VH12-Mϕ. VH12-Mϕ showed early expression of phospho-STAT3 (Ser 727) 15–60 min after LPS treatment, whereas WT-Mϕ showed the same level of phosphorylation only 60 min after LPS stimulation (Fig. 5D), thus suggesting a faster and preferential activation of STAT3 in VH12-Mϕ.

To directly assess the role of IL-10, WT-Mϕ were co-cultured with B1 cells in the presence or absence of IL-10 neutralizing Ab for 36 h, followed by washing and stimulation with LPS for 4 h. The addition of IL-10 neutralizing Ab to the B1 cell/Mϕ co-culture induced an almost twofold increase in the suppressed *Tnfa* expression observed in B1-cell-polarized Mϕ (Fig. 5E). ELISA data for TNF-α production also confirmed this trend (Fig. 5E, inset). Neutralizing IL-10 also substantially reduced expression of the TRIF-dependent genes *Ccl5* and *Cxcl10* and the M2-marker, *Il1ra* in Mϕ co-cultured with B1 cells (Fig. 5E). Incubation with isotype-matched control Ab had no significant effect (data not shown).

To further testify the role of B1-cell-derived IL-10 in polarization of Mϕ, we performed experiments involving the

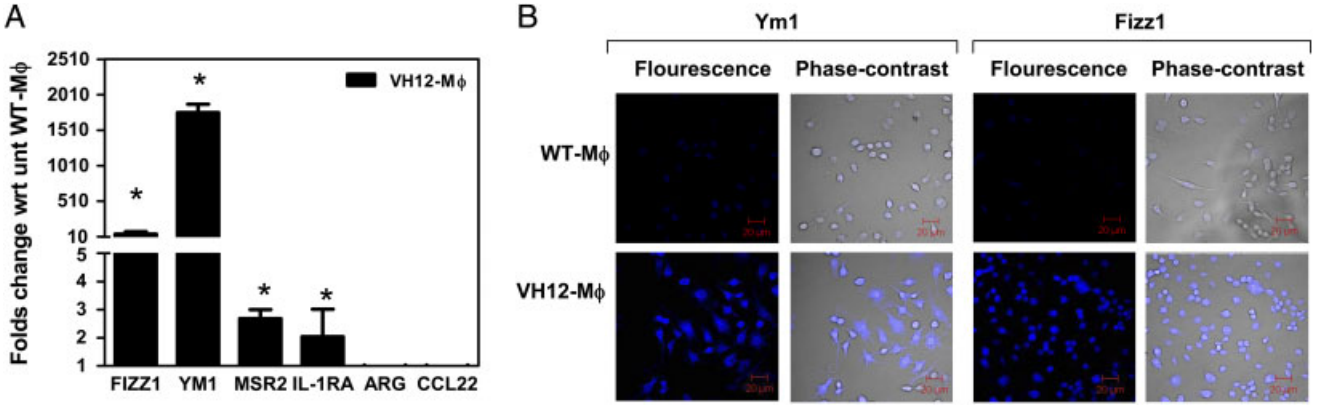

Figure 4. *In vivo*-polarized Mϕ from VH12 mice show a M2 phenotype. (A) Mϕ from WT or VH12 mice were analyzed for the expression of typical M2 marker genes by qPCR. Black columns represent gene expression in VH12 Mϕ shown as fold change with respect to untreated WT Mϕ ("Unt WT Mϕ). Data show mean ± SEM from three independent experiments, $^{*}p<0.02$, WT *versus* VH12. (B) Mϕ from WT or VH12 mice were grown on cover slips and labeled with fluorescent-tagged Ab recognizing the indicated M2-associated molecules. Fluorescence panel shows immunofluorescence for the indicated molecules (blue), while the phase-contrast panel shows phase-contrast image of these cells overlayed with the respective fluorescent stainings. All images were acquired on a Zeiss LSM 510 META confocal microscope. Results are representative of three independent experiments.

co-culture of WT-Mϕ with IL-$10^{-/-}$ B1 cells. WT-Mϕ co-cultured with IL-$10^{-/-}$ B1 cells showed a marked reduction in *Il10* expression as compared with those co-cultured with WT B1 cells, upon LPA simulation (Fig. 5F, upper panel). Similarly, expression of the TRIF-induced gene, *Ccl5* as well as the M2 gene, *Il1ra* was abrogated in Mϕ co-cultured with IL-$10^{-/-}$ B1 cells instead of WT B1 cells (Fig. 5F middle and lower panel). This observation provided genetic evidence for the requirement of B1-cell-derived IL-10 in driving *Il10* expression and polarization of Mϕ.

We also performed a reverse experiment where WT B1 cells were co-cultured with IL-$10^{-/-}$ Mϕ in order to assess the relative contribution of Mϕ-derived IL-10 in their polarization. Figure 5F (upper panel) confirms lack of *Il10* transcript in the IL-$10^{-/-}$ Mϕ, as expected. Figure 5F (middle and lower panels) shows comparable *Ccl5* and *Il1ra* expression between LPS-treated WT-Mϕ and IL-$10^{-/-}$ Mϕ, following co-cultured with WT B1 cells. Similarly, no significant difference in the modulation of other genes such as *Tnfa* or *Ccl3* was noted between the WT-Mϕ and the IL-$10^{-/-}$ Mϕ, when co-cultured with the WT B1 cells (data not shown). These observations undermine the contribution of Mϕ-derived IL-10 in driving Mϕ polarization in these co-culture experiments. On the contrary, the results from the IL-$10^{-/-}$ B1-cell co-culture experiments clearly suggested a crucial role for B1-cell-derived IL-10 in driving the polarization of Mϕ.

B1-cell-derived IgM does not contribute to the polarization of Mϕ

B1 cells are efficient producers of IgM and Mϕ express Fcμ receptor, which serves as a receptor for this immunoglobulin. We wanted to evaluate whether IgM released by B1 cells influenced the M2 polarization of these Mϕ when co-cultured with B1 cells. For this purpose, we treated WT-Mϕ *in vitro* with purified IgM and subjected them to LPS or IL-4 treatment to induced M1 and M2 polarization, respectively. Treatment of WT-Mϕ with IgM alone induced a modest expression of the proinflammatory genes such as *Tnfa*, *Ccl3* and *Il1b* (Fig. 6A). However, on stimulation with LPS, WT-Mϕ treated with or without IgM showed similar expression of *Tnfa*, *Ccl3* and *Il1b* suggesting that IgM did not have any appreciable influence on the expression of M1 polarizing genes (Fig. 6A). Expression of *Il10* was similar for IgM-treated or untreated Mϕ under basal and LPS-stimulated conditions. In contrast, when WT-Mϕ were treated with or without IgM and subjected to IL-4-induced M2 polarization, the expression of M2-genes *YM1*, *FIZZ1* and *MGL2* was significantly inhibited in the IgM-treated Mϕ as compared with the Mϕ, which did not receive IgM treatment (Fig. 6B). *Il1ra* expression remained unaffected by the IgM treatment. Furthermore, Mϕ treated with IgM alone did not induce any significant modulation in the expression of *Ym1*, *Fizz1*, *Mgl2* and *Il1ra* (Fig. 6B). Collectively, these results do not support a likely role for IgM in driving M2 polarization in Mϕ.

B1 cells can polarize Mϕ in tumors

Several reports have shown tumor-associated Mϕ (TAM) to be an M2-polarized population and to play a pro-tumoral role [7, 9, 13]. Our results suggest B1 cells to polarize Mϕ into a M2-like phenotype similar to that of fibrosarcoma-derived TAM [7]. We wanted to investigate whether B1 cells could also drive an M2-like polarization of Mϕ *in vivo* in a tumor setting.

B16 cells were injected s.c. together with purified B1 cells or control cells (consisting of splenic B2 cells, mainly). After 11 days, when the tumors became palpable, the experimental group received intratumoral injection of B1 cells, whereas the control group received control splenic B2 cells. On day 14, mice were sacrificed, TAM were enumerated by flow cytometry and purified to measure their polarization status following *ex vivo* stimulation with the M1-stimulus, LPS or the M2 stimulus, IL-4.

We did not observe any significant difference in the absolute numbers of TAM in the two groups of mice (Supporting Information Fig. 2). However, LPS inducibility of the pro-inflammatory genes *Tnfa*, *Ccl3* and *Il1b* was significantly reduced in TAM from B1-cell-treated tumors compared with TAM from the control group (Fig. 7A). *Cxcl10* and *Ccl5* levels were comparable between the groups (Fig. 7A). Notably, a dramatic upregulation of the M2 marker genes *Ym1*, *Fizz1*, *Il1ra*, *Mgl1* and *Mgl2* (basally and after *ex vivo* treatment with IL-4) was observed in TAM from B1-cell-treated tumors compared with TAM from the control group, demonstrating their preferential M2-like phenotype (Fig. 7B). Thus, our results show that B1-cell transfer could indeed induce an M2-like polarization of Mϕ *in vivo* in a tumor setting.

We also performed an additional experiment where B16 tumors were grown either in WT mice or in B-cell-deficient μMT mice. As compared with TAM from WT mice, those from the μMT mice showed higher expression of the proinflammatory genes *Tnfa*, *Il1b* and *Ccl3* but downregulated expression of *Il10*, upon LPS stimulation, which suggested their predominantly M1 phenotype, in the absence of B cells (Supporting Information Fig. 3).

Discussion

The concept of Mϕ polarization [8–12] and the use of gene expression profiling to characterize *in vitro*-polarized Mϕ have been reported in several studies [8, 33, 34]. However, relatively little is known about Mϕ polarization *in vivo* and in particular, how interaction with other immune cell types contributes to modulation of Mϕ phenotypes.

Although the role of innate immune cells in polarizing the adaptive immune response is well established [8], the potential of cells of adaptive immunity to polarize innate immune cells is less known. Our current observation of B-cell-driven polarization of Mϕ both *in vitro* and *in vivo* raises such a possibility. Our initial observation that peritoneal Mϕ from B-cell-deficient μMT mice showed higher inflammatory responses to LPS stimulation than their WT counterparts triggered our interest in investigating

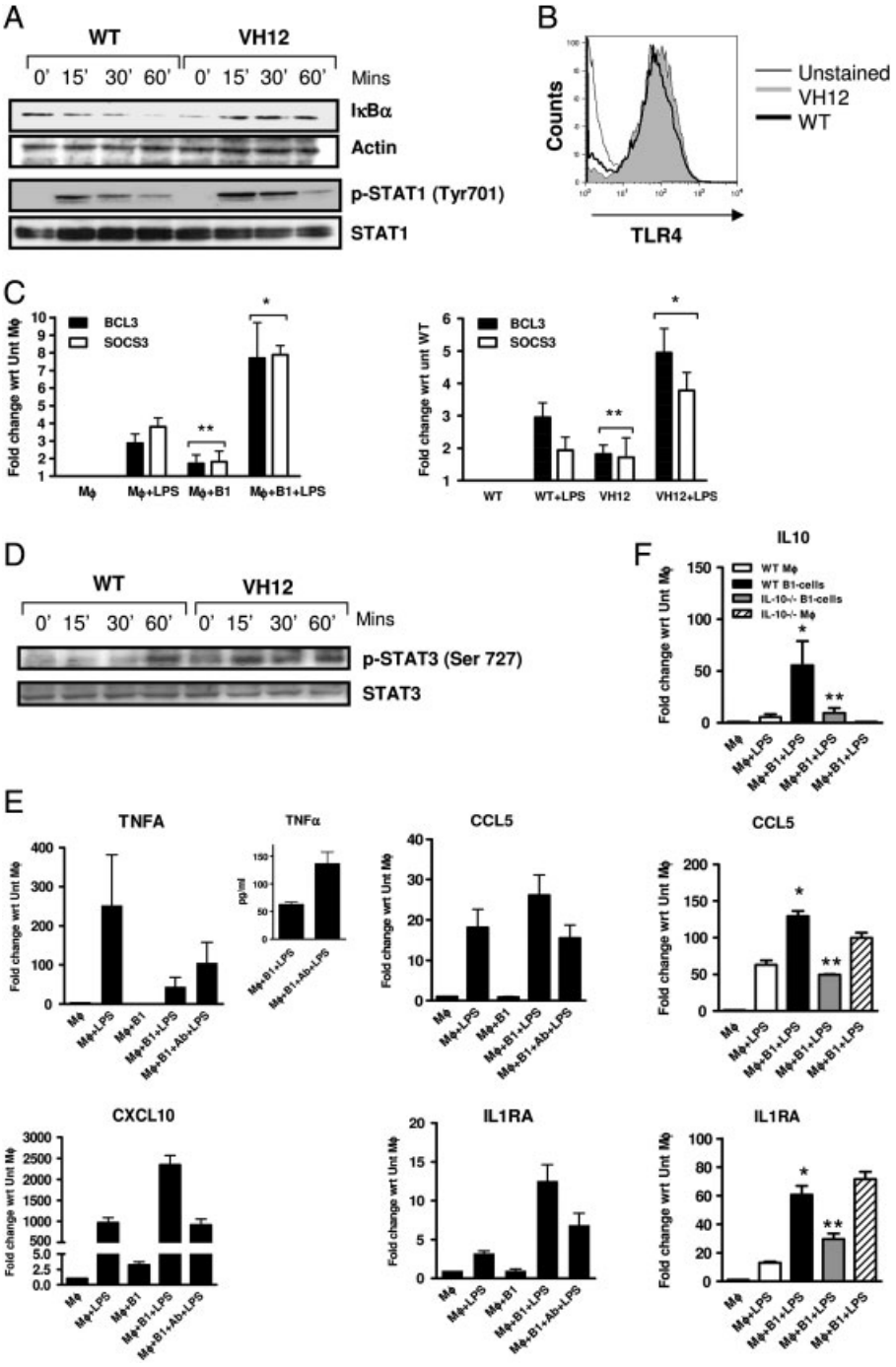

Figure 5. Molecular phenotype of B1-polarized Mϕ. (A) Mϕ from WT or VH12 mice were stimulated for indicated time periods with LPS (100 ng/mL) and assayed for the expression of signaling molecules by Western blotting. Actin and STAT1 expression serve as loading controls (B) TLR4 expression gated on F4/80-positive Mϕ from WT and VH12 mice. (C) Expression of IL-10-inducible genes *Bcl3* and *Soc3* in WT Mϕ/B1 cell co-cultures (left panel) or in Mϕ from WT and VH12 mice (right panel), under unstimulated or 4 h LPS (100 ng/mL) stimulated conditions. qPCR expression of the indicated genes is shown. *p<0.05, Mϕ+LPS *versus* Mϕ+B1+LPS; **p<0.05, Mϕ *versus* B1+Mϕ; *p<0.05, WT+LPS *versus* VH12+LPS; **p<0.05 WT *versus* VH12 (D) Immunoblot showing STAT3 activation in WT and VH12 Mϕ treated with 100 ng/mL LPS for indicated time points. Equal loading is shown by STAT3 expression. (E) IL-10-neutralization in WT Mϕ/B1 cell co-culture experiments. Mϕ were cultured alone or with B1 cells in the presence or absence of anti-IL-10 neutralizing Ab (4 μg/mL, indicated by "Ab"). Expression of selected cytokine genes following an additional 4 h LPS (100 ng/mL) stimulation of these Mϕ is shown. *Inset* shows ELISA data for TNF-α production in the above experiment. (F) LPS-induced expression of the indicated genes in WT-Mϕ co-cultured with WT or IL-$10^{-/-}$ B1 cells. Expression of these genes is also shown from IL-$10^{-/-}$ Mϕ co-cultured with WT B1 cells. Gene expression data shown in (C), (E) and (F) are represented as fold change with respect to untreated WT Mϕ ("Unt Mϕ"). *p<0.05 with respect to WT-Mϕ+LPS; **p<0.05 with respect to WT-Mϕ+WT B1 cells+LPS. Gene expression data shown in (C), (E) and (F) are represented as fold change with respect to untreated WT Mf ("Unt Mf").

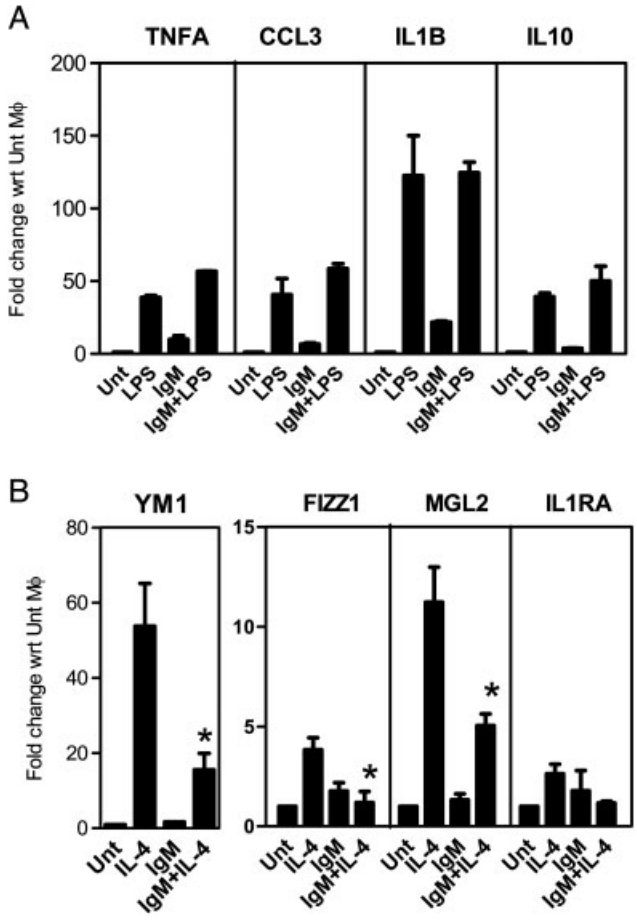

Figure 6. Expression of M1 and M2 polarization genes in IgM-treated Mϕ. Mϕ from WT mice were treated with purified mouse IgM (5 μg/mL) and either left alone or polarized to M1 and M2 states using LPS (100 ng/mL) and IL-4 (10 ng/mL) treatments, respectively. Cells were lysed at 4 h after the treatments and RNA used for qPCR for the expression of typical (A) M1 and (B) M2 polarization genes. Gene names are indicated on the top of each panel. Gene expression is represented as fold change with respect to untreated Mϕ ("Unt Mϕ"). Data show mean ± SEM from three independent experiments. *$p<0.05$, Mϕ+IL-4 *versus* Mϕ+IgM+IL-4.

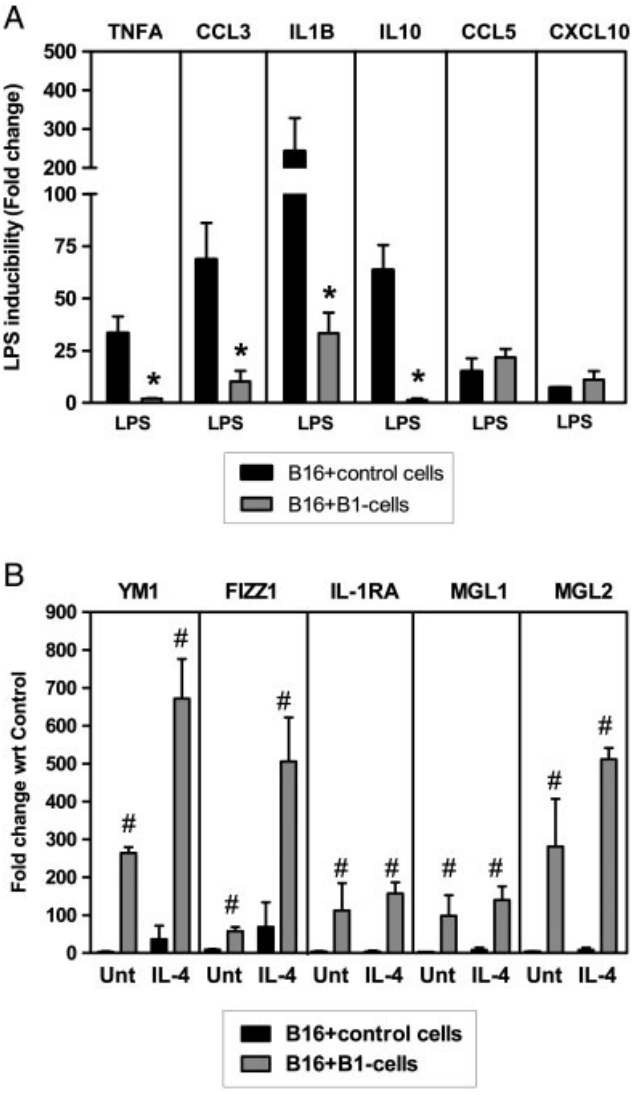

Figure 7. B1-cell-induced polarization of TAM. (A) qPCR analysis of proinflammatory genes in *ex vivo* LPS (100 ng/mL)-stimulated TAM from control cell or B1-cell-treated B16 melanoma at day 14. Data represent LPS inducibility of the indicated genes expressed as fold change. Gene names are indicated on the top of each panel. *$p<0.05$, LPS-treated TAM from B16+B1 cells treatment group *versus* LPS-treated TAM from B16+ control cells treatment group, Mann–Whitney *U*-test; (B) qPCR analysis of M2-specific genes in *ex vivo* IL-4 (10 ng/mL)-stimulated TAM isolated from the tumors indicated in (A). Gene expression is presented as fold change with respect to control (*i.e.* untreated TAM from B16+ control cell treatment group). Gene names are indicated on the top of each panel. Unt: untreated TAM; IL-4: IL-4-treated TAM. ♯ $p<0.03$, IL-4-treated TAM from B16+B1 cell treatment group *versus* IL-4-treated TAM from B16+ control cell treatment group, Mann–Whitney *U*-test. Data show mean ± SEM of three independent experiments.

whether B cells had a role in modulating Mϕ polarization (Fig. 1). We demonstrated that B1 cells but not B2 cells, co-cultured with peritoneal Mϕ polarized them to a phenotype characterized by impaired expression of LPS-induced pro-inflammatory genes (*e.g.* *Tnfa*, *Ccl3* and *Il1b*), with upregulation of the anti-inflammatory gene *Il10* (Fig. 2). This phenotype was also observed in peritoneal Mϕ from VH12 mice, which preferentially develop B1 cells, suggesting an *in vivo* polarization of Mϕ by B1 cells (Fig. 3). Mϕ isolated from the spleen of VH12 mice, but not those derived from the bone marrow, exhibited a similar phenotype, indicating that this phenomenon was restricted to those anatomical compartments where B cells and Mϕ co-exist (*e.g.* peritoneum and spleen) (Supporting Information Fig. 5).

Detailed characterization of the *in vivo*-polarized VH12-Mϕ revealed their M2-like phenotype. This was evident from their defective expression of proinflammatory genes, with high expression of the anti-inflammatory gene *Il10* (Fig. 2) and M2 markers like *Ym1*, *Fizz1*, *Msr2* and *Il1ra* (Fig. 4). However, another unique feature of the VH12-Mϕ phenotype was the upregulation of *Ifnb* expression and IFN-inducible genes such as *Ccl5*, *Cxcl10* (Fig. 3). This phenotype is similar to that of TAM from a murine fibrosarcoma model, reported earlier [7]. In this

study, the molecular basis of the M2-like TAM phenotype was correlated to a defective NF-κB but a functional TRIF/STAT1 pathway [7]. Similarly, VH12-Mϕ also possessed a defective NF-κB activation, but a functional TRIF/STAT1 pathway (Fig. 5). This molecular phenotype explains their defective proinflammatory gene expression and overexpression of IFN-inducible genes (*e.g*. *Ccl5* and *Cxcl10*). The co-expression of IL-10 and IFN-inducible chemokines in VH12-Mϕ may result from enhanced TRIF signaling that has been recently implicated in the regulation of IL-10 expression through IFN-β and TRAF3 [35, 36]. However, further molecular characterization would be needed to clarify this point.

The significance of our observation on B1-cell-induced polarization of Mϕ is particularly interesting in the context of the recent findings by Coussens and colleagues [19, 37]. Using a K14-HPV16 mouse model of epithelial carcinogenesis, these authors show that B cells drive malignant progression by regulating the recruitment, composition and pro-tumoral properties of circulating and resident leukocytes (such as mast cells, dendritic cells and Mϕ). These effects were mediated through the activation of Fcγ receptors (FcγR) on the myeloid cells by IgG [37]. Although Mϕ play a key role in tumor progression, the molecular pathways which regulate their tumor promoting properties are not well understood [4, 5, 9, 13, 38, 39]. In this context, evidence of B cells regulating Mϕ recruitment and their function in tumors present an intriguing perspective. In our B16 melanoma mouse model, the presence of both Mϕ and B1 cells was observed in the tumors (Supporting Information Fig. 2). Interestingly, intratumoral injection of B1 cells polarized the TAM to a more M2-like phenotype. These TAM were characterized by significantly downregulated expression of M1 genes *Tnfa*, *Ccl3* and *Il1b*, but higher levels of well-known M2 markers *Ym1*, *Fizz1*, *Il1ra*, *Mgl1* and *Mgl2*, upon stimulation with M1 or M2 stimuli, respectively (Fig. 7). Consistent with the pro-tumoral role of M2-polarized TAM [9, 13], we also investigated whether there were any differences in the tumor growth between the B1-cell-injected and the control tumors. Preliminary observation show a trend toward increased tumor growth for the B1-cell-injected tumors, compared with the control group, although not statistically significant when seen in a limited temporal window (Supporting Information Fig. 4B). A role for B cells in B16 melanoma model is further supported by the studies of Perricone *et al.* [40] where they show enhanced efficacy of melanoma vaccines in the μMT mice lacking B cells. Similarly, B cells may also play a role in human breast cancer progression, where the presence of infiltrating plasma cells was linked to poor prognosis [41]. Our data from the B16 melanoma study extend the paradigm of B1-cell-induced polarization of Mϕ *in vivo* to tumor settings. These observations are the first to implicate a specific B-cell subset (*i.e*. B1 cells) in driving the M2 polarization of TAM. Further investigation is needed to clarify how these cells modulate various aspects of TAM function in experimental tumor models.

Finally, our investigations into the mechanistic basis of B1-cell-induced Mϕ polarization showed the crucial role of B1-cell-derived IL-10. This was evident from (i) the overexpression of IL-10-inducible genes such as *Socs3* and *Bcl3* in WT-Mϕ co-cultured with B1 cells as well as VH12-Mϕ, (ii) the activation of STAT3 (required for IL-10R signaling) in VH12-Mϕ, (iii) the ability of IL-10 neutralizing Ab to reverse B1-cell-induced polarization of WT-Mϕ in the co-culture experiments and (iv) the ability of B1 cells to constitutively release IL-10 [31]. In addition, given that IL-10 is produced constitutively by B1 cells as well as by B1-cell-polarized Mϕ, we determined the relative contribution of these two sources of IL-10 in the modulation of Mϕ functions. Results from our Mϕ co-culture experiments with IL-$10^{-/-}$ B1 cells demonstrated a direct role of B1-cell-derived IL-10 in promoting *IL10* expression by Mϕ and their consequent M2 polarization (Fig. 5F). The role of IL-10 in polarizing Mϕ to an M2 phenotype is well documented in diverse *in vivo* settings, including a number of tumors such as chemically induced fibrosarcoma [7] and Lewis lung carcinoma [42]. But, the role of B cells in inducing this process and the unique M2-like phenotype described here has not been reported previously.

Other mechanisms for B1-cell-induced Mϕ polarization may exist. Mantovani [20] hypothesized that IgG immune complexes released by B cells could possibly induce the M2-polarization of Mϕ in the tumors. This concept was recently validated in a mouse squamous carcinoma model, where B-cell-derived autoAb (*e.g*. IgG) activate FcγR on TAM to mediate their pro-tumoral and M2-like polarization [37]. Similarly, mouse Mϕ and human monocytes activated by LPS in the presence of immune complexes display an immunosuppressive phenotype [11, 33], similar to that of the TAM from fibrosarcoma [7] and our B1-cell-polarized Mϕ. However, it is important to note that B1 cells predominantly release multivalent IgM which do not bind to the FcγR. Instead, IgM bind to the Fcμ receptor also expressed on Mϕ [43, 44]. Therefore, we investigated whether B1-cell-derived IgM could induce M2 polarization of Mϕ. IgM treatment failed to show any modulation in the M1 polarization of Mϕ (Fig. 6A). In fact, IgM-treated Mϕ showed marked downregulation of many M2 genes such as *Ym1*, *Fizz1* and *Mgl2* when they were polarized to a M2 phenotype (Fig. 6B). These results do not support a likely role for IgM in mediating the B1-cell-induced polarization of Mϕ.

In conclusion, our study demonstrates for the first time the ability of B1 cells to polarize Mϕ to a unique, but M2-like phenotype both *in vitro* and *in vivo*. Furthermore, we demonstrate that this paradigm is operative *in vivo* in pathological settings like tumors, where B1 cells skewed TAM toward a pro-tumoral M2-like phenotype. However, future studies need to be carried out to clarify the relevance and mechanism of B-cell-mediated regulation of Mϕ response in other pathological conditions. If proven, this could lead to the targeting of B cells as a means for therapeutic intervention in such disease conditions. In support, Perricone *et al.* [40] suggest a therapeutic regimen with depletion of B-lymphocytes that may be beneficial to cancer vaccine therapy, in their preclinical B16 melanoma study.

Materials and methods

Reagents

All murine cells were cultured in DMEM (GIBCO Life Technologies, NY, USA) containing 4500 mg/L D-glucose and L-glutamine without sodium pyruvate and sodium bicarbonate. Medium was supplemented with 10% fetal bovine serum (HyClone, UT) and 100U/mL penicillin–streptomycin. The following reagents were used for cell treatment: LPS from *Escherichia coli* 055:B5 (Sigma, St. Louis, MO, USA), anti-IL-10 neutralizing Ab (Pierce-Endogen), purified mouse IgMκ (Bethyl Laboratories, TX, USA) and recombinant human IL-4 (Peprotech, UK).

Mice

C57BL/6, μMT, IL-10-deficient (IL-$10^{-/-}$) and CD45.1 mice were obtained from Jackson Laboratory, NE, USA, and maintained in our animal facility. BALB/c and V_H12f transgenic mice [28] were maintained in our animal facility. All mice used were between 6 and 8 wk of age and in accordance with the institutional guidelines as well as approved protocol by the IACUC committee, Biological Resource Center (A*STAR), Singapore.

Purification and isolation of B cells

To obtain a pure population of B1 cells, peritoneal cavity washout of V_H12f (VH12) mice was seeded onto a tissue culture dish for 2–3 h to remove adherent Mϕ. Thereafter, the nonadherent cells were retrieved and checked for purity using flow cytometry. More than 90% of these cells were peritoneal B1 cells, characterized by a $B220^{lo}IgM^{hi}$ staining pattern. In some specific experiments (as indicated in the *Results* section), B1 cells were also isolated from WT and IL-$10^{-/-}$ mice according to the protocol described above. B2 cells were isolated from splenocytes of WT mice by MACS, using negative selection with anti-CD43 microbeads (Miltenyl Biotech, Germany). The purity of B2 cells obtained was $>85\%$ as assessed by flow cytometry analysis. They were characterized by a $B220^{hi}IgM^{lo}$ staining.

Preparation of peritoneal Mϕ

Peritoneal exudate cells were harvested from WT, VH12 μMT or IL-$10^{-/-}$ mice and peritoneal Mϕ purified according to the protocol described earlier [7]. Monolayers were $>95\%$ Mϕ, assessed by labeling with Ab against CD11b and F4/80 antigen and analyzed by flow cytometry. Unless mentioned, all Mϕ experiments were performed with resident peritoneal Mϕ.

B16 melanoma model and the isolation of tumor-associated Mϕ

Mice were inoculated s.c. in the left- and right-hind flanks with a total of 1×10^5 B16 melanoma cells expressing luciferase (Xenogen, CA, USA). Tumor take was monitored by bioluminiscence imaging using an Ivis Spectrum device (Xenogen) as well as by caliper measurements over 14 days. Cell transfer experiments were performed on two mice groups *viz.* (i) control group were treated with control cells (*i.e.* mainly splenic B2 cells) and (ii) experimental group were treated with B1 cells according to the regime indicated below. Briefly, 1.5×10^5 control cells or B1 cells from CD45.1 transgenic mice were co-injected s.c. with tumor cells on day 0, followed by another injection of 0.5×10^5 cells intratumorally on day 11 (Supporting Information Fig. 4A). Solid tumors were disaggregated with 0.3% w/v collagenase (*i.e.* 1mg/mL collagenase A+0.1mg/mL DNase I, Roche, Switzerland) in serum-free medium for 30 min at 37°C, and TAM were extracted as described earlier [7]. Purity and phenotype of infiltrating TAM and B cells were assessed by flow cytometry using F4/80/CD11b and CD19/B220/IgM/CD45.1 staining, respectively.

qPCR

Cells were lysed with Trizol (Life Technologies, Invitrogen) and total RNA was prepared according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed and used for qPCR analysis on a ABI-PRISM 7500 detection System (PerkinElmer, Norwalk, CT, USA) as described earlier [7].

ELISA

Cell-free supernatants from untreated or LPS (100ng/mL)-treated cells were tested for the indicated cytokines/chemokines (TNF-α, IL-1β, IL-10, CCL5 (QuantiKine Kits, R&D Systems Minneapolis, MN, USA); IFN-β and CCL3 (RayBio® Mouse ELISA kits, RayBiotech, GA)) using ELISA Kits according to the manufacturers' instructions.

Immunoblotting

Mϕ from WT and VH12 mice were treated as indicated in the *Results* section and protein extracts were prepared for immunoblotting as described earlier [7]. The following Ab were used for immunoblots: anti-phospho-STAT1(Tyr701) and anti-phospho-STAT3(Ser727) from Cell Signaling Technology, MA, USA; anti-IκBα, anti-STAT1 and anti-STAT3 from Santa Cruz Biotechnologies, CA.

Flow cytometry

Cells were stained with the indicated fluorochrome-conjugated Ab for 30 min on ice. After washing in phosphate-buffered saline

containing 3% fetal bovine serum and 0.01% NaN_3, the cells were analyzed on a FACSAria (Becton Dickinson, CA, USA) using Diva and FlowJo Software. The following Ab used in flow cytometric analyses were obtained from eBioscience (San Diego, CA, USA): anti-IgM (R6-60.2), anti-B220 (RA3-6B2), anti-CD45.1, anti-CD11b, anti-CD19 and anti-F4/80. Anti-TLR4 PE-conjugated Ab and all isotype-matched Ab were purchased from BD Biosciences, San Diego, CA, USA.

Immunocytochemistry

Immunocytochemistry was performed according to the protocol described earlier [7]. Peritoneal Mϕ from WT and VH12 mice were grown on coverslips and stained with rabbit anti-mouse Ym1 and Fizz 1, followed by Alexa-405 conjugated anti-rabbit Ab. Slides were then visualized on a Zeiss LSM 510 META confocal microscope. Ym1 and Fizz1 Ab were kind gifts from Professor J. E. Allen, School of Biological Sciences, University of Edinburgh, UK.

Statistical analysis

We used a two-tailed unpaired *t*-test for statistical comparisons for most of the experiments. However, for those experiments where Mann–Whitney *U*-test was used to compare group data, and this is indicated in the figure legends. In all cases differences were considered to be significant when $p<0.05$.

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Abbreviations: **μMT-Mϕ:** Mϕ from B-cell-deficient μMT mice · **FcγR:** Fcγ receptors · **qPCR:** quantitative PCR · **TAM:** tumor-associated Mϕ

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