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ORIGINAL ARTICLE

Evaluation of trained immunity by β-1, 3 (D)-glucan on murine monocytes *in vitro* and duration of response *in vivo*

Pablo Garcia-Valtanen[1], Ruth Marian Guzman-Genuino[1], David L Williams[2], John D Hayball[1,3] and Kerrilyn R Diener[1,3]

The β-1, 3 (D)-glucan (β-glucan) present in the cell wall of *Candida albicans* induces epigenetic changes in human monocytes resulting in primed macrophages exhibiting increased cytokine responsiveness to reinfection. This phenomenon is referred to as trained immunity or innate immune memory. However, whether β-glucan can reprogramme murine monocytes *in vitro* or induce lasting effects *in vivo* has yet to be elucidated. Thus, purified murine spleen-derived monocytes were primed with β-glucan *in vitro* and assessed for markers of differentiation and survival. Important macrophage cell markers during monocyte-to-macrophage differentiation were downregulated and survival enhanced due to partial inhibition of apoptosis. Increased survival and not the β-glucan training effect explained the elevated production of tumour necrosis factor-α (TNFα) and interleukin-6 (IL-6) induced by subsequent lipopolysaccharide (LPS) challenge. *In vivo*, 4 days after systemic administration of β-glucan, mice were more responsive to LPS challenge as shown by the increased serum levels of TNFα, IL-6 and IL-10, an effect shown to be short lived as enhanced cytokine production was lost by day 20. Here, we have characterised murine macrophages derived from β-glucan-primed monocytes based on their surface marker expression and for the first time provide evidence that the training effect of β-glucan *in vivo* declines within a 3-week period.
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Trained immunity was coined to describe the ability of the innate arm of the mammalian immune system to activate mechanisms by which immune cells, after a first pathogen insult, increase their ability to protect against reinfection from the same or different pathogen.[1,2] While trained immunity has long been known to operate in invertebrate,[1] vertebrate[3–8] and plant species,[9] it is only in recent years that the molecular mechanisms that lead to trained immunity in mammalian cells have been described. These studies have focused mainly on natural killer cells[6,10,11] and monocytes.[8,12,13] For instance, binding of the surface receptor dectin-1 to the pathogen-associated molecular pattern (PAMP) β-1, 3 (D)-glucan (β-glucan),[14] commonly present on the cell wall of fungi, has been shown to trigger epigenetic reprogramming in human monocytes mainly through changes in histone trimethylation at H3K4.[8,13] Macrophages derived from these β-glucan-primed monocytes (BG-Mφ) exhibit a degree of training or memory and respond with increased release of inflammatory cytokines to subsequent infections with related or unrelated pathogens.[2,8,12,13,15–19]

The bulk of the data on the *in vivo* effects of β-glucan in murine models has been generated mainly in experiments carried out with *Candida albicans* organisms. Mice treated with sublethal strains of *C. albicans* are partially or totally protected against a subsequent infection with lethal strains of *C. albicans*, *C. tropicalis* or, more importantly, the unrelated pathogenic bacterium *Staphylococcus aureus* up to 14 days after *C. albicans* exposure.[19] This protection was mediated by the plastic-adherent population of mouse splenocytes, and resulted in a number of circulating polymorphonuclear leucocytes, including monocytes, remaining elevated in the blood for up to 20 days.[19] This protection has been shown to be accompanied by epigenetic changes in peritoneal macrophages and act independently of adaptive immunity.[8,16]

While sublethal *C. albicans* infections have provided a valuable means to study trained immunity in murine models, the extent of the specific contribution of β-glucan to the activation of trained immunity *in vivo* and the length of its effect still remains poorly understood. Moreover, the *in vivo* effects of β-glucan cannot be inferred from experiments where whole *C. albicans* have been used to induce trained immunity as *C. albicans*-infected animals are inherently exposed to other foreign microbial components. For instance, chitin from the cell wall of *C. albicans* enhances resistance to anti-fungal agents,[20] and is also a potent trained immunity activator.[21] Similarly, other

[1]Experimental Therapeutics Laboratory, Hanson and Sansom Institute for Health Research, School of Pharmacy and Medical Science, University of South Australia, Adelaide, SA, Australia; [2]Department of Surgery, Center of Excellence in Inflammation, Infectious Disease and Immunity, Quillen College of Medicine, East Tennessee State University, Johnson City, TN, USA and [3]Robinson Research Institute and Adelaide Medical School, The University of Adelaide, Adelaide, SA, Australia
Correspondence: Dr KR Diener, Robinson Research Institute and Adelaide Medical School, The University of Adelaide, Frome Road, North Tce, Adelaide, SA 5005, Australia.
E-mail: kerrilyn.diener@adelaide.edu.au
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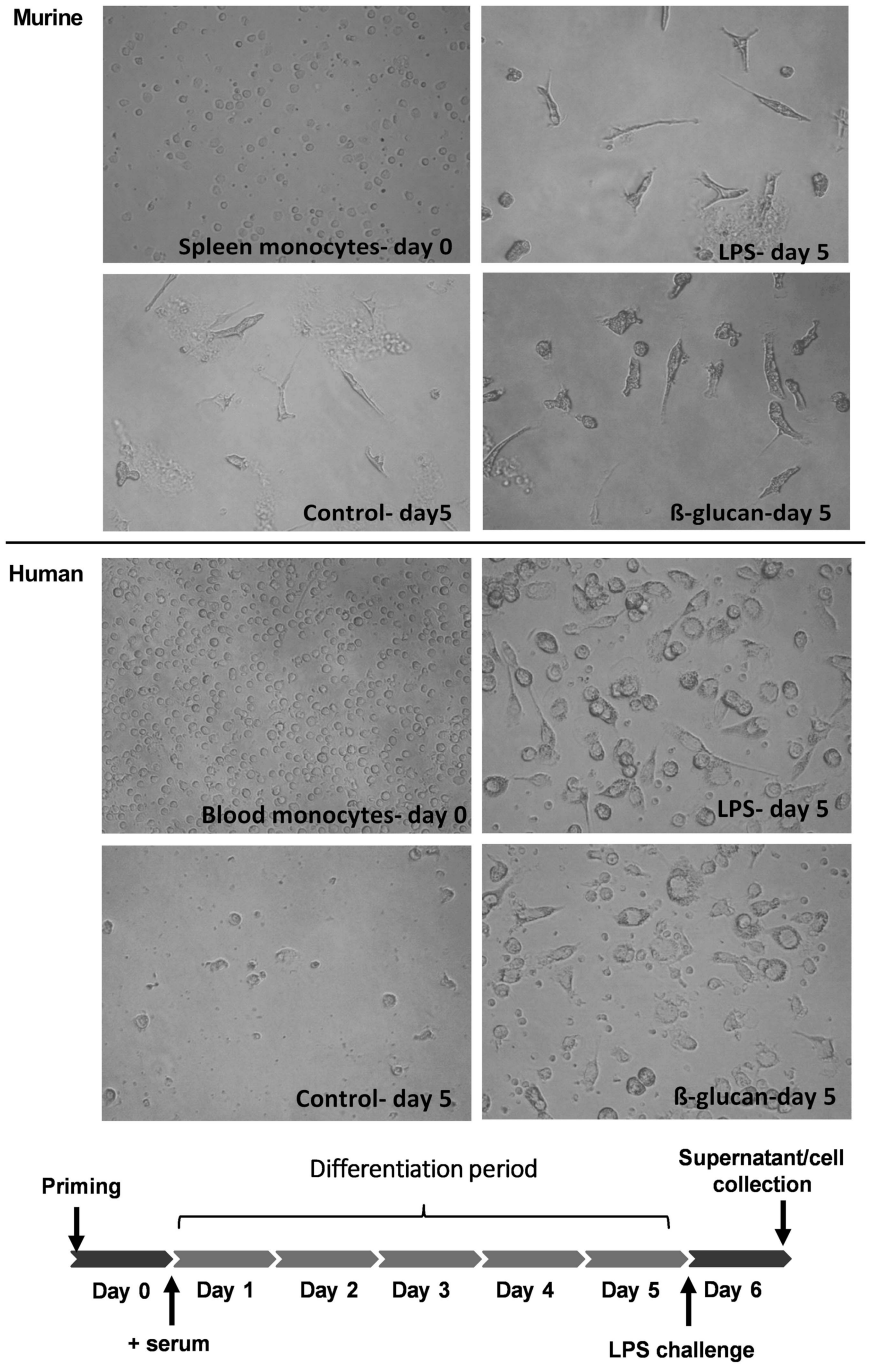

Figure 1 *In vitro* differentiation of murine and human monocytes after the addition of serum to cell culture media. Murine (top panels) and human monocytes (bottom panels) were isolated, treated and cultured following the schedule indicated in the timeline diagram (bottom), with images captured at day 0 (untreated) and 5 days later after priming with LPS, β-glucan or RPMI. All pictures were taken using the same magnification objective (×40), and are representative of six others.

components of the cell wall of *C. albicans* exert immunosuppressive functions during infection in murine models.[22,23] These are likely to mask or enhance β-glucan-induced effects *in vivo* and *in vitro*, including its ability to induce trained immunity. Furthermore, in previous studies where purified β-glucan was used to activate trained immunity *in vivo*, its effects beyond 7 days after its administration were not detailed;[12,15,17] thus, it is difficult to unequivocally ascertain whether the *in vivo* responses to infection in animals pretreated with β-glucan are exclusively the result of lasting innate immune memory (i.e., trained immunity) or a combined response due to the overlap of the innate responses to both β-glucan and the subsequent immune challenge.

In this study, we investigated the ability of *C. albicans*-purified β-glucan to prime murine spleen-derived monocytes *in vitro*. We found that the differentiation of monocytes after a priming period with β-glucan produced large cells with a morphology consistent with macrophages and lower expression levels of the cell surface markers F4/80, CD11b, CD11c and MHC II, relative to control Mφ. Subsequent stimulation of BG-Mφ with lipopolysaccharide (LPS)

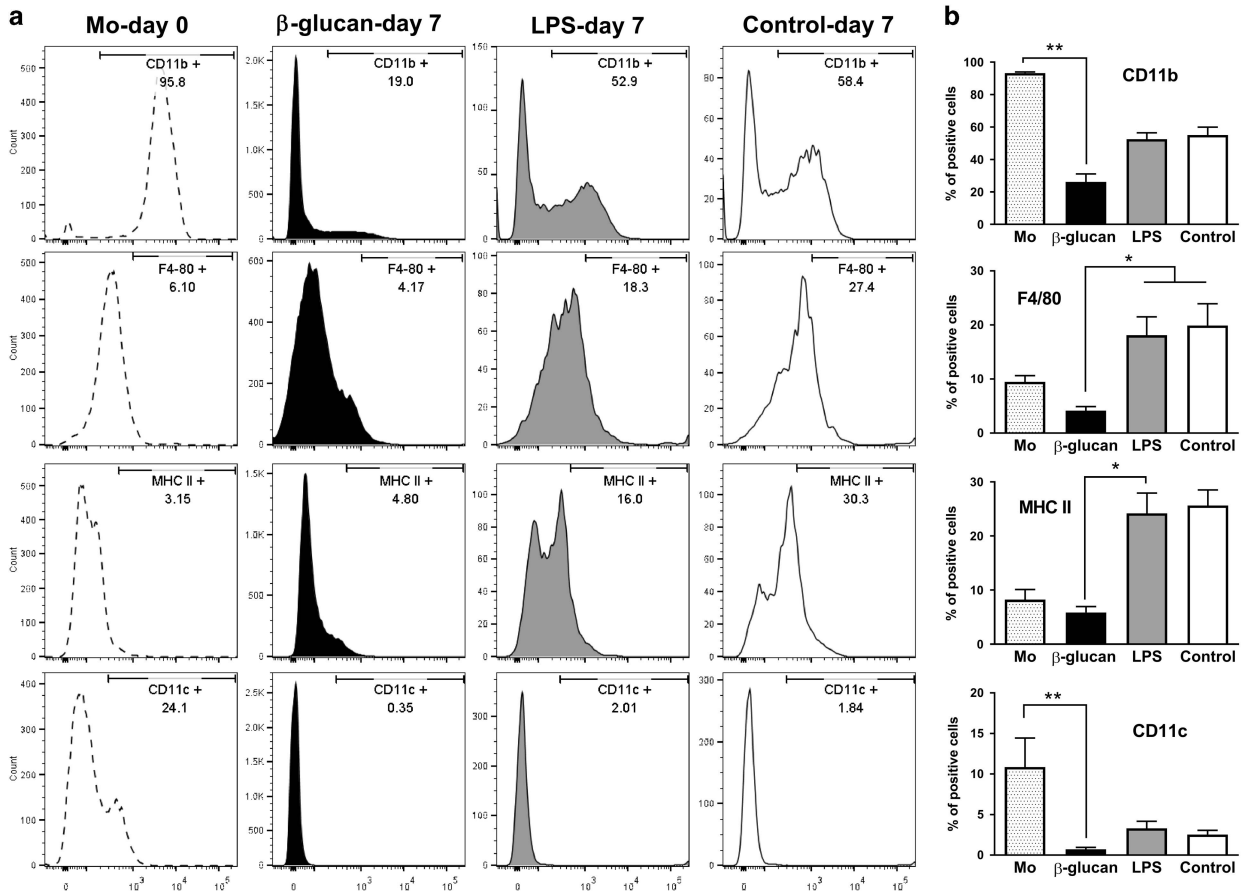

Figure 2 β-Glucan-trained macrophages express relatively low levels of phagocytic cell surface markers. Undifferentiated monocytes (Mo, day 0) or differentiated macrophages (day 7) were collected and stained with antibodies against CD11b, F4/80, MHC II and CD11c and subsequently assessed by flow cytometry. (**a**) Representative histogram plots showing the expression of the different cell surface markers in undifferentiated Mo (day 0) or day 7 differentiated macrophages initially primed with β-glucan (black), LPS (grey) or RPMI (white, control). (**b**) Percentage of positive cells for each of the four markers evaluated in monocytes and differentiated macrophages. Spleen monocytes from five mice were analysed in independent experiments for all makers. Bars and error bars represent average and s.e.m. values, respectively. * and ** *P*-values $\leqslant$0.05 and 0.01, respectively.

resulted in enhanced secretion of tumour necrosis factor-α (TNFα) and interleukin-6 (IL-6); however, this could be largely explained by a greater number of cells surviving in culture over a 6-day period, a finding that was recapitulated in human peripheral blood mononuclear cell (PBMC)-derived monocytes. Moreover, increased survival of murine monocytes coincided with a relative reduction in apoptosis after β-glucan treatment. *In vivo*, β-glucan-trained mice challenged with LPS 4 days later resulted in increased production of TNFα, IL-6 and IL-10 serum cytokines, a response not seen after 20 days, suggesting that the β-glucan priming effect *in vivo* is not sustained beyond 3 weeks in adult mice and therefore suggesting that β-glucan-mediated trained immunity is transitory.

RESULTS

Splenic monocytes primed with β-glucan in culture differentiate into macrophage-like cells

Since β-glucan has only been shown to induce trained immunity in human monocytes *in vitro*, purified murine splenic monocytes or human PBMC-derived monocytes were cultured with β-glucan, LPS or cell culture media alone for 24 h. To ensure that results were comparable to previous published studies, purified β-glucan was obtained from the same source, produced in the same laboratory using the same methodology.[5,13] After an initial priming period, cells were subjected to a differentiation process driven by the addition of normal murine or human serum to the cell cultures using the same protocol as described previously[5] (see timeline in Figure 1). Irrespective of the primary stimulus, all cell treatment groups underwent profound morphological changes by day 5 of the assay. In both cases (mouse and human), round-shaped monocytes (day 0) differentiated into cells with increased size, and often elongated morphology on day 5, with all exhibiting a classical mature macrophage morphology irrespective of their origin. Whereas differences in morphology among treatments was not as prominent in the murine assays, human cells treated with LPS or β-glucan (LPS-Mφ and BG-Mφ) were generally larger than control cultured macrophages (Mφ) (Figure 1).

Splenic monocytes primed with β-glucan express relatively low levels of macrophage markers upon differentiation

Recent studies have found that β-glucan exposure influences the cell surface marker phenotype of human BG-Mφ,[5,13] but, to date, the expression of macrophage-relevant surface markers on murine BG-Mφ has not been studied. Therefore, purified naïve splenic monocytes (day 0) and Mφ after LPS challenge (day 7) were stained with antibodies against CD11b to assess whether the expression of this marker, highly expressed in mouse splenic monocytes,[24] was modulated as a result of the differentiation process. Additionally, the

cells were stained with anti-CD11c and anti-F4/80 antibodies to evaluate whether the differentiated cells adopted a predominantly dendritic cell or macrophage phenotype, respectively. Finally, MHC class II expression was investigated to evaluate the antigen-presenting ability of the differentiated cells. As expected, monocytes on day 0 expressed high levels of CD11b and low expression of F4/80, MHC II or CD11c (Figure 2a).[24] After priming and differentiation, the number of CD11b-expressing cells decreased in LPS-Mφ and Mφ from 93% to 52% and 55%, respectively, with the decrease more pronounced in BG-Mφ cultures (93–26%) (Figure 2b). Differentiation increased the expression of F4/80 by 99% and 10% in LPS-Mφ and Mφ cultures, respectively, while expression in BG-Mφ decreased by 55% when compared with the initial expression levels in naïve monocytes. Similarly, MHC II was upregulated on the surface of LPS-Mφ and Mφ (from 88% on day 0 to 24 and 25%, respectively), but slightly decreased in BG-Mφ (6%). All treatments resulted in the downregulation of CD11c; however, BG-Mφ exhibited the lowest expression (<1% of the cells were CD11c positive). Median intensity fluorescence values for all marker and treatment groups are summarised in Supplementary Table 1.

Priming with β-glucan results in increased cell viability in murine and human monocytes *in vitro*

During cell surface marker analysis, the number of live singlet cells identified by FSC-weight/FSC-height and SSC-weight/SSC-height gating and lack of DAPI (4',6-diamidino-2-phenylindole) staining (see gating strategy; Supplementary Figure 1a) exhibited considerable variability depending on the initial priming stimulus. On day 7 in all murine assays, BG-Mφ were more abundant than LPS-Mφ or control Mφ (Figure 3a), despite initial cell numbers being variable between individual mice (Supplementary Table 2). This suggested that β-glucan may have facilitated the survival of differentiating monocytes during the culture period. To determine whether these observations were also true for human blood monocytes treated with β-glucan, similar analyses were performed. As it was possible to isolate more monocytes per volunteer than per mouse, the number of live cells at day 1 after the priming period, and day 6 after the differentiation period, was evaluated. On day 1, live β-glucan-treated monocytes were more abundant than control monocytes, as were those cultured with LPS (Figure 3b). This early difference in cell numbers was sustained during the differentiation phase, as on day 6, after monocytes had differentiated into macrophages, BG-Mφ numbers were still significantly higher than control Mφ. Moreover, monocyte cultures from volunteers 1, 3 and 6 also proliferated in the first 24 h in response to β-glucan (Supplementary Table 2). Taken together, these results suggest that stimulation with β-glucan has a prosurvival effect on monocytes from both human and mouse.

To further investigate how β-glucan may facilitate survival of murine monocytes *in vitro*, the level of apoptosis was evaluated at different time points during the differentiation period. For this, purified murine monocytes were stained on days 0, 1 and 4 of culture with Annexin V (AnnV) and propidium iodide (PI). On day 1, after the priming period and before initiation of the differentiation period, the percentage of $AnnV^+PI^+$ (i.e., late apoptotic and/or necrotic) in the β-glucan-treated cultures was consistently lower than that of the LPS and control groups (quadrants Q2 in Figures 3c and d), and not different to that observed at day 0 before culture. In contrast, more than 50% of LPS-treated and control cells were $AnnV^+PI^+$ after the priming period (Figure 3d). Dot plots suggested that cultured monocytes first became apoptotic, and then proceeded to a late apoptotic/secondary necrotic phenotype within this time. This was confirmed by staining cells for the expression of caspase-3/7, effector caspases present in late stages of cell apoptosis processes,[25] which indicated to different extents, positive caspase-3/7 staining in all treatment groups (Supplementary Figure 1b). Interestingly, the proportion of monocytes at different stages of apoptosis during this 24 h period was not the same for each treatment group. Cultures treated with β-glucan exhibited an increased proportion of live ($AnnV^-PI^-$) cells at the start of the differentiation period (Figure 3e), and an increased percentage of $AnnV^+PI^-$ cells, generally indicative of cells in early apoptosis, at the same time point (Figure 3f). Taken together, this suggested that β-glucan slowed the rate at which monocytes entered the apoptosis pathway, thus leaving these cultures at the start of the differentiation period with a larger number of viable $AnnV^-PI^-$ cells in comparison with LPS or control treatment. It is also possible that a proportion of the $AnnV^+PI^-$ cells may not actually be apoptotic but rather bind AnnV due to translocation of phosphatidylserine to the outer membrane as part of processes related to membrane receptor selection, as has been previously described for B cells,[26] and therefore contribute to the elevated numbers of $DAPI^-$, live cells in this group (Figure 3a). By day 4 all treatments showed low numbers of $AnnV^+PI^+$, indicating that the cells that initially survived went on to differentiate into macrophages (Figure 3d).

Increased survival of β-glucan-treated monocytes dictates the amount of TNFα or IL-6 released in response to LPS

BG-Mφ derived from human monocytes has been shown to induce epigenetic changes resulting in the secretion of increased amounts of TNFα and IL-6 after stimulation with LPS.[5,8,12,13] By using the same protocols and source of β-glucan, we have recapitulated this increased cytokine production here in human monocytes (Figure 4a). In a similar manner, murine BG-Mφ cultures challenged with LPS resulted in a 2.0- and 3.6-fold increase in TNFα and IL-6 compared with LPS-Mφ and Mφ (Figures 4c and e, respectively). However, when total cytokine data was normalised by the number of live cells detected per well in each treatment, the previous difference in TNFα and IL-6 cytokine expression was negated (Figures 4d and f). Surprisingly, normalising human TNFα expression to the number of viable human monocytes per treatment also resulted in the same effect (Figure 4b), indicating that BG-Mφ release of inflammatory cytokines is, to a certain extent, affected by β-glucan-induced survival in both species, mouse and human.

Enhanced cytokine responses in β-glucan-trained mice declines within 3 weeks

Since our *in vitro* results did not unequivocally answer the question of whether murine BG-Mφ exhibit higher sensitivity to subsequent LPS challenge, and since all reports to date only show trained immunity effects in mice *in vivo* within a four to seven day window, the effects of β-glucan *in vivo* was more comprehensively assessed. For this, C57Bl/6 J mice were systemically administered two 1 mg doses of β-glucan as described previously.[12,17] Control mice were injected with isovolumetric endotoxin-free phosphate-buffered saline (PBS). Then, at 4 days (short-term) or 20 days (long-term) after β-glucan administration, mice were challenged *in vivo* with LPS and the serum levels of TNFα, IL-6 and IL-10 measured 2.5 h after challenge (see timeline; Figure 5a). In the short-term experiment, serum cytokine levels in trained mice (β-glucan-treated) were significantly higher for TNFα (5.7-fold), IL-6 (2.4) and IL-10 (3.1) in comparison with PBS-treated mice (Figure 5b). Control mice in the long-term experiment exhibited similar cytokine production levels after LPS stimulation at 20 days to that of control mice in the short-term

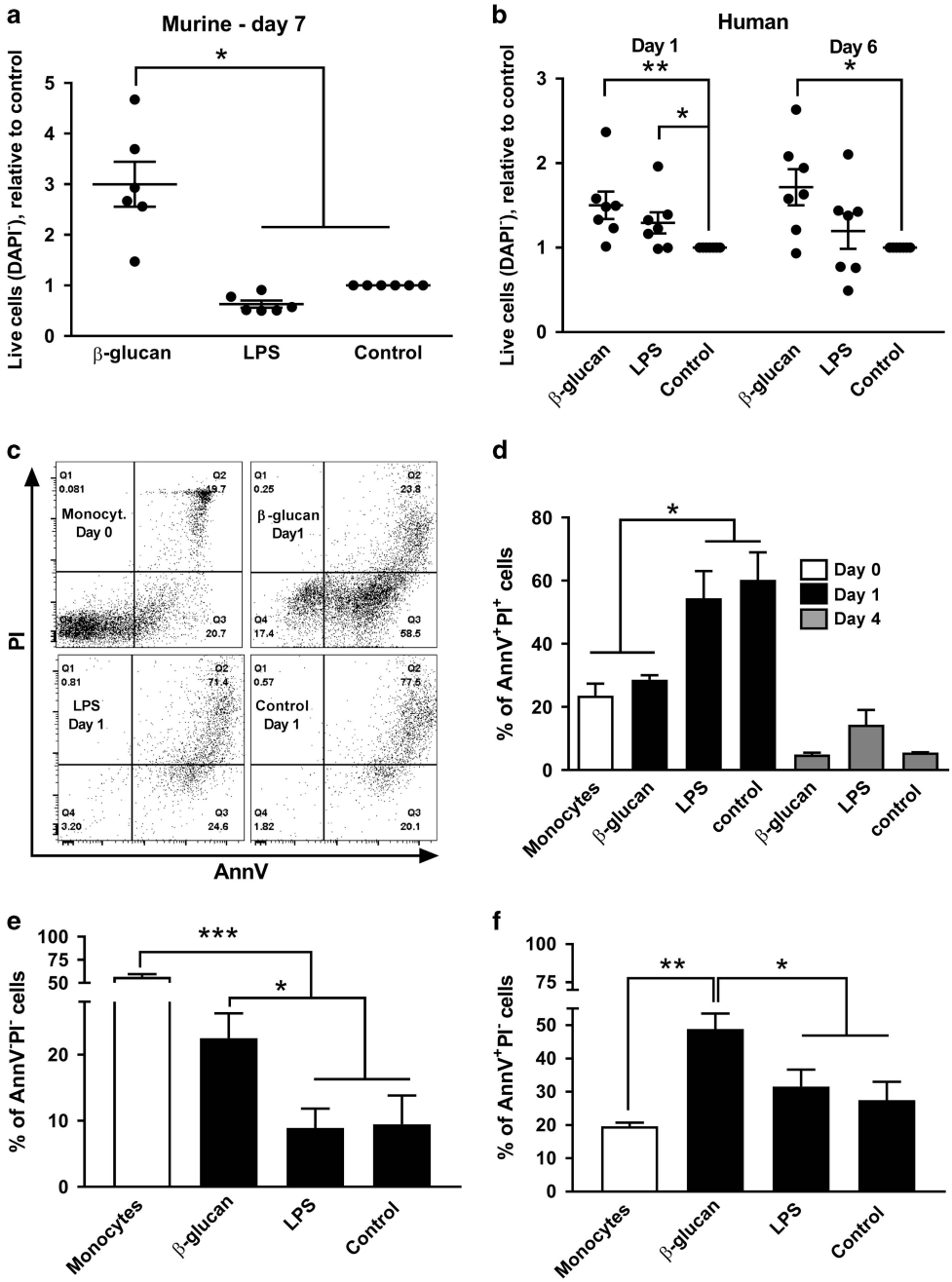

Figure 3 β-Glucan facilitates the survival of differentiating monocytes *in vitro*. (**a**) Fold change in live cells (normalised against control cells) after priming, differentiation and LPS challenge (day 7) in the murine *in vitro* assays ($n=6$), and (**b**) healthy volunteers ($n=8$) on days 1 and 6 under the same culture conditions and treatments. (**c**) Dot plots representing the apoptotic status of murine monocytes on day 0 (untreated) and treated monocytes on day 1. Cells were stained with FITC-conjugated AnnV and PI. Late apoptotic and/or necrotic cells are gated in the top right quadrant of each of the dot plots ($AnnV^+PI^+$). (**d**) Combined $AnnV^+PI^+$ data, (**e**) $AnnV^-PI^-$ data and (**f**) $AnnV^+PI^-$ data obtained from three independent experiments at different time points during the differentiation assays. Bars and error bars represent averages and s.e.m., respectively. * and ** *P*-values $\leqslant 0.05$ and 0.01, respectively.

experiment (Figure 5c). However, in contrast to trained mice in the short-term experiment, trained mice did not exhibit significantly increased serum cytokine levels, indicating a loss of heightened inflammatory responses to endotoxemia within a 3-week period.

DISCUSSION

The present study describes the *in vitro* effects of β-glucan priming on macrophages (BG-Mφ) derived from monocytes isolated from the spleen of C57Bl/6 J mice as well as its effect *in vivo* after administration by intraperitoneal injection. We found that β-glucan partially protected cultured monocytes from apoptosis and this, in turn, led to both increased cell numbers and secreted inflammatory cytokine levels after challenge with LPS. *In vivo*, we demonstrated that the heightened response of β-glucan-trained mice to endotoxemia only ensues when β-glucan is administered a few days before LPS challenge, providing evidence that the trained phenotype might be transient.

Monocytes act as a myeloid precursor reservoir of tissue-resident macrophages and antigen-presenting dendritic cells.[27] *In vitro*, in the

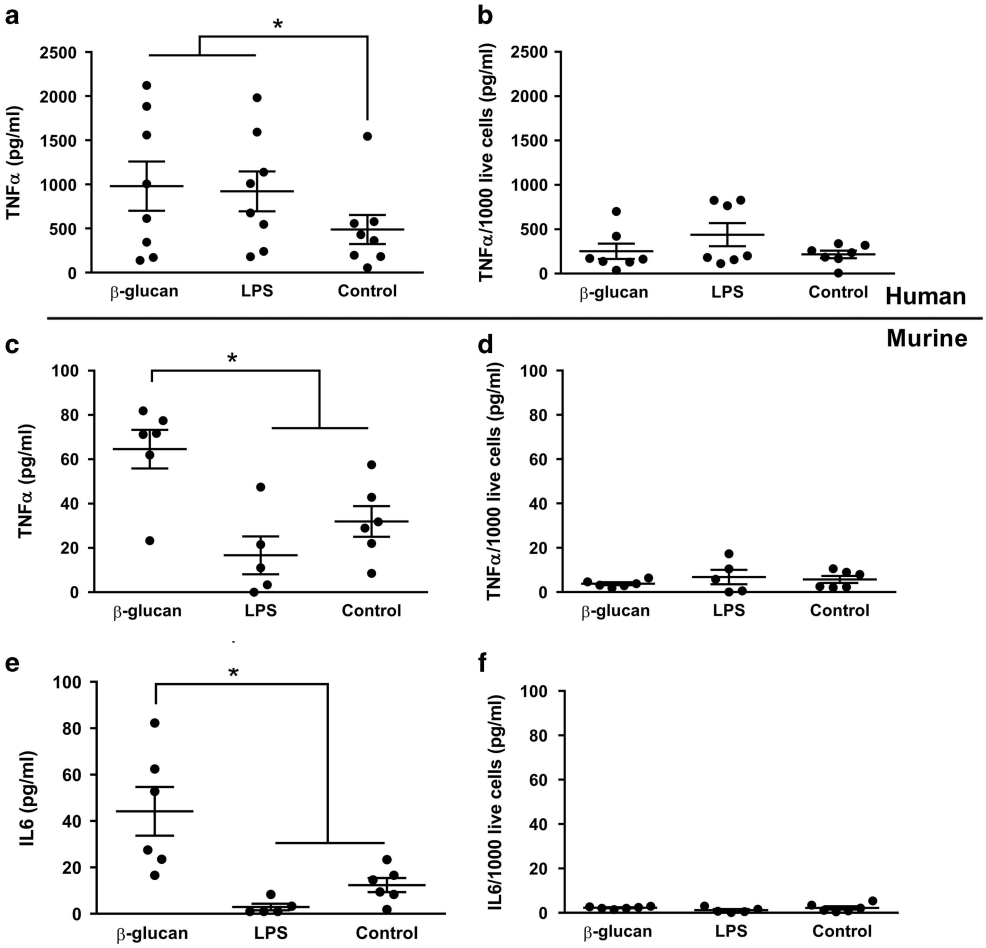

Figure 4 Increased survival of β-glucan-trained macrophages determines increased TNFα and IL-6 levels *in vitro*. (**a**) Total and (**b**) live cell number normalised amount of TNFα released into cell supernatants by human macrophages after challenge with 10 ng ml^{-1} of LPS. (**c**) Total and (**d**) normalised murine TNFα released into supernatants. (**e**) Total and (**f**) normalised IL-6 was measured in the same supernatants. **P*-values ⩽0.05.

presence of serum, human blood monocytes differentiate into macrophages after a few days in culture. Moreover, if primed with β-glucan the resulting macrophages (BG-Mφ) exhibit increased responsiveness to bacterial PAMPs such as LPS.[5,8,12,13] Using β-glucan from the same source and laboratory and identical experimental procedures as previous human studies, murine monocytes, in the presence of mouse serum, differentiated from small round monocytes into large elongated cells consistent with macrophage or dendritic cell morphology. This differentiation also occurred irrespective of the priming stimulus- (LPS or β-glucan) producing cells with similar morphology.

At the molecular level, priming with β-glucan induces epigenetic reprogramming that leads to, among other changes, a phenotype of human BG-Mφ that exhibits a distinct surface marker profile.[5] In murine BG-Mφ originated from splenic monocytes, the expression of F4/80 in BG-Mφ was significantly lower than that of LPS-Mφ and control Mφ, suggesting that the initial β-glucan stimulus blocks or inhibits the expression of F4/80. The marker F4/80 is highly expressed in macrophages from different murine tissues.[28] By contrast, murine peritoneal macrophages infected with Bacillus Calmette-Guerin (BCG) express significantly lower levels of F4/80 than their non-infected counterparts.[29] Similarly to β-glucan, BCG was found to induce epigenetic changes in monocytes that lead to trained immunity.[30] Our results indicate that downregulation of F4/80 in BG-Mφ as well as in BCG-infected Mφ could be a shared trait among certain trained macrophage phenotypes. Additionally, β-glucan downregulated the expression of MHC II, whereas BCG vaccination upregulates this marker in macrophages.[29] BCG, a whole-cell pathogen-based vaccine, is known to induce antigen presentation[31,32] and lead to the development of long-lasting protective immunity against *Mycobacterium tuberculosis*.[33] Purified β-glucan is a single PAMP among the many expressed in the cell wall of fungal species and, therefore, is not likely to favour an antigen-presenting cell function, at least not to the same extent as BCG. On the other hand, downregulation of CD11c in BG-Mφ suggests that β-glucan steers the monocyte differentiation process away from dendritic cell-like phenotypes and functions, which include antigen-presenting functions and could partially explain why MHC II is also downregulated.

Similarly to the previous makers, CD11b (a cell surface integrin), highly expressed in naïve monocytes, was markedly downregulated in BG-Mφ. This integrin is directly involved in facilitating the function of pattern recognition receptors such as TLR3[34] and TLR4,[35] which sense the PAMPs polyinosinic:polycytidylic acid (poly I:C) and LPS, respectively. Thus, downregulation of CD11b could influence the ability to respond to such PAMPs, and perhaps other stimuli, and modulate the immune function of BG-Mφ. In contrast with our results in mice, human BG-Mφ have been shown to upregulate CD11b, compared with control Mφ.[5] Differences in the expression of

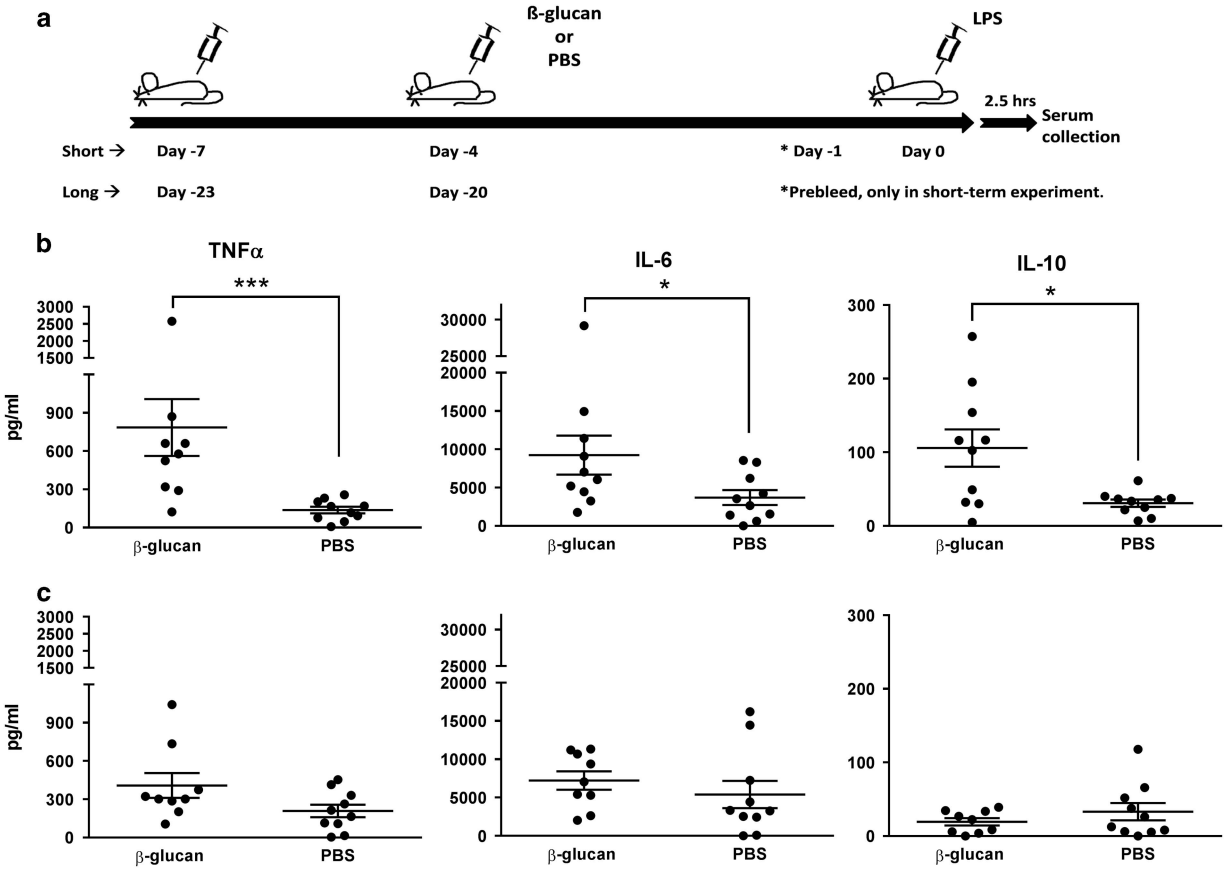

Figure 5 The enhanced *in vivo* response in β-glucan-trained mice declines in the first 3 weeks. (**a**) Timeline of the β-glucan dosage schedule in the murine *in vivo* experiments. (**b**) Serum levels of TNFα, IL-6 and IL-10 in β-glucan- or PBS-treated mice in the short-term experiment and (**c**) in the long-term experiment. At least nine mice per group and two independent experiments were performed in all cases. Horizontal and error bars represent average and s.e.m. values. * and ****P*-values ⩽0.05 and 0.001, respectively.

this receptor in BG-Mφ among different species might determine the magnitude of subsequent responses to PAMPs, and therefore their ability to resolve secondary infections in immune trained organisms.

The flow cytometric analysis of each individual mouse experiment showed a relatively increased survival in BG-Mφ during the monocyte-to-macrophage differentiation period, compared with LPS-Mφ and Mφ, and this was also evident with human blood monocytes. Consistent with enhanced cell viability, the percentage of dead apoptotic cells after the priming period was significantly lower in the β-glucan-treated monocyte cultures. In culture, monocytes and other short-lived immune cells rapidly undergo apoptosis in a process mediated by long non-coding RNA in the absence of soluble factors such as granulocyte–macrophage colony-stimulating factor, IL-3 or IL-5.[36] This is consistent with high apoptosis-mediated cell death seen in control (not primed) as well as LPS-treated monocytes. On day 4, low levels of dead apoptotic cells were observed in all groups, presumably because the necessary soluble factors had been released and accumulated in the supernatants during this period of time. In line with these results, several *in vivo* studies have shown that treatment with β-glucan (from *C. albicans* and other sources) or a sublethal *C. albicans* infection can increase the number of tissue-resident and blood polymorphonuclear leucocytes in different murine species[15,17,19] and in cancer patients.[37] *In vivo*, however, we believe that apoptosis due to lack of soluble factors is less likely to occur since a larger number of cell types are present in most organs and can, potentially, be the source of such soluble factors. Thus, more research is needed to determine whether the *in vitro* assay to investigate monocyte trained immunity-like responses described here and in previous studies[5,8,12,13,21] provides an accurate reflection of these responses *in vivo*.

One of the key characteristics of the human trained BG-Mφ phenotype is its increased ability to release proinflammatory cytokines in response to subsequent challenges with PAMPs and whole pathogens,[5,8,12,13] results that were recapitulated in this study using peripheral blood monocytes from healthy volunteers. This increase in inflammatory cytokines (TNFα and IL-6) was also observed in supernatants from murine BG-Mφ in response to LPS. However, having observed that β-glucan priming resulted in increased cell survival, we reanalysed the cytokine data taking this into account. Interestingly, when total cytokine release data was normalised by the number of live cells per well in each treatment, no differences between BG-Mφ and the control groups (LPS- Mφ and Mφ) were observed. In both species the enhanced inflammatory response of BG-Mφ appeared to be a reflection of the increased Mφ numbers available to respond to LPS. Based on our results we believe that to different extents, other studies that did not account for the increased number of cells in culture due to treatment with β-glucan[5,8,12,13] might have over-estimated the capacity of these cells to release inflammatory cytokines *in vitro*.

A week after systemic β-glucan administration to mice, TNFα and IL-6 were significantly increased in trained mice after challenge with LPS. Using the same time frame and dose of β-glucan, others have

subsequently demonstrated that such trained mice are more resistant to infection with the pathogenic bacterium *S aureus*.[12,15] However, in a longer experiment, we demonstrated that mice that had received the same dose of β-glucan 20 days before challenge with LPS did not show increased serum cytokine responses. Therefore, on the one hand, our short-term experiments (in combination with previous studies[12,15]) strongly suggest that after a few days of systemic administration of β-glucan, the murine immune system is more efficient in responding to LPS. Similarly, TNFα and IL-6 serum levels are significantly elevated in mice trained with heat-inactivated *C. albicans* 7 days before challenge with LPS.[8] On the other hand, the long-term experiment showed that serum TNFα and IL-6 in trained mice fell to PBS-injected control mice levels, suggesting that the murine-trained phenotype might be transient or rapidly changing in its ability to elicit inflammatory responses. Similarly, after a rapid increase in blood polymorphonuclear leucocytes and monocytes and spleen cellularity caused by systemic administration of a sublethal strain of *C. albicans*, these cell numbers declined by day 20 after stimulation with *C. albicans*.[19] Thus, the decline in cytokine production observed in trained mice at 20 days post-treatment and previous studies that show that a decline in monocytes occurs at the same time point after identical treatment[19] suggest that increases in cytokine production in trained mice could be linked to, at least to an extent, to increases in cell numbers, presumably monocyte/macrophage cells. We also provided evidence that *in vitro* this is likely to be the mechanism that results in higher readouts of inflammatory cytokines in BG-Mφ. Additionally, serum IL-10 was also increased in trained mice in the short-term experiment, but when measured in the *in vitro* training experiments, this cytokine was not present at detectable levels (data not shown). This suggests that increased serum IL-10 levels *in vivo* are produced by cells other than BG-Mφ to control exacerbated inflammatory responses in trained mice. Conversely, in the long-term experiments, where the inflammatory response (TNFα and IL-6) to endotoxemia was similar in trained and control mice, IL-10 serum levels were also similar, which further supports the hypothesis that the β-glucan effect does not persist in the trained mice.

Taken together, these results open an interesting question about the duration and nature of trained immunity mediated by β-glucan in murine models. Often, trained immunity is described as the memory of the innate immune system.[2,18] In fact, trained immunity phenotypes that share traits with the β-glucan-trained phenotype, such as that generated by the BCG vaccine, seem to have long-lasting effects in humans.[3,7] Based on our *in vivo* cytokine results and previous reports that studied changes in immune cell numbers *in vivo* after stimulation with β-glucan,[19] it could be argued that the training effect of β-glucan peaks at around 2 or 3 weeks after its administration. β-glucan-induced trained immunity relies on epigenetic changes[8,12,13] and the duration of the innate immune memory might be limited by the lifespan and turnover of the trained cell population (i.e., BG-Mφ). It is expected that future studies looking at the long-term effects of β-glucan on the innate immune system, such as this one, will provide the basis to form a more accurate picture of how trained immunity is induced and affects the immune response to secondary infections in murine models. *In vivo* challenge experimental models to test the ability of the murine β-glucan-trained phenotype to survive infection will be key to provide answers to these questions.

METHODS

Animals

Male C57Bl/6 J (6–8 weeks) mice were bred in-house at the Reid Animal Facility, University of South Australia, and housed in individually ventilated cages under standard specific-pathogen free conditions with food and water provided *ad libitum*. All animal experiments were carried out with dual approval from the University of South Australia and The University of Adelaide Animal Ethics Committees, and conducted in accordance with National and Institutional ethical and regulatory guidelines.

Antibodies and cell staining reagents

The antibodies APC-anti-mouse/human CD11b (BioLegend, San Diego, CA, USA; cat. no. 101212), Alexa Fluor 488-anti-mouse MHC II (I-Ab) (BioLegend; cat. no. 116410), biotin-anti-mouse CD11c (BioLegend; cat. no. 117303), streptavidin-APC-Cy7 (BD, San Jose, CA, USA), anti-mouse CD16/32, that is, Fc block, (eBioscience, San Diego, CA, USA; cat. no. 14-0161-86), PE-anti-mouse F4/80 (eBioscience; cat. no. 12-4801-82) and DAPI (4′,6-diamidino-2-phenylindole; Sigma-Aldrich, St Louis, MO, USA) were used in cell staining assays.

Monocyte isolation and stimulation experiments

A commercial kit of antibody-conjugated magnetic beads was used to negatively select and isolate naïve monocytes from the spleens of C57Bl/6 mice. Isolation was carried out according to the instructions provided by the manufacturer. Cells consistent with both monocyte morphology (as described elsewhere) and high CD11b and low CD11c, Ly71 and MHC II (I-Ab) surface expression were obtained.[24] Then, monocytes were resuspended in RPMI cell culture medium (Sigma-Aldrich) supplemented with 2 mM L-glutamine, 10 mM HEPES, 12 μg ml^{-1} penicillin and 16 μg ml^{-1} gentamycin (the Media Production Unit at the Royal Adelaide Hospital) and allowed to adhere to the bottom of the wells in 96-well plates for 1 h at 37 °C. After this period, the media were removed and replaced with 100 μl of the same supplemented culture medium (control cells), medium containing 5 μg ml^{-1} of β-glucan (from *C. albicans*[38]) or 100 ng ml^{-1} of LPS (*E. coli* strain 0111:B4; Sigma-Aldrich). After 24 h, the treatments were washed away with prewarmed (37 °C) RPMI media and replaced with 150 μl RPMI in the same supplemented culture medium containing 10% mouse pooled sera. Then, cells were allowed to differentiate in the wells for 5 days. After the differentiation period, cell culture media were removed and cells were challenged by adding supplemented culture medium containing 10 ng ml^{-1} of LPS. At 24 h after the challenge with LPS, supernatants were collected for cytokine assessment by Bead Array Assays (mouse) or Enzyme-linked Immunosorbent Assays (human) and cells were harvested for cell surface marker staining and analysis.

For the human *in vitro* assays, PBMCs were isolated from blood from healthy volunteers using Lymphoprep (StemCell Technologies, Vancouver, BC, Canada) standard procedures. Then, PMBCs were plated in 96-well plates at a density of 5×10^5 cells per well in a total volume of 100 μl in supplemented RPMI media and incubated at 37 °C. After 1 h, the cells were washed three times with prewarmed PBS (37 °C) to eliminate non-adherent cells. The remaining cells were used in the stimulation protocols as described above. Human PBMC-derived monocytes were also consistent with monocyte morphology and high CD11b expression (data not shown). Images of the monocytes were taken using Olympus IX51 fluorescence microscope (Olympus Corporation, Tokyo, Japan) and processed using the software cellSens Entry v.1.13 (Olympus Corporation).

Cell surface marker staining and analysis by flow cytometry

On day 7 in the *in vitro* murine assays and days 1 and 6 in the human assays, cells were collected for cell surface marker staining and posterior analysis by flow cytometry. Briefly, cells were incubated with Tryple Select (100 μl per well), an enzymatic disassociation reagent, for 10 min at room temperature. Under the conditions used here, Tryple Select did not have any effect in the amount of positive cells or intensity of marker-associated fluorescence for any of the markers analysed in this study (Supplementary Figure 2). Immediately after this, cells were resuspended in 2 ml of PBS containing 2% FBS (FACS staining buffer), spun down at 300 *g* for 5 min at 4 °C and resuspended again in 100 μl FACS staining buffer containing 2.5 μg ml^{-1} of anti-mouse CD16/32 (Fc block). After a 15 min incubation at 4 °C, 100 μl of FACS staining buffer containing 1 μg ml^{-1} of anti-mouse CD11b, 10 μg ml^{-1} of anti-mouse CD11c, 1 μg ml^{-1} of anti-mouse Ly71, 1.25 μg ml^{-1} anti-mouse MHC II (I-Ab) and 1 μg ml^{-1} DAPI was added to

each tube and the mixes were incubated for 20 additional minutes at 4 °C. Then, 2 ml of FACS staining buffer were added to all samples and each tube was spun down at 300 *g* for 5 min at 4 °C. Finally, cells were resuspended in a 10% neutral-buffered formalin solution and kept in the dark at 4 °C until analysed. The same protocol was used for human monocytes. These cells were stained anti-human 1 μg ml^{-1} CD11b and 1 μg ml^{-1} DAPI.

Flow cytometric data acquisition was carried out in BD FACSAria Fusion (BD). Flow cytometric data was analysed using the software FlowJo v.10.0.7 (FlowJo, LLC, Ashland, OR, USA).

Mouse cytokine detection by bead array-based detection methods

A Bead Array Kit (custom LEGENDplex panel; BioLegend) was used to detect TNFα, IL-6 and IL-10 based on sandwich ELISA principles. Cytokine concentration was determined using standard curves obtained using recombinant cytokine standards provided in the kit. Bead signal readouts were obtained using the flow cytometer BD FACSAria Fusion (BD).

Human TNFα detection by ELISA

Human TNFα was evaluated using recombinant TNFα (ELISA standard) (BioLegend), LEAF Purified anti-human TNFα (BioLegend), biotin-anti-human TNFα (BioLegend), streptavidin-HRP (horse radish peroxidase; Rockland, Limerick, PA, USA) and SIGMAFAST OPD (*o*-phenylenediamine dihydrochloride) tablets (Sigma-Aldrich). Briefly, high-binding 96-well plates were coated with 50 μl of the capture antibody and kept at 4 °C overnight. After washing, the wells were blocked with 200 μl of 2% bovine serum albumin-PBS for an hour. Samples and standards were incubated in the wells for 3 h and then detected using 50 μl of the biotin-conjugated antibodies. Streptavidin-HRP antibodies were added after washing. The presence of cytokines was visualised using OPD tablets as a colorimetric substrate for HRP. The plates were then read at 490 nm using the MultiSkan FC (Thermo Fisher Scientific, Waltham, MA, USA) plate reader.

Normalisation of *in vitro* TNFα and IL-6 results

In the human experiments, PBMCs from each volunteer were seeded as described above in four different plates corresponding to day 0, 1, 6 and 7. At each time point, cells were harvested and stained with DAPI. Counts for DAPI-negative cells (live cells) were used to obtain live cell numbers at the different time points (Supplementary Table 2). Day 6 live cell numbers were used to normalise the secreted TNFα values in human assays. Similarly, day 7 data were used to normalise TNFα values obtained in the murine assays. DAPI fluorescence and cell counts were evaluated by flow cytometry. For normalisation, the following formula was used in both murine and human assays:

$$\text{Normalised TNF}\alpha\left(\text{pg ml}^{-1}\right) = \text{TNF}\alpha\left(\text{pg ml}^{-1}\right)/[\text{live cells perwell}/1000]$$

Evaluation of apoptosis in murine monocytes

Apoptosis and cell death was assessed using the The ApoDETECT Kit (Life Technologies, Carlsbad, CA, USA) and analysed by flow cytometry. Briefly, cells were stimulated following the same procedure as in the stimulation experiments. On day 0 (before stimulation), day 1 (24 h after stimulation) and day 4 (3 days into the differentiation period) cells were harvested and incubated with 3 μl AnnV-FITC in binding buffer for 10 min, and then washed and resuspended in binding buffer before the addition of 2 μl of PI (20 μg ml^{-1}). Cells were recorded as early apoptotic (AnnV^{+}PI^{-} cells), late apoptotic and/or necrotic (AnnV^{+}PI^{+} cells) and necrotic (AnnV^{-}PI^{+} cells). Cells that are negative for AnnV-FITC and PI were recorded as live and healthy. Flow cytometric data was analysed using FlowJo v.10.0.7 (FlowJo).

The CellEvent Caspase-3/7 Green Probe Reagent (Life Technologies) was used to stain murine monocytes on day 1 of the assays following the manufacturer's instructions.

In vivo β-glucan stimulation experiments and challenge with LPS

Following the schedules described in Figure 5a, mice were injected intraperitoneally with two doses of 400 μl of a suspension of β-glucan in PBS or 400 μl of PBS (control mice). The concentration of β-glucan was adjusted so that mice received 1 mg of β-glucan in each injection. In the short-term experiment, blood was obtained from the facial vein of the mice on day 1 to assess for pre-existing levels of TNFα, IL-6 and IL-10 in the serum. On day 0, all mice were challenged intraperitoneally with 400 μl of a solution containing 250 ng of LPS in PBS. At 2.5 h after challenge, blood from each mouse was obtained by cardiac bleeding and collected in MiniCollect Z serum Sep tubes (Greiner Bio-One, Kremsmünster, Austria) for serum separation by centrifugation. Serum samples were stored at − 20 °C until used.

Statistical analysis

Graphs and statistical analysis were carried out using the GraphPad Prism6 (GraphPad Software, La Jolla, CA, USA). Murine *in vitro* apoptosis data was compared using analysis of variance (Fisher's *least significant difference* test) for multiple comparisons. Three independent experiments were performed with cells from a total of four mice. The Wilcoxon's rank test (paired data, one-tailed) was used to analyse the *in vitro* cytokine and cell number data obtained in both murine and human assays. In the *in vitro* assays, five to six mice were assayed in four independent experiments and blood from eight donors were assayed in three independent experiments.

In the *in vivo* assays, nine to ten mice were used in all groups in both the short- and long-term experiments. The Friedman test (paired data, one-tailed) was used to analyse the murine *in vitro* cell marker data. In these assays, five mice were assayed in three independent experiments for each of the cell marker. The degree of statistical significance and sample size is indicated in each figure.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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