β-glucan-induced innate immune memory distinctively affects macrophage activation in response to differential environmental cues

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Abstract

Advances in the field of immunological memory demonstrate that innate immune cells can recall a previous encounter – the innate immune memory. *In vitro*, exposure of human primary monocytes to the fungal β-glucan enhances their pro-inflammatory responsiveness towards several pathogens. During infection, circulating monocytes infiltrate tissues where, following conditioning by local environment, they differentiate and polarise into different types of macrophages. Hence *in vivo* interaction of β-glucan with innate cells would occur in a complex environment. Understanding the potential of β-glucan to induce innate immune memory in complex physiological environments is crucial for future translational research.
Recapitulating different physiological conditions *in vitro* we found that β-glucan imprinting does not always enhance responsiveness and function of macrophages but can also reduce it. In this study, we show that upon both GM-CSF- and M-CSF-mediated polarisation, imprinting by β-glucan leads to less differentiated macrophages with a convergent functional phenotype.
Altogether, these observations provide insightful and crucial knowledge that will help apprehending the *in vivo* high potential of β-glucan-induced innate memory in different pathological contexts.

Introduction

For a long time, it was assumed that immunological memory was an exclusive hallmark of adaptive immune response. However, this binary classification of mammalian immune memory is now outdated. Advances in the field of immunological memory demonstrate that innate immune cells can recall a previous encounter with fungal β-1,3-glucan to have a modified immunological response – the innate immune memory. Over the past few years, we and others have established that β-glucan imprinting of human primary monocytes cultured in human serum enhances their pro-inflammatory responsiveness towards several other pathogens that are not necessarily fungal-related. While these studies on monocytes have been crucial to demonstrate innate immune memory and identify underlying mechanisms, bloodstream classical monocytes are not themselves effector cells during infection, but rather mostly circulating intermediates. Indeed, circulating monocytes infiltrate peripheral tissues, where they are recruited and contribute to effective control and clearance of viral, bacterial, fungal, or protozoal infections. In tissues, following conditioning by local growth factors, pro-inflammatory cytokines, and microbial products, they differentiate into macrophages. Depending on the microenvironmental stimuli, macrophages undergo activation or "polarisation" along a wide spectrum of phenotypes (1). Hence the *in vivo* interaction of β-glucan with the innate cells would occur in a complex environment. Recently, by recapitulating different physiological environments *in vitro* we uncovered a novel property of innate immune memory, in which β-glucan imprints on differentiating macrophages an inhibitory effect on NLRP3 inflammasome activation and subsequent mature IL-1β secretion that is independent of previously described transcriptional regulation mechanisms (2). This underscores that we do not fully comprehend the effects of β-glucan imprinting on polarising and differentiating macrophages. Understanding the potential of β-glucan to induce innate immune memory in a complex physiological environment is crucial for future translational research that would harness the beneficial effects of innate immune memory.

We therefore resumed our recently published protocol (summarised in Figure 1 and Supplementary Figure 1) and comprehensively investigated the effects of β-glucan imprinting on polarising macrophages. We focused on the two best studied and opposing terminally differentiated macrophage subsets: granulocyte-macrophage colony-stimulating factor (GM-CSF)- and macrophage colony-stimulating factor (M-CSF)-induced monocyte-macrophage lineage differentiation both *in vivo* and *in vitro* (3).

GM-CSF-differentiated human macrophages are polarised to classically activated macrophages, also known as M1-like macrophages, which harbour immune-stimulatory properties against infections with high expression of pro-inflammatory cytokines, high ROS production, and cytotoxic function against tumour cells (4). M-CSF-differentiated human macrophages are polarised to alternatively activated, or M2-like, macrophages, described as an anti-inflammatory cell type, with low cytotoxic function therefore potent pro-tumoral activity but with high tissue-remodelling and repair activity (5).

In this study, we show that not only does β-glucan imprinting enhance responsiveness and function of macrophages but under certain conditions can reduce it. Responses to secondary challenges were increased by β-glucan imprinting in M-CSF-polarising macrophages but decreased in GM-CSF-polarising cells. Hallmarks of terminally matured macrophages were all distinctively affected by β-glucan, leading towards a common type of less differentiated

macrophages. β-glucan-induced innate memory in macrophages skewed their terminal differentiation through the Dectin-1 receptor and the master regulators IRF5 and IRF3. Altogether, these observations provide insightful and crucial knowledge that will help apprehending the *in vivo* high potential of β-glucan-induced innate memory in different pathological contexts.

Results and Discussion

The inflammatory cytokine response to secondary infections of M-CSF- and GM-CSF-macrophages are oppositely regulated by β-glucan-induced innate immune memory.

Past literature focusing on innate immune memory induced by β-glucan in human monocytes has used the protocol depicted in **Supplementary Figure 1A**. In this study we focused our research using the protocol depicted in **Figure 1A** and **Supplementary Figure 1B and C** to study two opposite types of *in vitro* induced macrophages, namely GM-CSF- and M-CSF-macrophages (also described as M1-like and M2-like, respectively) (4-6).

Previous works that have studied the innate immune memory (7-10), and more specifically the enhanced secretion of IL-6 and TNFα upon secondary stimulation, a hallmark of *trained immunity*, have used isolated human monocytes from PBMCs and maintained them in human serum only. Human serum contains low but detectable amounts of M-CSF (around 2 to 10 ng/mL (11)) and human monocytes isolated from PBMCs can differentiate into macrophages with 10-20 ng/mL of M-CSF. It is therefore likely that macrophages obtained in human serum only are comparable to M-CSF-macrophages.
As expected for M-CSF-derived macrophages (2), β-glucan imprinting increased their pro-inflammatory status and responses to gram-positive and gram-negative bacteria challenges in a dose-dependent manner (**Figure 1B**). Surprisingly however, β-glucan imprinting of GM-CSF-differentiating macrophages decreased their responsiveness, resulting in less inflammatory macrophages (**Figure 1B**). Although past literature demonstrated that β-glucan inhibits spontaneous human monocyte apoptosis (12), we have previously established that β-glucan together with growth factors does not alter the viability of macrophages (2). In addition, we further ascertained that β-glucan does not alter survival of M-CSF- and GM-CSF-macrophages in this study (**Supplementary Figure 2A, B**). Monocytes stimulated by β-glucan do not release GM-CSF but secrete M-CSF (from 20 pg/mL up to 100 pg/mL depending on the amount of β-glucan) (12). The effect of β-glucan on M-CSF-differentiating macrophages can therefore not be due to an imbalance in GM-CSF concentration. On the other hand, the secretion of M-CSF caused by β-glucan could affect the differentiation of GM-CSF-macrophages. However, neither physiologically secreted nor high doses of exogenous M-CSF affected the cytokine responses of GM-CSF-macrophages to secondary bacterial stimulation (**Supplementary Figure 2C**). Hence the effect of β-glucan-induced innate immune memory on differentiating macrophages was neither due to an effect of β-glucan on the viability of the macrophages nor to an

imbalanced M-CSF or GM-CSF concentration possibly caused by β-glucan stimulation. β-glucan reprogramming therefore differentially imprints pro-inflammatory GM-CSF- and anti-inflammatory M-CSF-macrophages towards a similar intermediate type of macrophage that presents both pro- and anti-inflammatory responses potentially making it more plastic in terms of the type of response required.

β-glucan reprogramming of macrophages results in common less differentiated macrophages regardless of the microenvironment

Cytokine expression upon stimulation is only one feature that distinguishes the types of macrophages studied. We therefore looked at other properties of macrophages using the strongest effective concentration of β-glucan for priming (10 μg/mL). We first analysed with microscopy the morphology of the cells, which is one of the described polarisation features distinguishing M-CSF- and GM-CSF-macrophages (13)(**Figure 2A**). The canonical round shape of GM-CSF-macrophages and spindle shape of M-CSF-macrophages were both affected by β-glucan imprinting. Both M-CSF- and GM-CSF-β-glucan-imprinted macrophages were similarly roundish with increased size and granularity compared to their non-primed counterparts (**Figure 2A**). We also investigated the expression of specific markers defining M-CSF- and GM-CSF-macrophages terminal differentiation. As such, IL-12 and CCL22 GM-CSF-markers decreased upon β-glucan-priming in GM-CSF-macrophages and increased in M-CSF-macrophages (**Figure 2B**). However, the GM-CSF marker CXCL9 decreased in both GM-CSF- and M-CSF-macrophages after β-glucan-induced innate immune memory (**Figure 2B**). Of interest, while the M-CSF-associated markers VEGF and CCL2 (14) decreased in M-CSF-macrophages after β-glucan priming, these two markers also decreased in GM-CSF-macrophages. Of note, the specific effects of β-glucan imprinting on the secretion of cytokines such as IL-12 and IL-6 (**Figure 1B**) in M-CSF- and GM-CSF-macrophages were also observed at the transcriptional level (**Figure 2B** and **Supplementary Figure 3**), excluding a post-translational regulation by β-glucan-reprogramming as we recently reported for the inflammasome-dependent IL-1β (2)**.** With respect to cell surface markers of macrophages, the LPS coreceptor CD14 is a monocyte-specific surface marker whose expression ultimately decreases upon differentiation into macrophages but remains more expressed in M-CSF-macrophages compared to GM-CSF-macrophages (15) (**Figure 2C**). β-glucan imprinting on differentiating macrophages did not alleviate this difference between M-CSF- and GM-CSF-cells, and in both cases β-glucan-imprinted macrophages presented a lower CD14 surface expression, compared to their non-primed counterparts. The GM-CSF-associated cell surface marker CD206 expression (16) decreased upon β-glucan-priming in GM-CSF-macrophages and increased in M-CSF-macrophages (**Figure 2C**), although the expression remains higher in GM-CSF-macrophages regardless of the priming. With respect to the M-CSF-associated cell surface marker CD163, β-glucan imprinting again did not alleviate the specific difference between M-CSF and GM-CSF but decreased the expression of CD163 in M-CSF-macrophages. Overall, it appears that β-glucan reprogramming confers a unique phenotype to differentiating macrophages. In conclusion, β-glucan-macrophages are all roundish with high granularity and

size. All M-CSF- and GM-CSF-specific markers of terminally differentiated macrophages we measured were decreased by β-glucan imprinting in M-CSF- and GM-CSF-macrophages respectively. Altogether these observations highlight that β-glucan reprogramming of differentiating macrophages decreases their terminal maturation marks, providing cells with larger plasticity and panel of reactivity to secondary input.

β-glucan reprogrammed macrophages exhibit new functional activities

We next determined whether the functions of macrophages were also affected by β-glucan reprogramming, given the modulation of their differentiation markers. M2-like macrophages show higher phagocytic affinities and capacities than M1-like macrophages (17), a process that we observed in our inactivated-bacteria phagocytosis assay (**Figure 3A**). Interestingly, β-glucan imprinting significantly reduced the quantity of phagocytosed bacteria by M-CSF-macrophages (**Figure 3A**). More specifically, less M-CSF-macrophages phagocytosed bacteria upon β-glucan priming (**Supplementary Figure 4A**) and β-glucan-treated-M-CSF-macrophages engulfed less bacteria than their naïve counterparts (**Supplementary Figure 4B**). In fact, resulting β-glucan-primed macrophages showed the same phagocytic capacities as GM-CSF-macrophages. We can conclude that β-glucan imprinting skews the differentiation of M-CSF-macrophages from their tissue scavenging purpose, one of their terminal primary functions.
M-CSF-macrophages are inducers of endothelial migration that is essential to angiogenesis, mainly through the production of VEGF, the most active endogenous pro-angiogenic factor in humans (18, 19). Given the observed modulation of VEGF by β-glucan imprinting (**Figure 2B**), we monitored the angiogenesis properties of our macrophages using conditioned media on HUVEC cells. As expected, we observed higher migration of HUVEC cells when cultured with conditioned media from M-CSF-macrophages compared to that of GM-CSF-macrophages (**Figure 3B, C**). β-glucan imprinting, which decreases VEGF expression in M-CSF-macrophages (**Figure 2B**) also decreased the migration-inducing properties of the conditioned media. Excessive VEGF and subsequent angiogenesis have been associated with atherosclerotic plaque progression (20, 21) and tumor angiogenesis (22). β-glucan imprinting might therefore decrease risk of cardiovascular disease or tumor progression. Of interest, β-glucan imprinting increased the migration properties of GM-CSF-macrophages conditioned media (**Figure 3B, C**) without increasing VEGF expression (**Figure 2B**). Angiogenesis plays critical roles in human physiology that range from reproduction and foetal growth to wound healing and tissue repair (23). Hence β-glucan imprinting could represent therapeutic opportunities to potentiate the beneficial functions of angiogenesis induced by GM-CSF-macrophages secretome, independently of VEGF.

ROS production is a hallmark of macrophage-mediated inflammatory response – ranging from antimicrobial activity, redox regulation of immune signalling to induction of inflammasome activation (24). As expected, ROS production was higher in GM-CSF-macrophages than M-CSF-macrophages (**Figure 3D, E**). β-glucan imprinting increased the capacity of M-CSF-macrophages to produce ROS to a similar level to that of GM-CSF-macrophages. β-glucan had

no additional effect on GM-CSF-macrophage production of ROS. Altogether these results demonstrate that β-glucan priming differentially affects M-CSF-macrophages and GM-CSF-macrophages. The differentially decrease or increase of specific functions lead towards macrophages with similar scavenging, angiogenesis and ROS signalling properties.

β-glucan imprinting skews the commitment of macrophages

We observed that β-glucan priming of macrophages in the first 24 hours affects their differentiated features 6 days after, which could be seen as either skewing or reverting differentiation. To test these hypotheses, we next assessed whether β-glucan imprinting rapidly skews the commitment of macrophages, reprogramming them from the onset towards priming, or whether it progressively reverts the polarisation over time. We measured the cytokine responses of the cells to heat-inactivated *S. aureus* stimulation as a hallmark of differentiation. Measurements were performed as usual, 6 days after β-glucan priming, but also before β-glucan imprinting (monocytes), a day after, and 3 days after (**Figure 4A**). First, without β-glucan input, it is interesting to note that none of the differentiating macrophages were polarised after only one day of incubation with growth factors with regards to the pro-inflammatory cytokines (**Figure 4B**, indicated as #). Polarisation started to be observable for the pro-inflammatory IL-12 and TNFα from day 3 and was maintained throughout the rest of differentiation up to day 6. For IL-6, although differentiating macrophages already expressed lower cytokines than monocytes from day 1, the polarisation required more time and was only observable at day 6. So altogether polarisation for pro-inflammatory cytokines is a progressive event that occurs over time. In contrast to pro-inflammatory cytokines, polarisation with respect to the anti-inflammatory cytokine IL-10 was observable from day 1 and maintained throughout the experiment. β-glucan imprinting never affected the very low IL-10 responses in GM-CSF-macrophages, but the decrease detected in M-CSF-macrophages is observable at the onset from day 1 (*) and is strengthened over time. With respect to pro-inflammatory signature, β-glucan priming affected both types of macrophages early and inhibited their responses to *S. aureus*. This inhibition at the onset was maintained for GM-CSF over the time of the experiment, culminating in the decreased responsiveness described above at day 6. However, for M-CSF, reprogramming effect with increased responsiveness only appeared after 6 days post-priming, only when control naïve M-CSF-macrophages reached their final polarisation features. Altogether, these results highlight that β-glucan affects the polarisation and differentiation processes of GM-CSF- and M-CSF-macrophages as soon as it has been detected by the cells, skewing their full differentiation in either direction. β-glucan-imprinted-macrophages are therefore less terminally differentiated, but not because of a reversion and rather because of an impediment of the primary processes of polarisation. The effect of β-glucan imprinting in early processes has already been reported with regards to inhibition of the early activation cascades of NRLP3 (2) and highlights that β-glucan is a very strong early modulator of innate immune cells.

β-glucan reprogramming affects the expression of the master regulators of macrophage polarisation IRF5 and IRF3

To determine how this early impediment of macrophage terminal differentiation occurs, we investigated the mechanism of action of β-glucan on the commitment of M-CSF- and GM-CSF-macrophages. β-glucan-induced innate immune memory acts on human primary monocytes (7) and macrophages (2) through the C-type lectin receptor Dectin-1. β-glucan imprinting reduced the TNFα responsiveness of GM-CSF macrophages whereas it exacerbated that of M-CSF-macrophages (**Figure 1B and 5A**). Blocking the Dectin-1 receptor with an anti-Dectin-1 antibody alleviated these opposite effects of β-glucan on GM-CSF- and M-CSF-macrophages (**Figure 5A**). Of note, Dectin-1 expression is highly up-regulated by GM-CSF (25). This increased amount of Dectin-1 receptor at the surface of GM-CSF-macrophages might not be fully blocked by the anti-Dectin-1 antibody, compared to M-CSF-macrophages, which could explain why the restoration of TNFα secretion in Dectin-1-inhibited-GM-CSF-macrophages is not total. β-glucan priming reduced the IL-10 responsiveness of M-CSF macrophages (**Figure 1B and 5A**). Blocking the Dectin-1 receptor with an anti-Dectin-1 antibody impended the β-glucan imprinting effect on M-CSF macrophages, which turned out to secrete similar IL-10 cytokine levels upon stimulation as their naïve control counterparts (**Figure 5A**). These results demonstrate that β-glucan-priming effects on polarising macrophages are mediated by Dectin-1 as already reported in human primary monocytes and macrophages (2, 7), asserting Dectin-1 as a master regulator of innate immune memory in human primary mononuclear phagocytes.
Members of the interferon-regulatory factor (IRF) family determine commitment to GM-CSF-M1-like or M-CSF-M2-like macrophages. The transcription factor IRF5 is the master regulator of M1 macrophage polarisation (26). As expected, IRF5 was highly expressed in human GM-CSF-M1-like-macrophages compared to M-CSF-M2-like macrophages (**Figure 5B**, **C**, (26)). Of interest, β-glucan imprinting of M-CSF-macrophages increased the expression of the GM-CSF-master regulator IRF5 to the same level as GM-CSF macrophages. Based on the CD4 T helper type 1 (Th1), Th2 and Th17 cells polarisation paradigm (27), M1 macrophages produce proinflammatory cytokines, mediate resistance to pathogens and drive a Th1/ Th17 response, whereas M2 macrophages produce anti-inflammatory cytokines, promote tissue repair and remodelling and drive a Th2 response. In M1 macrophages, IRF5 dictates the expression of proinflammatory genes such as IL-12 whilst repressing anti-inflammatory genes like IL-10, driving a potent Th1/Th17 response (26). Here, β-glucan reprogramming of M-CSF-M2-like-macrophages increased IRF5 expression, with a concomitant increase in IL-12 expression and decrease in IL-10 expression, therefore driving the β-glucan-M-CSF-macrophages towards setting up an environment for a CD4 Th1/ Th17 response.
The transcription factor IRF3 is predominantly activated in M-CSF-M2-like macrophages (28) and is associated with anti-inflammatory microenvironments and contributes to the polarisation towards a M2 macrophage phenotype (29). Indeed, IRF3 was found to be more expressed in our M-CSF-macrophages compared to GM-CSF-cells (**Figure 5B**, **C**). Interestingly again, β-glucan imprinting decreased the expression of IRF3 in M-CSF-macrophages. Altogether, β-glucan reprogramming affected the specificity of macrophage polarisation through Dectin-1

and the modulation of the expression of the master regulators of the macrophage polarisation IRFs.

Deciphering how β-glucan specifically imprints polarising macrophages in complex environments provides a fundamental knowledge bringing us a step further towards exploiting the *in vivo* potential of β-glucan-induced innate memory.

Materials and Methods

Cell isolation. Human PBMCs were separated on Ficoll-Paque (GE Healthcare) by density centrifugation of heparinized blood from healthy donors ("Etablissement Français du Sang" EFS, Paris, France). Monocytes were purified by positive selection using the CD14 MicroBeads, human (Miltenyi Biotec). The purity evaluated by flow cytometry was routinely >90%.

Macrophage differentiation. Monocytes were seeded at 10^6 cells/mL in RPMI 1640, supplemented with 2 mM GlutaMax, 50 µg/mL gentamicin and 1 mM sodium pyruvate (Gibco) and incubated 24h with GM-CSF (25 ng/mL, 130-093-865, Miltenyi Biotec) or M-CSF (25 ng/mL, 216-MC-025, R&D Systems) in the presence or absence of purified β-glucan from *Candida albicans* (10 µg/mL unless otherwise stated; depyrogenated and kindly provided by Dr. David L. Williams, East Tennessee State University). After the first 24 hours, the cells were washed with warm PBS and incubated for 5 more days in RPMI containing 10% FBS (F7524, Sigma-Aldrich) in the presence of GM-CSF or M-CSF. The medium was changed once at day 3. Cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

For Dectin-1 inhibition, anti-Dectin-1 mAb (10 µg/mL, ab82888, abcam) or IgG1 Isotype control (10 µg/mL, 401402, Biolegend) was added 30 min prior to β-glucan stimulation and maintained in culture medium for 24h. After this period, cells were differentiated as described above.

For evaluation of M-CSF effect on β-glucan imprinting, monocytes were seeded at 10^6 cells/mL in RPMI 1640, supplemented as described previously and incubated 24h with GM-CSF (25 ng/mL) in the presence of M-CSF (1000 pg/mL, 100 pg/mL or 50 pg/mL), or β-glucan (10 µg/mL) or medium as control. Then, the cells were washed with warm PBS and incubated for 5 more days in RPMI containing 10% FBS in the presence of GM-CSF. The medium was changed once and the cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

Viability assay. Monocytes were seeded in black 96-well plates and differentiated in macrophages as described above. At day 6, one set of macrophages was treated with saponin 0.05% for 15 minutes (positive control for PI). Then, the cells were washed three times with warm PBS and incubated with fluorescein diacetate (8 µg/mL, Sigma-Aldrich) and propidium iodide (20 µg/mL, Sigma-Aldrich) diluted in RPMI 1640 for 5 minutes in the dark. Then cells were washed three times with warm PBS and were imaged using an EVOS FL system (Thermo Fisher Scientific, Ex: 470nm, Em: 525nm for GFP and Ex: 530nm, Em: 592nm for PI) and PI fluorescence was measured with a Synergy H1 microplate reader (Ex: 520nm, Em: 610nm; BioTek Instruments).

Cytokine assay. Macrophages were differentiated as described above and, at day 6 (unless otherwise stated), were stimulated with heat-inactivated *Staphylococcus aureus* ($5x10^7$ cells/mL) or heat-inactivated *Escherichia coli* ($1x10^5$ cells/mL) for 24 hours in RPMI. IL-6, TNF-α, IL-12p40 (R&D Systems) and IL-10 (BioLegend) levels were determined by enzyme-linked immunosorbent assays (ELISA) on cell culture supernatants, according to the

manufacturer's instructions. Absorbance was measured with a Synergy H1 microplate reader (BioTek Instruments).

Reactive oxygen species measurement. Monocytes were seeded in white 96-well plates and differentiated as described above. At day 6, macrophages were stimulated with 500 ng/mL of PMA (phorbol 12-myristate 13-acetate, Sigma-Aldrich) diluted in HBSS (Hank's Balanced Salt Solution – Gibco). At the same time, Luminol (1mM; Sigma-Aldrich), also diluted in HBSS, was added, and light emission was measured every 2 minutes for a period of 120 min using the Synergy H1 microplate reader (Biotek Instruments, USA). The light emission levels are expressed as RLU (Relative Light Units = luminescence measured by the luminometer).

Phagocytosis assay. To evaluate the phagocytic activity of macrophages, pHrodo Green inactivated *S. aureus* BioParticles conjugate was used (P35367, Life Technologies). Monocytes were seeded in a flat clear bottom black 96-well plate (3603, Corning) and differentiated as described above. On the sixth day of macrophage differentiation, pHrodo BioParticles were resuspended in Live Imaging Solution (Life Technologies), sonicated and incubated with macrophages (5 µg/mL final concentration). The plate was centrifuged 300g for 2 minutes to synchronise the interaction between the BioParticles and macrophages and incubated for 1 hour at 37 ºC. After the incubation time, fluorescence was measured with a Synergy H1 microplate reader (Ex: 505 nm, Em: 535 nm; BioTek. Instruments, USA). The fluorescence emission levels are expressed as RFU (Relative Fluorescence Units).
Phagocytosis was also measured by flow cytometry: monocytes were seeded in a 12-well plate and differentiated as described above. On the sixth day of macrophage differentiation, the macrophages were stimulated with pHrodo BioParticles as described above, for 30 minutes at 37ºC. Then cells were detached from the plate using Accutase solution (Sigma-Aldrich) at 37ºC for 15 minutes followed by manual shaking and repeated pipetting. Acquisition was performed on a LSR Fortessa (BD Biosciences) and *S. aureus* BioParticles were detected in the FITC channel. Analysis was performed using FlowJo Software (Treestar, Ashland). Phagocytosis rate was quantified as the percentage of cells positive for the *S. aureus* BioParticles (%FITC+) and phagocytosis efficacy, or the average amount of bacteria per cell, was quantified as the Mean Fluorescence Intensity (MFI) for *S. aureus* BioParticles within FITC-positive cells.

RNA isolation and real-time PCR analysis. Macrophages were differentiated as described above and, at day 6 were stimulated for 16 hours with heat-inactivated *S. aureus* ($5x10^7$ cells/mL) or for 3 hours with heat-inactivated *E. coli* ($1x10^5$cells/ml) in RPMI, and total RNA was isolated from macrophages using TRIzol reagent (Invitrogen). RNA quality was monitored by spectrophotometric analysis. Total RNA (1 µg) from each sample was used for reverse transcription with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies). Real-time PCR was performed in ABI Step One Plus Real-Time PCR System (Applied Biosystems, Life Technologies) using the TaqMan 2× Universal Master Mix and the following TaqMan Gene Expression Assays: IL-12, Hs01011518, CCL22, Hs01574247; CCL2, Hs00234140; CXCL9, Hs00171065; VEGFA, Hs00900055; IL-6, Hs00174131 (Applied Biosystems, Life Technologies). Each sample was assayed in triplicates.

Thermal cycling conditions were as follows: denaturation at 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Fold change of the cytokine genes in the samples was calculated using the $2^{-\Delta\Delta CT}$ method. All values were normalised to endogenous control β-Actin (Hs99999903; Applied Biosystems, Life Technologies) and were expressed in arbitrary units.

Flow cytometry. Monocytes were seeded in a 12-well plate and differentiated as described above. At day 6, macrophages were detached from the plate using Accutase solution (Sigma-Aldrich) at 37ºC for 15min followed by manual shaking and repeated pipetting. Fc receptors were blocked by incubation with FcR Blocking Reagent (Miltenyi Biotech) and staining was performed using antibodies purchased at Miltenyi Biotech: anti-CD163 PE (clone REA812, Cat# 130-112-128,), anti-CD206 PE-Vio770 (clone DCN228, Cat# 130-100-152) and anti-CD14 VioGreen (clone REA599, Cat# 130-110-524). Cells were also stained with Fluorescence Minus One (FMO) mixes containing the relevant isotype control: REA control, clone REA293, coupled with VioGreen (Cat# 130-113-442), or PE (Cat#130-113-438), or mouse IgG1 PE-Vio770 (clone IS5-21F5, Cat# 130-113-202). Acquisition was performed with a LSR Fortessa (BD Biosciences) after fixation of cells with 4% Formaldehyde Solution at room temperature protected from light. Analysis was performed using FlowJo Software (Treestar, Ashland).

Transmigration assay. Monocytes were seeded in a 24-well plate and differentiated as described above. On the sixth day of macrophage differentiation, cells were washed once with warm PBS and stimulated, for 24 hours, with heat-inactivated *Staphylococcus aureus* ($5x10^7$ cells/mL) resuspended in Endothelial medium (C-22210, PromoCell) complemented with 10% of fetal bovine serum. After stimulation time, the conditioned medium was placed in the bottom chamber of a transwell system (pore size 5 μm, Costar). HUVECs cells ($2.5x10^5$ cells/mL - PromoCell), resuspended in free endothelial medium, were placed in the top chamber and the system was incubated for 24 hours in a humidified atmosphere of 5% CO_2 at 37°C. Then, the chamber was washed twice with warm PBS, the cells were fixed with 4% Formaldehyde Solution at 37 ºC for 15 minutes. Then, the cells were permeabilized with 0,5% Triton X-100 for 10 minutes at room temperature and stained 10 minutes in the dark with DAPI (Thermo Fisher Scientific). To only count the cells that migrated through the pores, the cells in the top chamber were eliminated with a swab. The transwell chambers were imaged using EVOS FL system (Ex: 360nm, Em: 447nm for DAPI, Thermo Fisher Scientific) and migrated cells were counted using ImageJ software (NIH, Bethesda, MD, USA).

Western blot. Monocytes were differentiated as described above and, after 6 days of differentiation, cells were stimulated with heat-inactivated *Staphylococcus aureus* ($5x10^7$ cells/mL) for 24 hours. After stimulation, total cellular protein extracts were obtained by lysing cells for 30 min at 4°C in lysis buffer (Pierce RIPA Buffer) in the presence of protease inhibitors (cOmplete Protease Inhibitor Cocktail, ROCHE). Samples were centrifuged for 20 min at 4°C, 13,000 rpm and supernatants were collected in fresh tubes. Proteins from cell extracts were resuspended in Laemmli Buffer 1X, boiled at 95°C for 7 min and resolved by SDS-PAGE. Then, proteins were transferred with an iBlot instrument (Life Technologies) to a nitrocellulose

membrane. Immunoblotting was performed using the following primary antibodies: anti-IRF3 (sc-33641, Santa Cruz Biotechnology), anti-GAPDH (sc-25778, Santa Cruz Biotechnology) and anti-IRF5 (20261S, Cell Signaling). Anti-mouse or anti-rabbit IgG, HRP-linked antibodies (NA931V and NA934V respectively, GE Healthcare) were used as secondary antibodies, and protein bands were visualized with ECL Prime (GE Healthcare) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) in a myECL imager (Thermo Fisher Scientific). Proteins were normalised for GAPDH using Thermo Scientific myImageAnalysis Software.

Statistic. The results were analysed using a Wilcoxon matched-pairs signed-rank test (unless otherwise stated). A p value below 0.05 was considered statistically significant. All data were analysed using GraphPad Prism software. Data are shown as mean ± standard error of the mean.

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Figures and Figure Legends

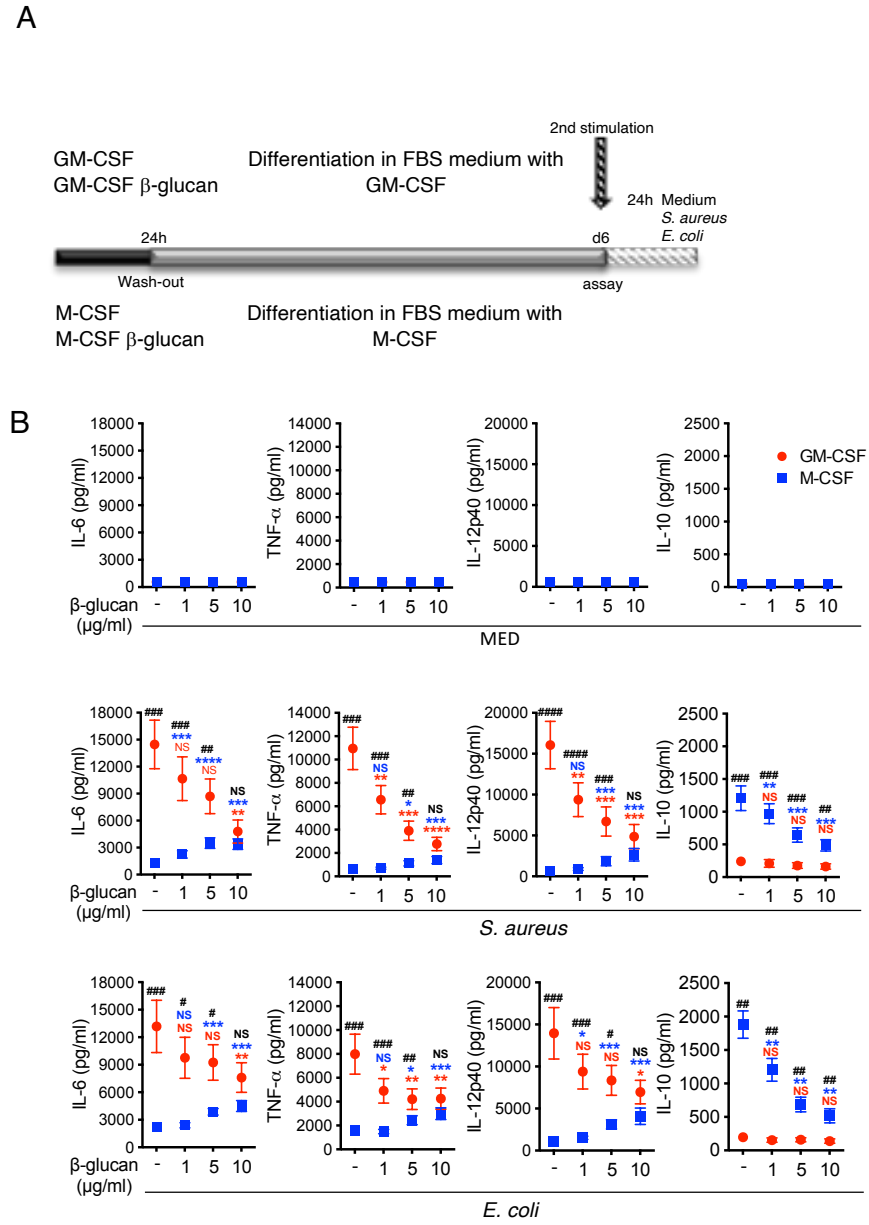

Figure 1. Distinct β-glucan imprinting on polarising macrophages. (A) Schematic overview of the methodology. Monocytes were preincubated with β-glucan or left untreated in a medium containing either GM-CSF or M-CSF. After 24 hours, the stimulus was washed away, and the cells were differentiated for an additional 5 days, after which the macrophages were re-stimulated. (B) Macrophages were re-stimulated with heat-inactivated *Staphylococcus aureus* or *Escherichia coli*. Culture supernatants were collected after 24h and concentrations of secreted TNF-α, IL-6, IL-12, and IL-10 were determined by ELISA. Graphs show the mean ± SEM of at least three independent experiments; $n = 11$; $*^{,\#}p < 0.05$, $**^{,\#\#}p < 0.01$, $***^{,\#\#\#}p<0.001$. Wilcoxon matched-pairs signed-rank test. (*: cells stimulated with β-glucan vs. non stimulated with β-glucan; #: GM-CSF vs. M-CSF-differentiated cells).

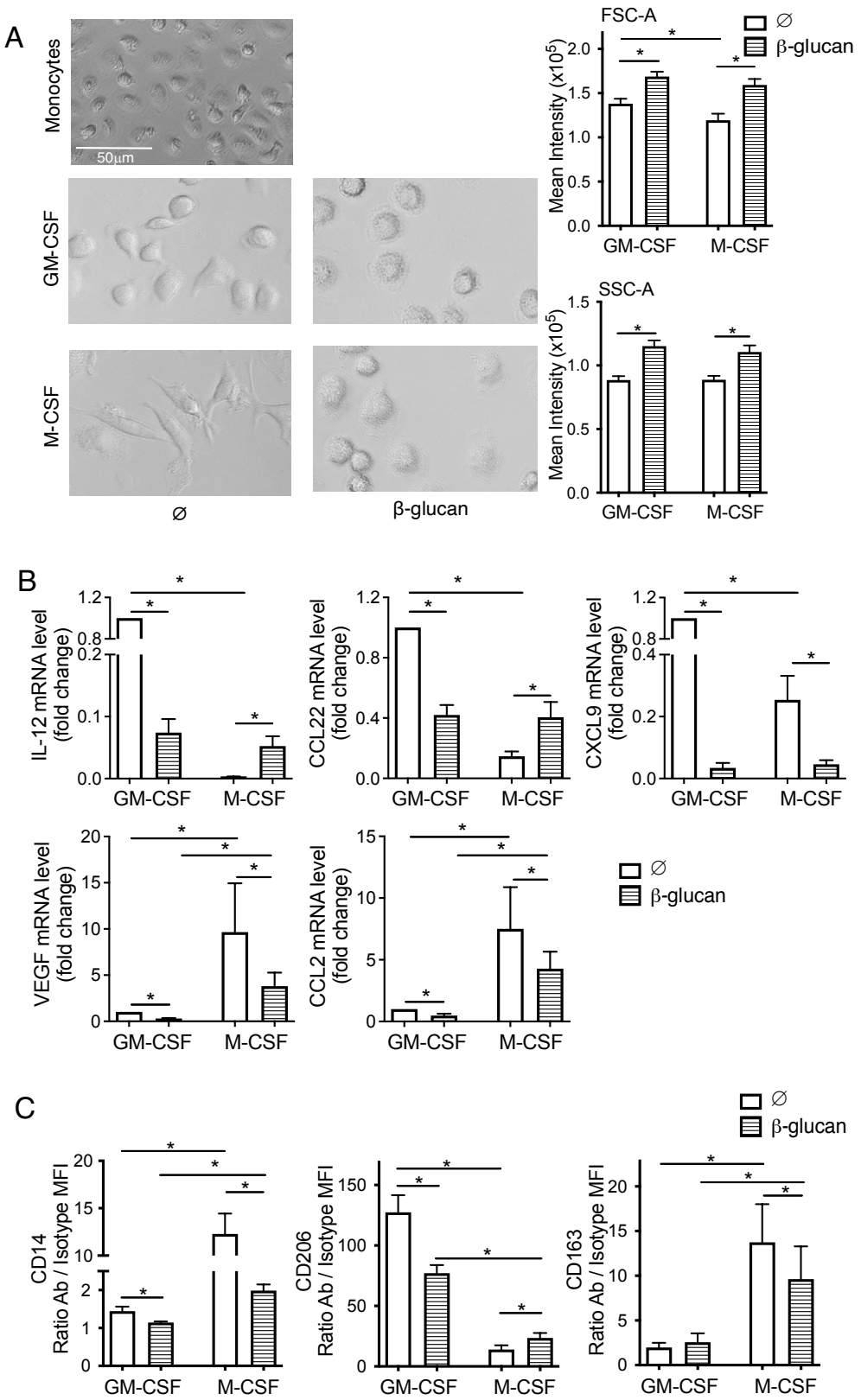

Figure 2. β-glucan modulate the terminal differentiation markers of macrophages. Monocytes were preincubated in the presence or absence of β-glucan and then differentiated with either GM-CSF or M-CSF. (A) Monocytes at day 0 and macrophages at day 6 were imaged to evaluate their morphology (left) or detached and analysed for size (FSC-A, top) and granularity (SSC-A, bottom) by flow cytometry (B) At day 6 of differentiation, macrophages

were re-stimulated for 18h with heat-inactivated *S. aureus* and IL-12, CCL22, CXCL9, VEGF and CCL2 gene expression was analysed by Real-Time quantitative PCR (RT-qPCR). Results were normalised to β-actin expression levels. The results obtained for the cells differentiated with GM-CSF in the absence of β-glucan were arbitrarily set at 1 to express the results as fold change. (C) At day 6 of the differentiation process, macrophages were stained for the indicated cell surface markers, or with FMO antibody mixes containing the relevant isotype controls, for flow cytometry analysis. Intensity of expression of a given marker was measured by doing the ratio of the full staining MFI over the isotype MFI ('ratio MFI'). Graphs show the mean ± SEM of at least three independent experiments. For A, n=6, B, n = 7; for C, n = 6-8; *$p < 0.05$, **$p < 0.01$. Wilcoxon matched-pairs signed-rank test.

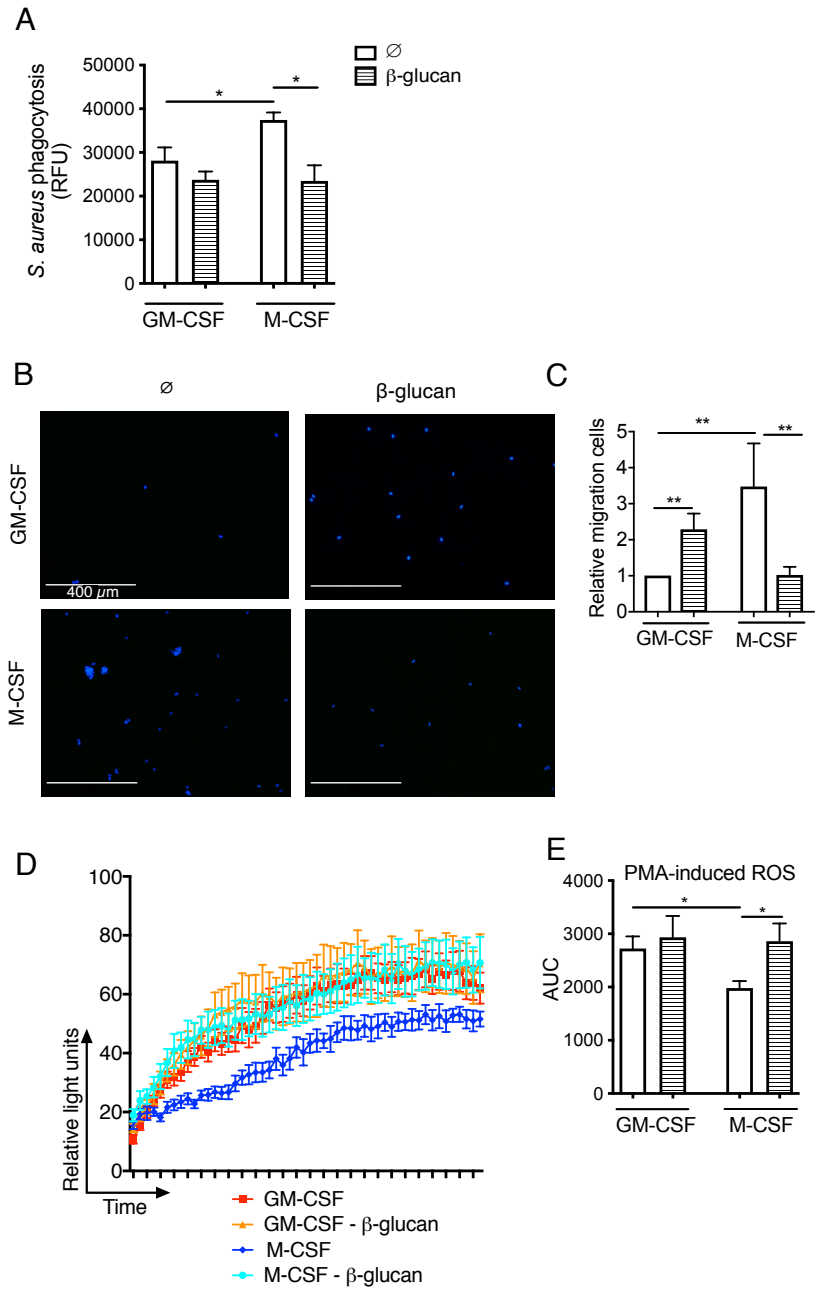

Figure 3. β-glucan imprinting affects functional activities in differentiating macrophages. Monocytes were preincubated in the presence or absence of β-glucan and then differentiated in macrophages with either GM-CSF or M-CSF. (A) After 6 days of culture, resulting macrophages were re-stimulated with pHrodo™ Green *S. aureus* Bioparticles™ for 1 hour. After the incubation time, phagocytosis was measured by reading the fluorescence and is expressed as relative fluorescence units (RFU). (B) After six days of differentiation, macrophages were stimulated with heat-inactivated *S. aureus* and, after 24 hours of incubation, the supernatant was placed in the bottom chamber of a transwell system. HUVEC cells were placed in the top chamber and the system was incubated for 24 hours. HUVEC migrated cells were stained with DAPI, imaged and counted. (C) Histograms of the HUVEC migration quantification. (D) After six days of differentiation, in order to measure ROS generation, macrophages were stimulated with PMA (500 ng/mL) and luminol was added immediately prior to acquisition and reading. ROS production was monitored by chemiluminescence for 2

hours and expressed as relative light units (RLU). Graphs show mean ± SEM corresponding to up to three independent experiments. (E) Histogram representation of the Area Under the Curve (AUC) of the data shown in D. Graphs show the mean ± SEM of at least three independent experiments. For A, $n = 8$; for C, $n = 8$; for D, E, $n = 7$; $*p < 0.05$, $**p < 0.01$. Wilcoxon matched-pairs signed-rank test.

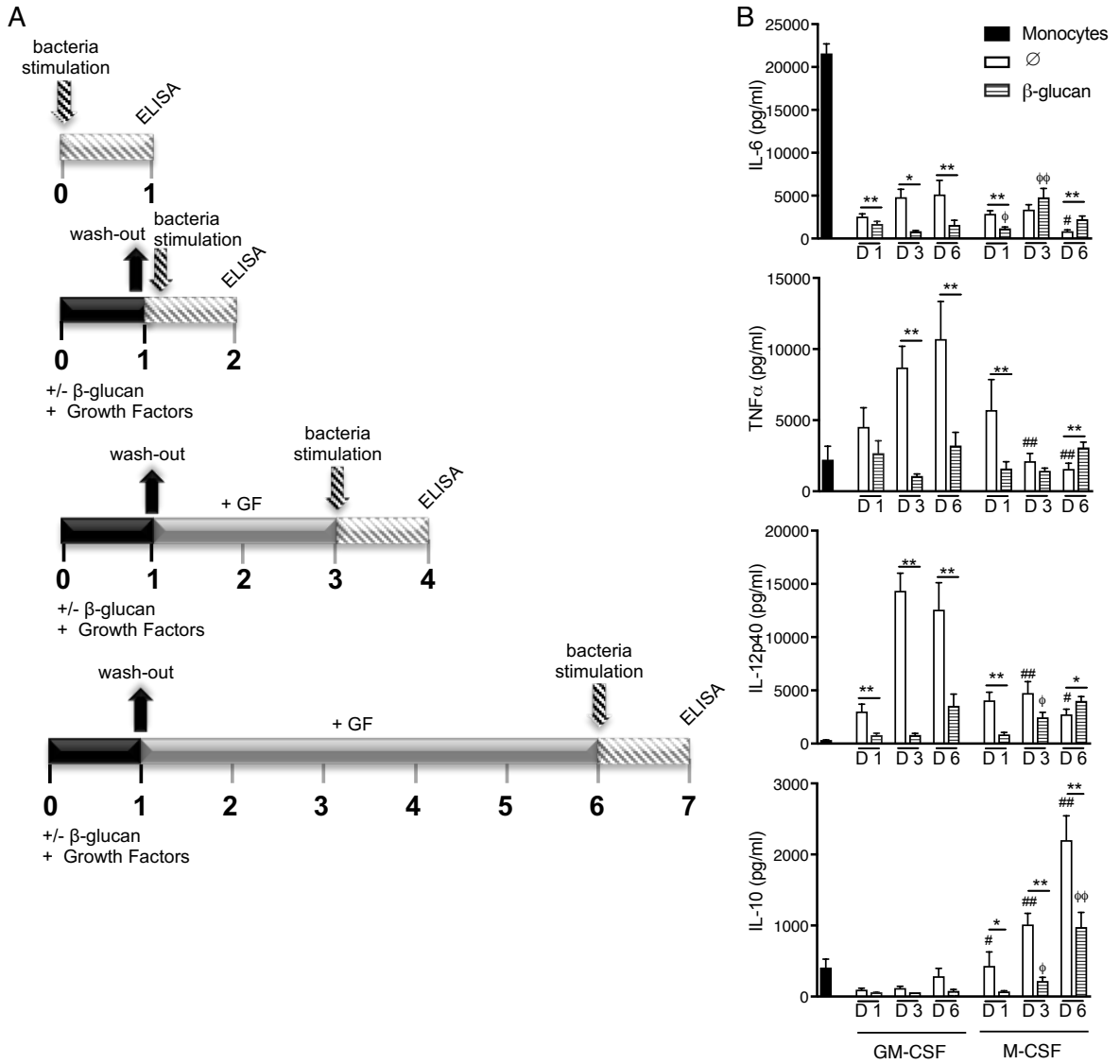

Figure 4. β-glucan imprinting modulates the release of cytokines during the differentiation process. (A) Schematic overview of the experiment. Four different timelines of differentiation and stimulation were designed to evaluate the response of the cells during the process. Culture supernatants were always collected after 24h of *S. aureus* stimulation. (i) Day 0: Monocytes were isolated and stimulated the same day with *S. aureus*. (ii) Day 1: Monocytes were differentiated with GM-CSF or M-CSF, in the absence or presence β-glucan for 24 hours. After this period, the system was washed before stimulation with *S. aureus*. (iii) Day 3: Monocytes were differentiated with GM-CSF or M-CSF, in the absence or presence β-glucan for 24 hours. After the first 24 hours, the system was washed away, and cells maintained in GM-CSF or M-CSF. On day 3 of the differentiation process the cells were stimulated with *S. aureus*. (iv) Day 6: Monocytes were differentiated with GM-CSF or M-CSF, in the absence or presence β-glucan for 24 hours. After the first 24-hour period, the system was washed away, and cells maintained in GM-CSF or M-CSF medium. On day 3 GM-CSF and M-CSF medium was refreshed. At day 6, the macrophages were stimulated with *S. aureus*. GF=growth factors. (B) Culture supernatants were collected after 24h of *S. aureus* stimulation at the different time-points depicted in (A) and concentrations of secreted TNF-α, IL-6, IL-12, and IL-10 were determined by ELISA. Graphs show the mean ± SEM of at least three independent experiments;

$n = 8$, $^{\#,*,\phi}p < 0.05$, $^{**,\#\#,\phi\phi}p < 0.01$. Wilcoxon matched-pairs signed-rank test. (#: GM-CSF vs. M-CSF-differentiated cells; *: cells that were stimulated with β-glucan vs. non stimulated with β-glucan; ϕ: GM-CSF-β-glucan vs. M-CSF-β-glucan-differentiated cells; all the comparisons are made between cells stimulated at the same day).

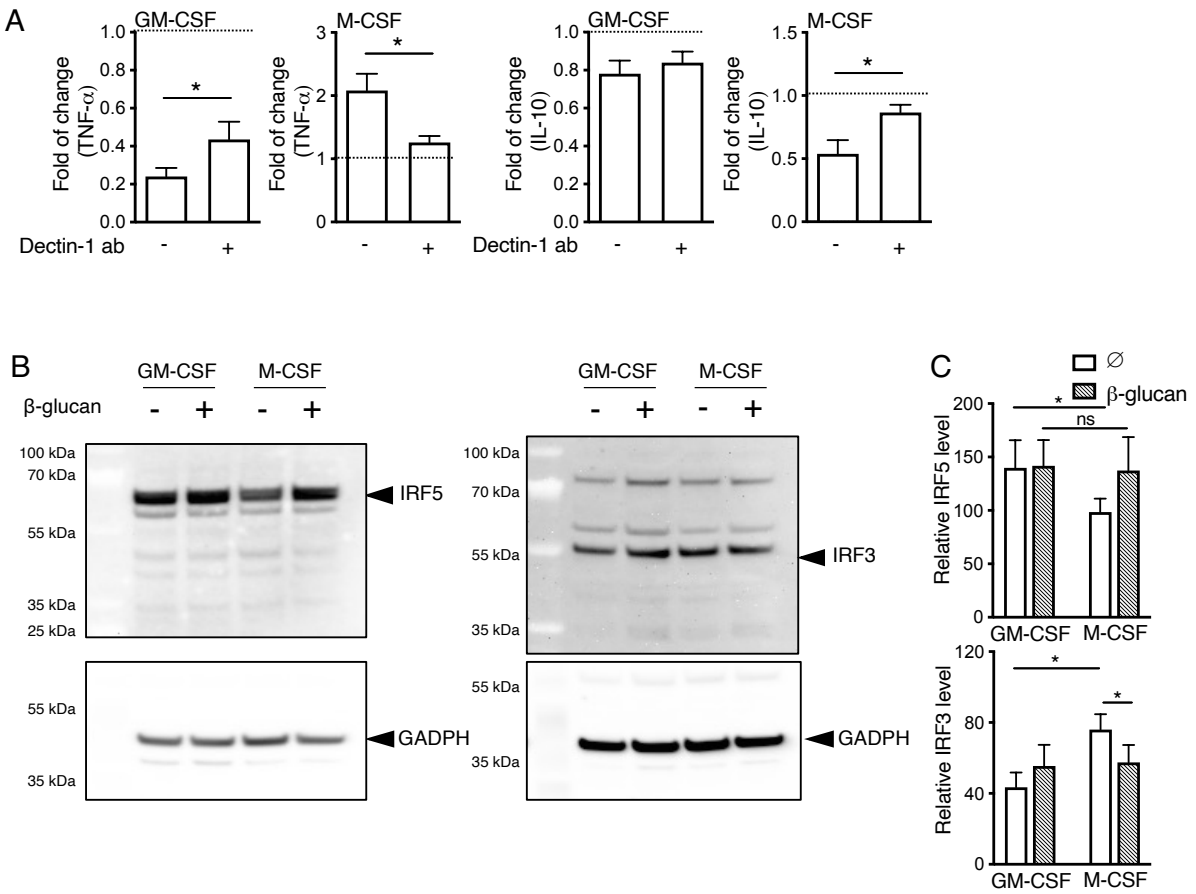

Figure 5. The effect of β-glucan treatment is mediated by Dectin-1 and IRFs. (A) Monocytes were pretreated for 30 minutes with an anti–Dectin-1 mAb or an IgG isotype control, then they were incubated with β-glucan or left untreated in a medium containing either GM-CSF or M-CSF. After 24 hours, the stimulus was washed away, and the cells were differentiated for an additional 5 days, after which the macrophages were restimulated with heat-inactivated *S. aureus*. Culture supernatants were collected following 24h restimulation and the concentrations of secreted TNF-α and IL-10 were determined by ELISA. The results obtained for the cells differentiated in the presence of β-glucan were normalised by its respective control; results above one indicate an increase mediated by β-glucan compared to control; results below one indicate a decrease mediated by β-glucan compared to control. (B) Monocytes were differentiated with GM-CSF or M-CSF and left untreated or stimulated with β-glucan for the first 24 hours. After this first period, the stimulus was washed out and cells were differentiated for another five days with GM-CSF or M-CSF, with the medium being refreshed once during this period. At day 6 of the differentiation process, macrophages were re-stimulated with *S. aureus* for 24 hours. Total cellular protein content was obtained and IRF5 and IRF3 expression was assessed by western blot. GAPDH was used as loading control. Blots are representative of five independent experiments. (C) Densitometric analysis of the blots shown in (B). Data represent mean values ± SEM of the analysis of at least three independent experiments. For A, $n = 5$; for C, $n = 7$; $*p < 0.05$. Wilcoxon matched-pairs signed-rank test.

Supplementary Materials for

β-glucan-induced innate immune memory distinctively affects macrophage activation in response to differential environmental cues

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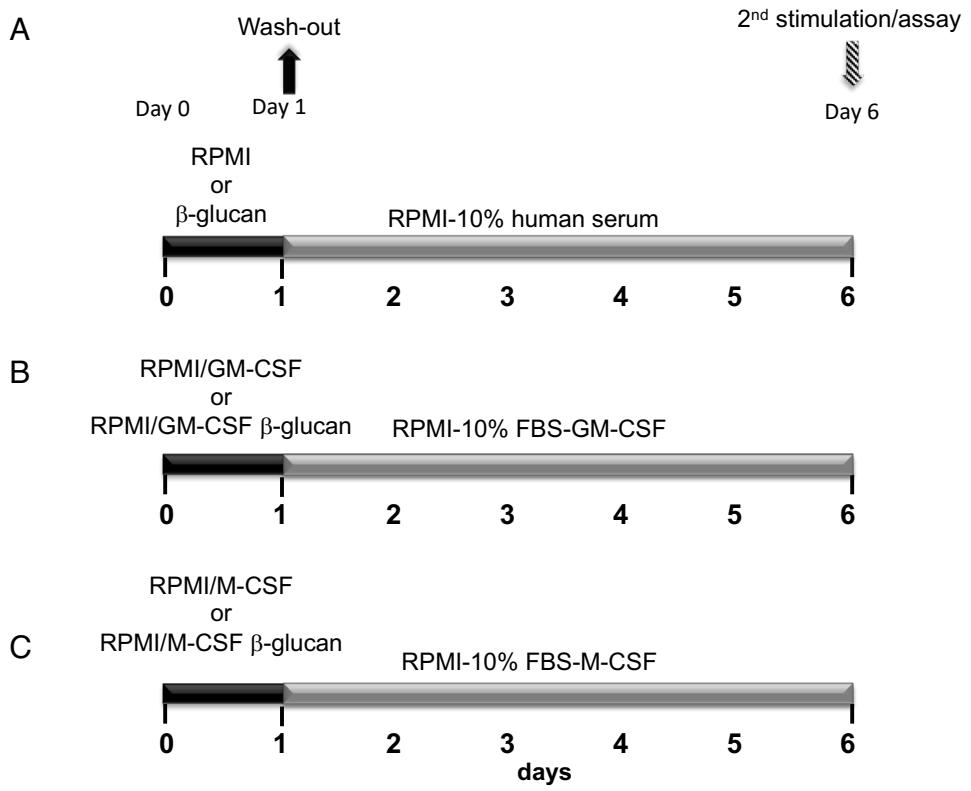
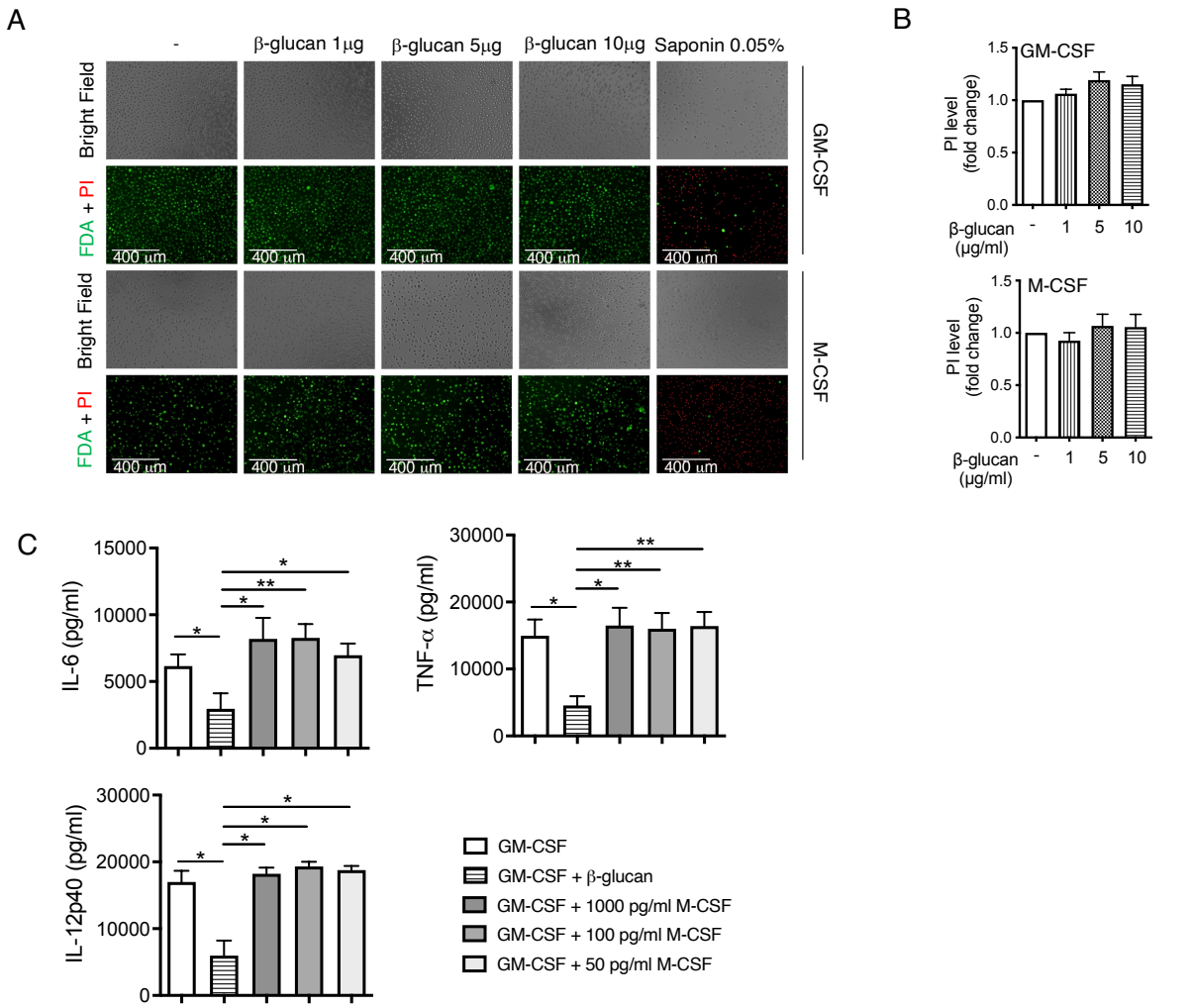
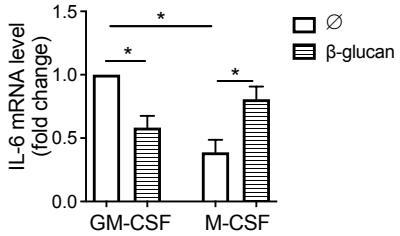
Supplementary Figure 1 to 4



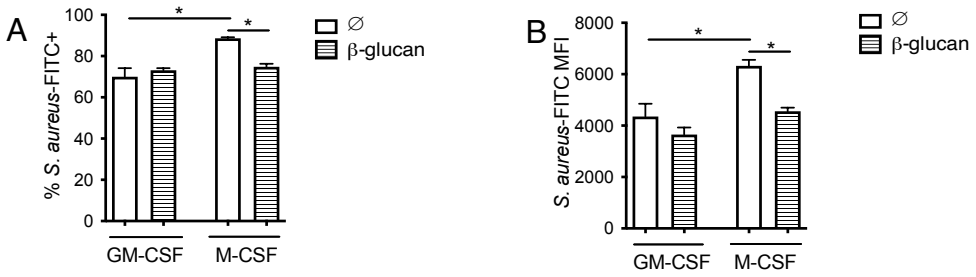
Supplementary Figure 1. Past and present *in vitro* β-glucan imprinting procedure of human primary cells. Schematic overview of the methodology used in previous studies and this study. (A) In former studies focusing on *trained immunity* induced by β-glucan, human primary monocytes were pre-exposed to culture medium only (without serum or growth factors), or with β-(1,3/1,6)-glucan for 24 hours (primary stimulation). After the 1st stimuli had been washed away, the cells were incubated for a week in culture medium supplemented with human pooled serum. Culture medium supplemented with serum was refreshed once during this second period. (B, C) In this study, human primary monocytes were differentiated with (B) GM-CSF (25 ng/mL) or (C) M-CSF (25 ng/mL). During the first 24 hours, human primary monocytes were pre-exposed to β-(1,3/1,6)-glucan (primary stimulation) or left untreated (in medium containing (B) GM-CSF or (C) M-CSF). After the 1st stimuli had been washed away, the cells were maintained in medium containing FBS and either GM-CSF (B) or M-CSF (C). Medium containing FBS and Growth Factors was refreshed once during this second period.



Supplementary Figure 2. β-glucan imprinting is not mediated by cell death or increased M-CSF secretion. (A) Monocytes were preincubated in the presence or absence of β-glucan and then differentiated with either GM-CSF or M-CSF. At day 6 of differentiation, live/dead rate was measured in the resulting macrophages by loading cells with Fluorescein diacetate (FDA) (green, live cells) and Propidium Iodide (PI) (red, dead cells). Saponin 0.05% was used as a positive control for the PI staining. (B) Quantification of PI level relative to the control. (C) Monocytes were preincubated with β-glucan or the indicated concentration of M-CSF in a medium containing GM-CSF. After 24 hours, stimuli were washed away, and the cells were differentiated for an additional 5 days in the presence of GM-CSF. At day 6 of differentiation, resulting macrophages were restimulated for 24h with heat-inactivated *S. aureus,* culture supernatants were collected, and the concentrations of secreted IL-6, TNF-α, and IL-12p40 were determined by ELISA. Graphs show the mean ± SEM of at least two independent experiments. For B, $n = 4$; for C, $n = 3$ $*p < 0.05$, $**p < 0.01$. Wilcoxon matched-pairs signed-rank test.



Supplementary Figure 3. Transcriptional regulation of the β-glucan imprinting on differentiating macrophages. Monocytes were differentiated with GM-CSF or M-CSF, in the absence or presence of β-glucan for the first 24 hours. After the first 24 hours, cells were maintained in GM-CSF or M-CSF medium which was changed once during this second period. At day 6 of differentiation, the macrophages were re-stimulated with heat-inactivated *E. coli*. After 3 hours of stimulation, RNA was extracted from macrophages and the expression of *IL6* evaluated by RT-qPCR. Graphs show the mean ± SEM of at least three independent experiments; $n = 7$; $*p < 0.05$, $**p < 0.01$. Wilcoxon matched-pairs signed-rank test.



Supplementary Figure 4. Phagocytosis rate and efficacy are both modulated by β-glucan-induced innate memory on polarising macrophages

Monocytes were preincubated in the presence or absence of β-glucan and then differentiated in macrophages with either GM-CSF or M-CSF. After 6 days of culture, resulting macrophages were re-stimulated with pHrodo™ Green *S. aureus* Bioparticles™ for 30 min. Cells were then detached and analysed by flow cytometry to determine (A) the percentage of stained cells and (B) the mean fluorescence intensity (MFI). Graphs show mean ± SEM; n = 3 independent experiments. Wilcoxon matched-pairs signed-rank test.