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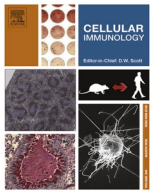

Short communication

VISTA as a ligand downregulates LPS-mediated inflammation in macrophages and neutrophils

Yu-Heng Vivian Ma [a,1], Amanda Sparkes [a,1], Shrayasee Saha [b], Jean Gariépy [a,b,c,*]

[a] Physical Sciences, Sunnybrook Research Institute, 2075 Bayview Ave., Room M7-436, Toronto, ON M4N 3M5, Canada
[b] Department of Pharmaceutical Sciences, University of Toronto, 2075 Bayview Ave., Room M7-434, Toronto, ON M4N 3M5, Canada
[c] Department of Medical Biophysics, University of Toronto, 2075 Bayview Ave., Room M7-436, Toronto, ON M4N 3M5, Canada

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ABSTRACT

VISTA has been proposed to function both as a ligand and a receptor to dampen immune responses, although the role of VISTA as a ligand on myeloid cells has been largely ignored. We observed that a VISTA receptor is rapidly expressed on the surface of macrophages and neutrophils upon exposure to lipopolysaccharides (LPS). Importantly, treating LPS-stimulated macrophages and neutrophils *ex vivo* with a high-avidity agonist of the VISTA receptor (VISTA.COMP) results in the downregulation of pro-inflammatory cytokines and the increased expression of immunoregulatory genes. Finally, the *in vivo* administration of VISTA.COMP attenuated the rise in circulating TNFα, IL-6, and IL-12p40 in LPS-treated mice.

1. Introduction

V-domain immunoglobulin suppressor of *T*-cell activation (VISTA) is described as an immune checkpoint molecule regulating *T*-cell activation. It is widely expressed within the hematopoietic compartment, with the highest expression found on myeloid cells [1–3]. Mechanistically, VISTA has been proposed to function both as a ligand and as a receptor [1–6], yet the majority of published studies focused on the differential expression of VISTA on myeloid cells and the consequences of agonizing or antagonizing VISTA itself. In particular, Bharai and colleagues reported that the overexpression of VISTA on monocytes enhanced cytokine secretion [7], while blocking VISTA on myeloid-derived suppressor cells (MDSCs) mitigates their suppressive activity [3]. More recently, VISTA has been found to have a profound impact on chemotaxis [8], and agonizing VISTA on myeloid cells dampens inflammatory immune responses [4]. None of the aforementioned studies examined the role of a VISTA receptor on myeloid cells. This is due, in part, to the lack of a definitive receptor identified for VISTA on murine myeloid cells. To facilitate VISTA receptor activation studies in the absence of Fc-related events, we previously developed a multimeric recombinant form of the extracellular domain of murine VISTA, termed VISTA.COMP [9]. This construct was engineered by fusing the IgV domain of VISTA to a short peptide corresponding to the pentamerization domain of the cartilage oligomeric matrix protein (COMP). The resulting VISTA pentamer proved superior to VISTA-Fc in terms of binding to the VISTA receptor on murine T cells and as a soluble factor able to dampen their proliferation and the production of pro-inflammatory cytokines [9]. Here, we investigated the mechanistic impact of exogenously treating myeloid immune cell populations with VISTA.COMP in an acute inflammatory setting, namely upon stimulating them *ex vivo* with lipopolysaccharides (LPS) in the context of a murine endotoxemia model.

2. Material and methods

2.1. Mice

Female C57BL/6 mice at 8–10 weeks of age (The Jackson Laboratory) were used throughout this study and housed at the Comparative Research Facility at Sunnybrook Research Institute (SRI; Sunnybrook Health Sciences Centre). All protocols were approved by the SRI Comparative Research Animal Care Committee, accredited by the Canadian Council of Animal Care.

Abbreviations: VISTA, V-domain immunoglobulin suppressor of T-cell activation; LPS, lipopolysaccharides; PEC, peritoneal exudates cells.

* Corresponding author at: Physical Sciences, Sunnybrook Research Institute, 2075 Bayview Ave., Room M7-436, Toronto, ON M4N 3M5, Canada.
E-mail address: jean.gariepy@utoronto.ca (J. Gariépy).

[1] These authors have contributed equally to this work and share first authorship.

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2.2. Macrophage and neutrophil collection

Brewer thioglycolate medium (3 %, 4 mL, autoclaved; MilliporeSigma) was injected intraperitoneally into mice to recruit macrophages and neutrophils to the peritoneal cavity. Mice were sacrificed either 1 day or 4 days later and peritoneal exudates cells (PECs) were harvested by lavage with PBS. PECs recovered after 4 days consisted mostly of macrophages, whereas PECs isolated 1 day after thioglycolate injection were predominantly neutrophils. The identity of the PECs was confirmed using flow cytometry with the following antibodies: APC/Cy7 anti-CD45, BV510 anti-CD11b, PE/Cy7 anti-Ly6G, Alexa Fluor 700 anti-Siglec F, mCherry anti-Ly6C, Pacific Bule anti-IA/IE, PerCP/Cy5.5 anti-CD11c, and FITC anti-F4/80 (BioLegend). Binding of recombinant mouse VISTA.COMP [9] was validated by incubating the isolated PECs with biotinylated VISTA.COMP or COMP (produced in house) at 4 °C for 1 h, followed by detection with a PE streptavidin conjugate (BioLegend). Stained PECs were detected using a BD LSR II flow cytometer maintained by The Centre for Flow Cytometry & Scanning Microscopy (CCSM) at SRI. Throughout the study, PECs were maintained at 37 °C and 5 % CO_2 in RPMI 1640 medium (Wisent) supplemented with 10 % heat-inactivated fetal bovine serum (Wisent), 1 % penicillin–streptomycin (Wisent), 0.05 mM 2-mercaptoethanol (MilliporeSigma), and 20 mM HEPES (Wisent).

2.3. RNA sequencing

Neutrophils and macrophages (PECs isolated 1 day or 4 days post-thioglycolate injection, respectively) were stimulated with 1 μg/mL LPS (*Escherichia coli* O111:B4; MilliporeSigma) and 15 μg/mL VISTA.COMP for 2 h. RNA content was isolated from the conditioned macrophages or neutrophils using the RNeasy Mini Kit (Qiagen), treated with DNA-free Kit (Life Technologies), and sent in for RNA sequencing at The Donnelly Sequencing Centre (University of Toronto). This sequencing data had been deposited in the ArrayExpress database at EMBL-EBI (https://www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-10856. Read counts were obtained with RNA Express on Illumina's BaseSpace (https://basespace.illlumina.com), which aligns RNA sequencing reads to reference mouse genome mm10 (GRCm38) using STAR. Differential gene expression was computed using DESeq2 v1.26.0 in R/Bioconductor. Enrichment of gene ontology terms and subgrouping within the biological processes were established based on the extent of experimentally observed up- and down-regulated genes using the algorithm GOseq v1.38.0 in R/Bioconductor. Revigo was used to trim down the list of gene ontology terms. Heatmaps were prepared with edgeR v3.28.1 and gplots v3.0.4 in R/Bioconductor.

For qPCR, RNA contents were isolated from the cells 1 or 2 h after stimulation using TRIzol Reagent (Thermo Fisher Scientific) and reverse-transcribed using high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific). qPCR was performed with the SensiFAST SYBR no-ROX kit (Meridian Bioscience) using gene-specific primers listed in Supplementary Table 1 (Integrated DNA Technologies). For cytokine analysis, supernatants were harvested 3 h after stimulation and the cytokine concentrations were determined by the LegendPlex kit (BioLegend).

2.4. Endotoxemia

LPS (10 mg/kg) and VISTA.COMP (30 mg/kg) were injected intraperitoneally into mice. After 90 min, mice were sacrificed, and blood was collected by cardiac puncture. Serum concentrations of selected cytokines were measured using the LegendPlex kit (BioLegend).

2.5. Binding ELISA

An ELISA-based approach was used to confirm that VISTA did not interact with itself. Briefly, ELISA plate wells (Thermo Fisher Scientific) were coated with murine VISTA.Fc (1 μg/ml; produced in house) and blocked with 0.5 % milk. VISTA.COMP (5 μg/ml) was then added and incubated for 1 h at room temperature. Binding was detected using an anti-his HRP conjugated antibody (MilliporeSigma) and the substrate TMB (3,3′,5,5′-tetramethylbenzidine; Thermo Fisher Scientific). Absorbance readings were recorded at 450 nm.

2.6. Statistics

Each experiment was performed with a minimum of 3 biological replicates (mice). Experiments using PECs were performed by splitting PECs isolated from one mouse into different stimulation groups to allow for paired analysis. Student's *t*-test (2-tail, paired for PEC experiments) was used to test significance between different groups (significance taken at $\alpha = 0.05$). For RNA sequencing results, a gene is considered to be differentially expressed when the adjusted *p*-value (Benjamini-Hochberg method) calculated by DESeq2 is less than 0.05.

3. Results

3.1. VISTA receptor is upregulated by LPS on macrophages and neutrophils

It has been recently demonstrated that during LPS-induced inflammation, VISTA acts as a receptor to reprogram macrophages [4]. In the present study, we investigated instead the role of VISTA as a ligand binding to a VISTA receptor on LPS-activated macrophages. A high-avidity form of the extracellular IgV domain of VISTA (VISTA.COMP) served as our ligand as it behaves as a true VISTA receptor agonist that lacks any Fc-related functions associated with the use of antibodies and VISTA.Fc constructs [9]. Macrophages were obtained as PECs harvested from mice 4 days after thioglycolate injection. The majority of the population was confirmed to be macrophages as defined by their expression of the marker F4/80 (Supplementary Fig. 1). Importantly, VISTA.COMP uniquely binds to F4/80^{++} macrophages after LPS stimulation (Fig. 1A).

In addition to macrophages, neutrophils represent another important cell population involved in LPS response [10]. To our knowledge, there is currently no data available with regard to the influence of exogenous VISTA on neutrophils. Therefore, we proceeded to perform the same experiment on neutrophils. PECs isolated from mice 1 day after thioglycolate injection were used, where the majority of the cells were confirmed to be neutrophils based on their Ly6G expression (Supplementary Fig. 1). As was the case with macrophages, VISTA.COMP binding was observed only on neutrophils (Ly6G^{+} cells) that were stimulated with LPS (Fig. 1B).

3.2. VISTA.COMP downregulates inflammatory responses in LPS-stimulated macrophages

To investigate the effect of engaging the VISTA receptor upregulated by LPS on macrophages (Fig. 1A), PECs harvested 4 days post-thioglycolate injection were stimulated with PBS, LPS (1 μg/mL LPS), LPS + VISTA (1 μg/mL LPS and 15 μg/mL VISTA.COMP), or VISTA (15 μg/mL VISTA.COMP) for 2 h, followed by RNA sequencing. Sets of genes that displayed the largest differences in expression between macrophages stimulated with LPS and those stimulated with LPS + VISTA were then classified into distinct biological processes using the grouping algorithm in GOseq. Specifically, heat maps were constructed where significantly differentially expressed genes (red: high expression; blue: low expression) were grouped under immune-related biological processes and ranked based on the difference in gene expression between macrophages stimulated with LPS and LPS + VISTA (Fig. 2). Notably, genes that were found to be significantly differentially expressed when comparing LPS- to LPS + VISTA-stimulated macrophages were not found to be affected in the absence of LPS stimulation (PBS- versus

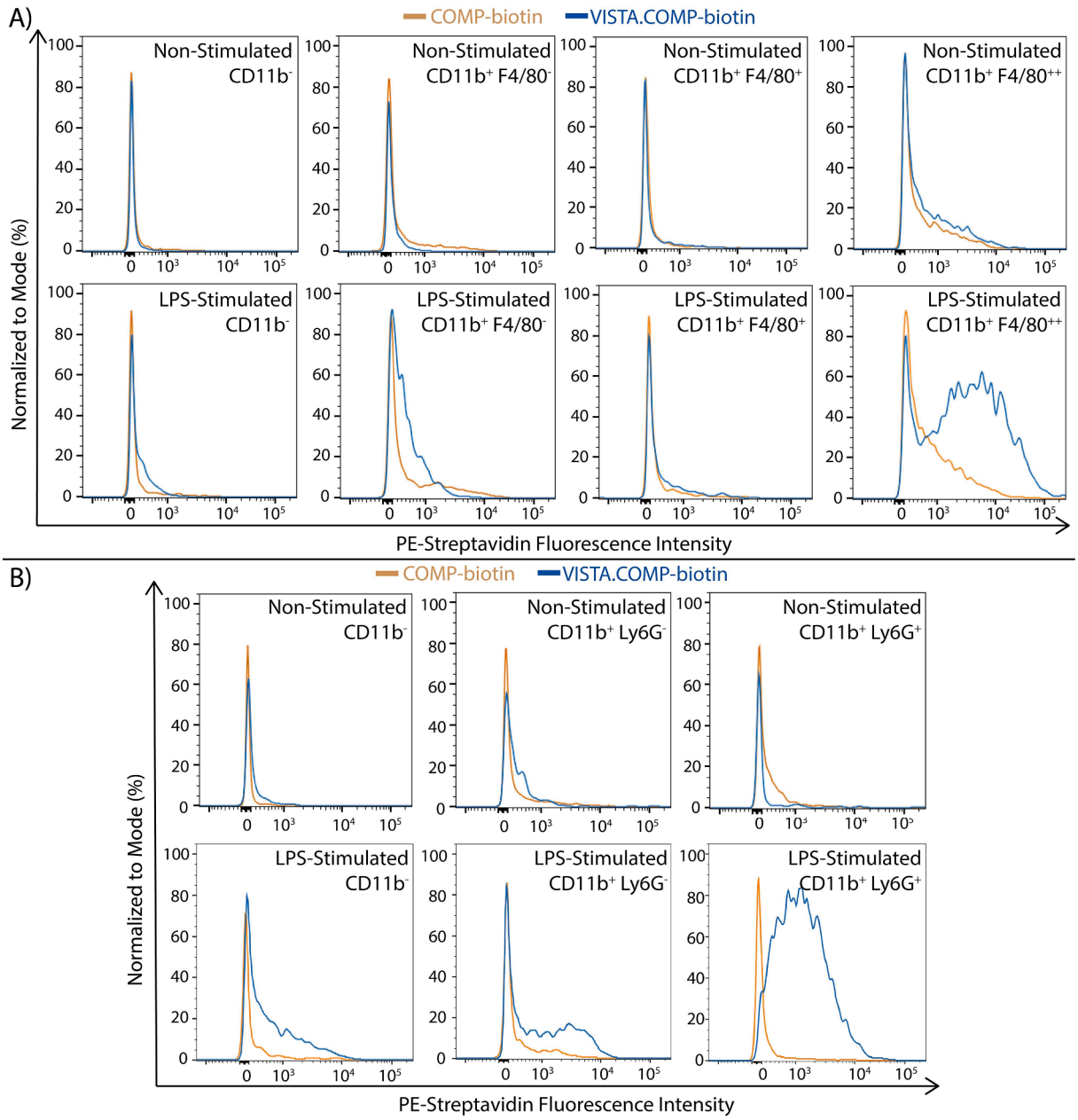

Fig. 1. Binding of VISTA.COMP to LPS-stimulated F4/80^{++} macrophages and Ly6G^{+} neutrophils. Flow cytometry was performed on PECs isolated (A) 4 days or (B) 1 day after thioglycolate injection and stimulated with or without 1 μg/mL LPS. PE-Streptavidin was used to detect the binding of biotinylated VISTA.COMP or isotype control (biotinylated COMP) on gated populations.

VISTA-stimulated). This finding suggests that a VISTA receptor is only present on macrophages following LPS stimulation, supporting the observation in Fig. 1A, in contrast to VISTA, which is similarly expressed on both stimulated and non-stimulated cells [11].

A number of genes were upregulated in LPS-stimulated macrophages by VISTA relative to the macrophages treated with LPS only (Fig. 2A). Importantly, the most represented biological processes associated with this set of upregulated genes were not linked to increased inflammatory or immune responses. Notably, genes associated with tissue remodeling and wound healing pathways were upregulated by VISTA in the LPS-stimulated macrophages, suggesting a reprogramming of these macrophages to a M2 subtype [12]. This conclusion is further supported by the upregulation of *tgfb1* [12]. The upregulation of *lif* also suggests an immunoregulatory subtype of macrophages [13]. Interestingly, although chemotactic genes were downregulated by VISTA in LPS-stimulated macrophages (Fig. 2B), genes involved in general locomotion appear to be upregulated with wound healing and tissue remodeling. In particular, *cxcr4*, a receptor involved in cell migration and known for its role in reprogramming macrophages into a M2 subtype in tumor microenvironments [14], was found to be upregulated in the LPS + VISTA-stimulated macrophages as compared to the LPS-stimulated macrophages.

As shown in Fig. 2B, the genes downregulated in macrophages treated with LPS + VISTA as compared to the LPS only group are involved in immune-related biological processes, such as inflammation and chemotaxis. Importantly, *il6,* a hallmark cytokine produced in response to LPS stimulation [15], is shown to be significantly reduced in the LPS + VISTA-treated group. Furthermore, *ccl24* and *cxcl1*, genes involved in multiple immune- and chemotaxis-related biological processes, were downregulated by VISTA in the LPS-stimulated macrophages. The levels of expression of these specific genes were subsequently verified by qPCR on additional biological replicates and

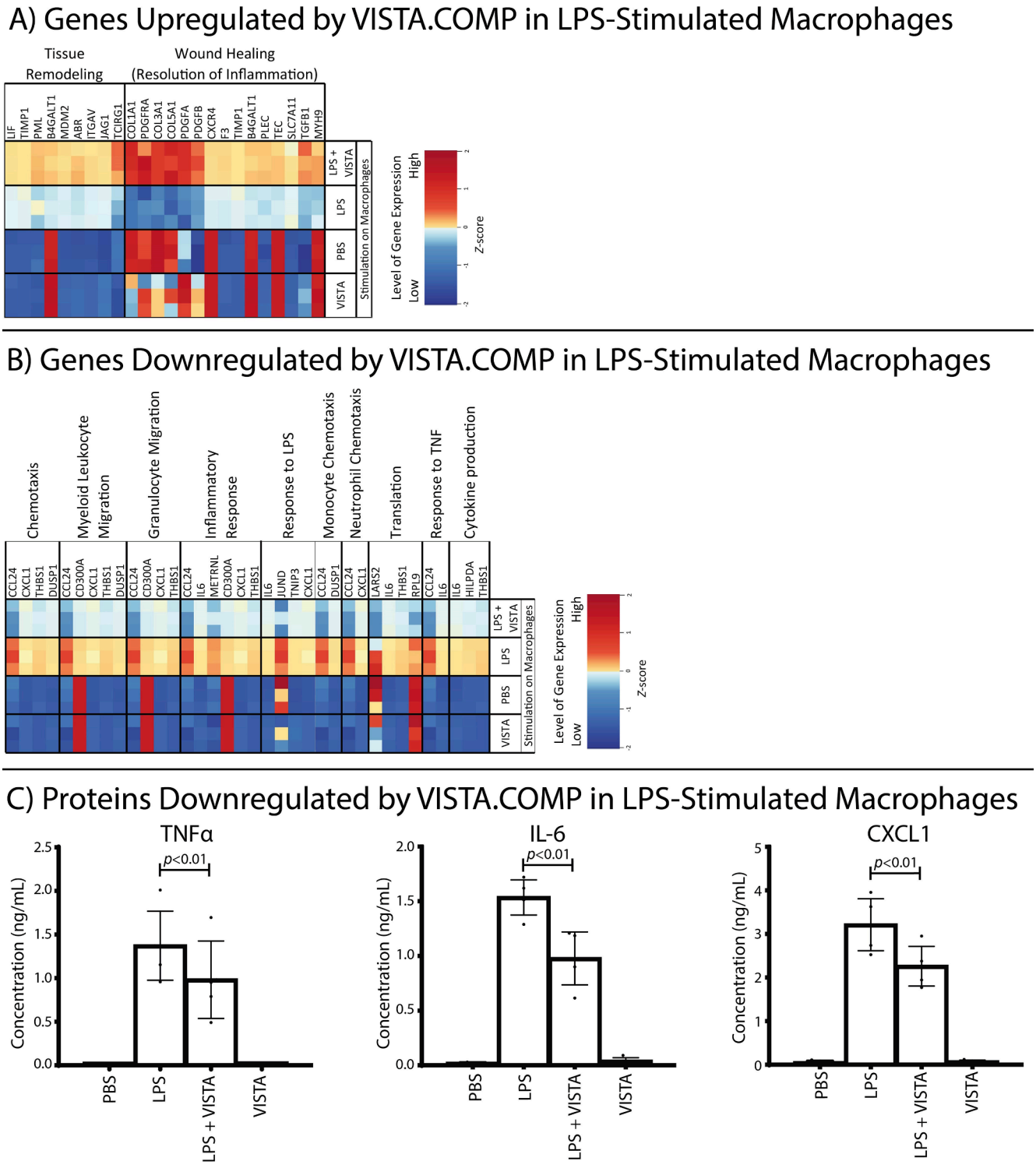

Fig. 2. Differential gene expression between macrophages stimulated *ex vivo* with LPS only or LPS and VISTA.COMP. RNA sequencing was performed on macrophages (PECs isolated 4 days post-thioglycolate injection) stimulated with PBS, LPS (1 μg/mL LPS), LPS + VISTA (1 μg/mL LPS and 15 μg/mL VISTA.COMP), or VISTA (15 μg/mL VISTA.COMP) for 2 h (n = 3 shown as individual squares on heat map). Heat map (red: high; blue: low expression) was shown on selected genes that were significantly (adjusted *p*-value < 0.05) (A) upregulated or (B) downregulated by VISTA.COMP treatment of LPS-stimulated macrophages, grouped under the most significantly altered immune-related biological processes. (C) LegendPlex results showing the concentrations of cytokines produced by macrophages stimulated with PBS, LPS, LPS and VISTA, or VISTA for 3 h. Data is shown as mean ± SD, each dot represents data collected from macrophages isolated from a single mouse.

shown to be significantly lower in the LPS + VISTA-treated group as compared to mice treated with only LPS (*il6* by 30 %; *ccl24* by 27 %; *cxcl1* by 10 %) (Supplementary Fig. 2). Moreover, although a down-regulation in *tnf* was not observed in LPS-stimulated macrophages after 2 h of treatment with VISTA.COMP, it has been shown by others to typically precede *il6* production in a standard response to LPS [15]. Therefore, we re-assessed *tnf* expression in macrophages at an earlier time-point, namely, after 1 h of stimulation. As expected, we found that

tnf expression was significantly reduced (by 56 %) in macrophages stimulated by LPS + VISTA as compared to those stimulated by LPS alone (Supplementary Fig. 2). To confirm that these changes in gene expression did result in changes in cytokine secretion, we measured the concentration of selected cytokines in the supernatant of macrophages, and found that concentrations of these cytokines were indeed lower in macrophages exposed to LPS + VISTA in comparison to macrophages that were exposed to LPS only (TNFα by 28 %; IL-6 by 36 %; CXCL1 by 30 %) (Fig. 2C).

Overall, VISTA.COMP (this study) and anti-VISTA antibody [4] affect the gene expression of LPS-stimulated macrophages in remarkably similar ways. Yet, binding ELISA between VISTA.COMP and VISTA.Fc shows that VISTA does not bind in a homotypic fashion to itself or to a structurally-related protein such as PD-1 (Supplementary Fig. 3), suggesting that signaling through VISTA and its receptor are two redundant but distinct pathways.

3.3. VISTA.COMP downregulates inflammatory responses in LPS-stimulated neutrophils

The effect of engaging the VISTA receptor expressed on LPS-stimulated neutrophils were observed by treating the PECs harvested 1 day after thioglycolate injection with PBS, LPS (1 μg/mL LPS), LPS + VISTA (1 μg/mL LPS and 15 μg/mL VISTA.COMP), or VISTA (15 μg/mL VISTA.COMP) for 2 h and gene expression levels were assessed by RNA sequencing. Heat maps were constructed to highlight genes exhibiting significant differences in expression between LPS- and LPS + VISTA-stimulated neutrophils (red: high expression; blue: low expression; ranked by magnitude of difference between LPS and LPS + VISTA groups). These genes were then regrouped under the most significantly altered immune-related biological processes using the grouping algorithm in GOseq (Fig. 3).

To investigate whether neutrophils responded to VISTA in a similar manner to macrophages, we first confirmed that genes found to be upregulated by VISTA in LPS-stimulated macrophages were also up

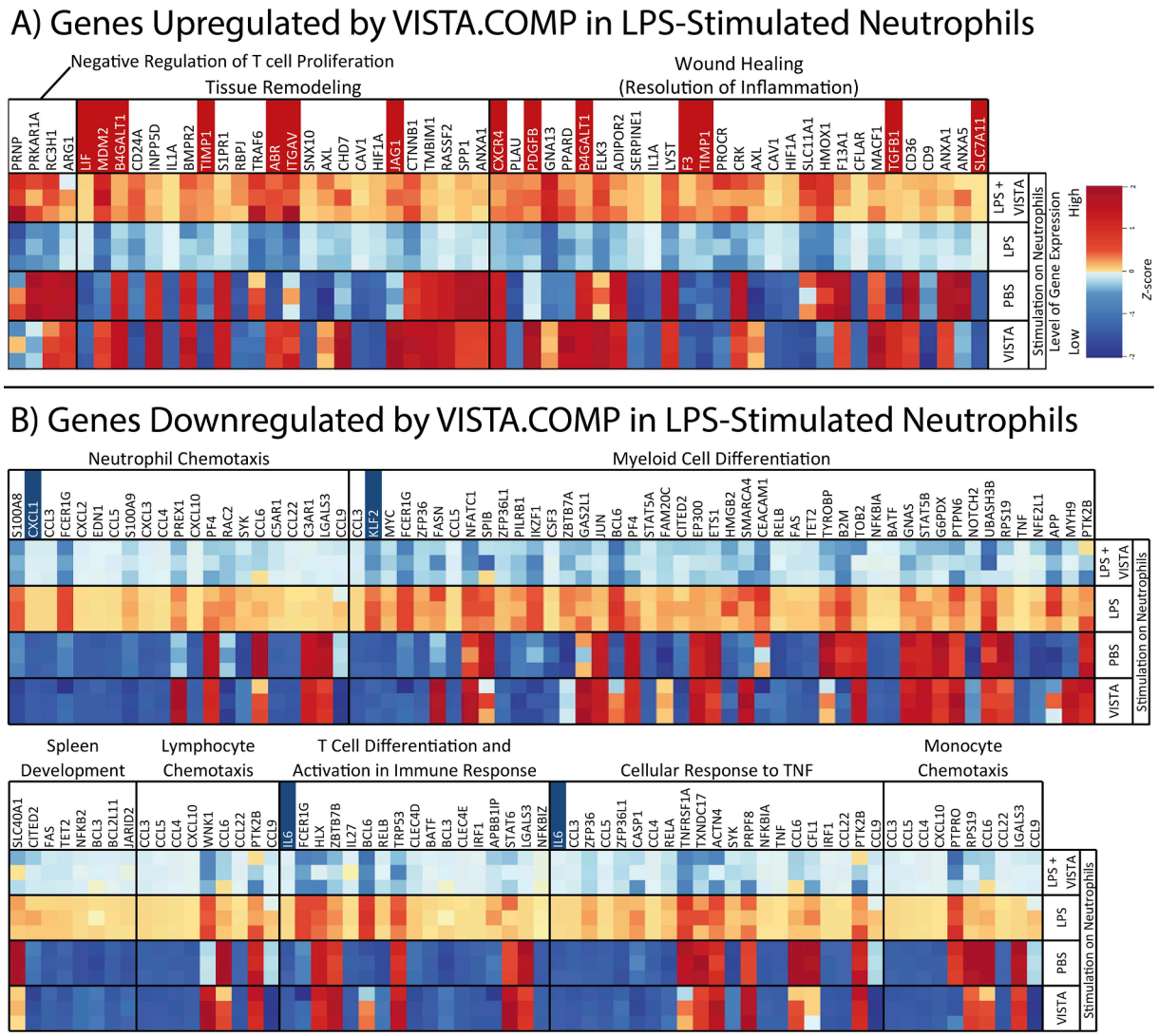

Fig. 3. Differential gene expression between neutrophils stimulated *ex vivo* with LPS only or LPS and VISTA.COMP. RNA sequencing was performed on neutrophils (PECs isolated 1 day after thioglycolate injection) stimulated with PBS, LPS (1 μg/mL LPS), LPS + VISTA (1 μg/mL LPS and 15 μg/mL VISTA.COMP), or VISTA (15 μg/mL VISTA.COMP) for 2 h (n = 3 shown as individual squares on heat map). Heat map (red: high; blue: low expression) was shown on selected genes that were significantly (adjusted *p*-value < 0.05) (A) upregulated or (B) downregulated by VISTA.COMP treatment of LPS-stimulated neutrophils, grouped under the most significantly altered immune-related biological processes. Gene names highlighted in red are genes significantly upregulated by VISTA.COMP in both LPS-stimulated macrophages and neutrophils; gene names highlighted in blue are genes that were significantly downregulated by VISTA.COMP in both LPS-stimulated macrophages and neutrophils.

regulated by VISTA in LPS-stimulated neutrophils. Indeed, genes linked to wound healing and tissue remodeling, along with key mediators of anti-inflammatory subtypes *lif* [13], *tgfb1* [12], and *cxcr4* [14], were upregulated by VISTA in LPS-stimulated neutrophils as well (Fig. 3A).

Furthermore, as projected, the expression of both *il6* and *cxcl1* genes were found to be significantly lower in LPS + VISTA-stimulated neutrophils as compared to LPS-stimulated neutrophils in RNA sequencing (Fig. 3B) and confirmed by qPCR (*il6* by 27 %; *cxcl1* by 38 %) (Supplementary Fig. 4). The expression of *tnf* was also downregulated by VISTA in LPS-stimulated neutrophils at this 2-hour time point (Fig. 3B). However, to be consistent with the time dependency of gene expression profiles observed in macrophages, the level of *tnf* gene expression was also measured after 1 h of stimulation. At this time point, the expression of *tnf* was significantly reduced (by 67 %) in neutrophils stimulated with LPS + VSITA relative to neutrophils stimulated with LPS alone (Supplementary Fig. 4). In addition, several other genes involved in immune-related biological processes, such as the differentiation and chemotaxis of myeloid cells and lymphocytes in general, were found to be significantly downregulated by VISTA in LPS-stimulated neutrophils as well. These results point to a pathway where VISTA signals neutrophils to downregulate their inflammatory response to LPS.

Overall, these results suggest that VISTA.COMP acts similarly on LPS-stimulated neutrophils to promote an immunoregulatory response.

3.4. VISTA.COMP reduces serum TNFα peak in a mouse model of endotoxemia

Given the suppressive activity of VISTA.COMP *ex vivo* in an acute inflammatory setting of LPS stimulation, we assessed its efficacy in the context of an *in vivo* model. Endotoxemia is a well-established *in vivo* model to assess therapeutic response following LPS insult. Since increase in TNFα is a hallmark of sepsis [16], treatments leading to a reduction of serum TNFα levels have been shown to protect mice against LPS-induced lethality [17]. As such, we measured the circulating levels of TNFα in the serum of mice treated with PBS, LPS (10 mg/kg LPS), or LPS + VISTA (10 mg/kg LPS and 30 mg/kg VISTA.COMP). As shown in Fig. 4, serum levels of TNFα were reduced by 49 % in animals treated with LPS + VISTA relative to LPS-treated mice. Furthermore, circulating levels of IL-6 and IL-12p40 were also shown to be reduced in the LPS + VISTA cohort, additionally supporting the suppressive activity of VISTA.COMP [18–19]. Of note, no measurable difference in IL-1β was observed at this time-point (data not shown).

4. Discussion

VISTA has been shown to function both as a ligand and a receptor to suppress *T*-cell functions [1–3,5–6]. More recently, an agonistic anti-VISTA antibody was shown to reprogram macrophages to regulate innate immunity [4]. Yet, the effect of VISTA behaving as a ligand on myeloid cells remains uncharacterized. In this study, we demonstrated that exogenous VISTA acts on a receptor upregulated by LPS on macrophages and neutrophils to promote an anti-inflammatory profile.

Interestingly, our findings parallel the pattern of dampening of pro-inflammatory cytokines observed when LPS-stimulated macrophages were treated with an agonistic anti-VISTA antibody [4]. Specifically, both the presence of exogenous VISTA on LPS-stimulated peritoneal macrophages (Fig. 2; Supplementary Fig. 2) and the use of anti-VISTA antibody on LPS-stimulated bone marrow-derived macrophages [4] reduce the expression of hallmark genes associated with LPS stimulation, *tnf* and *il6* [15]. We also noted a downregulation in CXC chemokine gene signatures and protein secretions that are associated with neutrophil chemotactic properties. Both treatments lead to the upregulation of immunoregulatory genes in LPS-stimulated macrophages, such as *lif* [14]. Although a limitation in the gene ontology analysis lies in the low number of genes affected by VISTA.COMP in LPS-stimulated macrophages (Supplementary Fig. 5), in particular the ones downregulated, the most significantly downregulated pathways (presented in Fig. 2) point towards the immunosuppressive nature of VISTA.COMP. These observations, combined with previous studies showing the similar immunosuppressive effect of both exogenous VISTA [1–3] and agonistic anti-VISTA antibodies [5,6] on T cells, suggest that VISTA acting as a ligand or a receptor evokes two redundant immunosuppressive pathways; potentially representing a self-regulation mechanism to control inflammatory responses. More studies in the context of VISTA as a self-regulation mechanism are warranted.

Interestingly, similar results were obtained when neutrophils were treated with VISTA.COMP. Neutrophils represent the most abundant circulating immune cell and are the first responders to several types of infection [10]. If left uncontrolled, activated neutrophils will contribute to tissue damage during inflammatory processes, positioning them as targets for therapeutic intervention. The ability of exogenously added VISTA.COMP to dampen the inflammatory and chemotactic responses of neutrophils would thus suggest its use to treat acute inflammatory disorders.

As such, the therapeutic potential of VISTA.COMP to target activated myeloid cells in an acute inflammatory setting was tested with an *in vivo* model of endotoxemia, in which macrophages and neutrophils are

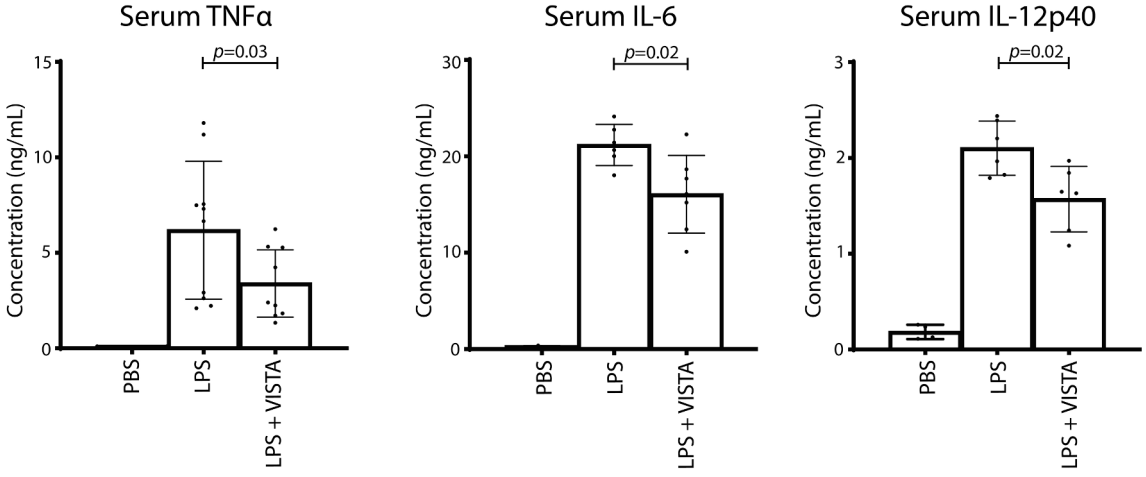

Fig. 4. VISTA.COMP suppressed LPS-stimulated TNFα, IL-6, and IL-12p40 production *in vivo*. Cytokine concentrations in serum were analyzed 90 min after C57BL/6 mice were injected intraperitoneally with PBS, LPS (10 mg/kg LPS), LPS + VISTA (10 mg/kg LPS and 30 mg/kg VISTA.COMP), or VISTA (30 mg/kg VISTA.COMP). Data is shown as mean ± SD, each dot represents a mouse in the experimental group (two trials).

documented to play key roles [10]. In Fig. 4, we showed that exogenously given VISTA.COMP significantly attenuated the increase in the characteristic monophasic spike in serum level of TNFα occurring upon LPS injection [15]. This reduction typically correlates with increased survival rates [17]. Furthermore, circulating levels of IL-6 and Il-12p40 were also shown to be reduced in the VISTA.COMP-treated mice. Similar to our observation, Rossato *et al.* previously reported that the anti-inflammatory cytokine IL-10 mediates the reduction in TNFα, IL-6, and Il-12p40, but not IL-1β [18]. Furthermore, Du and Siram additionally linked the suppression of LPS-induced IL-12p40 to the induction of *tgfb* [19], a gene we found to be upregulated in the LPS + VISTA cohort. Although we cannot definitively conclude that VISTA.COMP will rescue these mice from LPS-induced lethality, these results highlight VISTA.COMP as a potential immunotherapeutic agent to manage inflammatory events *in vivo*. In addition, we had previously shown that VISTA.COMP is capable of downregulating TNFα and IL-6 in ConA-induced hepatitis [9], further supporting the anti-inflammatory therapeutic potential of VISTA.COMP.

Overall, it is evident that more studies are necessary to unravel the exact mechanism by which VISTA, as a ligand, carries out its suppressive activity. One major limiting factor, in this context, is the nature of the VISTA receptor on murine macrophages and neutrophils. Several candidates have been proposed by previous studies [20]. VSIG3 has been identified as a ligand. However, it has not been shown to be expressed by hematopoietic cells, nor did our gene expression studies show VSIG3 to be expressed by resting or activated murine macrophages and neutrophils. Additionally, VISTA has been reported to bind to PSGL-1 in a pH-dependent manner. We did not find an increase in *psgl1* on activated murine macrophages and neutrophils. In addition, we have observed that VISTA functions at neutral pH, as similarly reported by another group [20], suggesting that the interacting partner of VISTA should be present and bind VISTA at neutral pH. Furthermore, Syndecan-2 has more recently been identified as a VISTA-interacting partner on human monocytes [21], but we do not have any supporting evidence to suggest that Syndecan-2 is the receptor in this context. Mass spectrometry following a pull-down with VISTA.COMP of LPS-stimulated RAW264.7 cells also yielded potentially interesting candidates, namely Semaphorin 3A/Neuropillin and Annexin A1 (data not shown). Semaphorin 3A and Neuropillin have been previously reported as binding partners for another B7 family member (B7H4) [22]. With regard to Annexin A1, in addition to being identified in our mass spectrometry analysis, the gene was found to be upregulated following LPS stimulation in our RNAseq analysis. Moreover, its expression is induced upon activation of macrophages and is known to play a role, albeit contextually dependent, in the down-regulation of inflammation [23]. To date, however, we have not been able to definitively prove the direct binding of VISTA to any of these recombinant proteins.

One key observation during this study, as shown in Fig. 1, is that the VISTA receptor is not constitutively expressed on resting macrophages and neutrophils, but is upregulated upon antigen (LPS in this case) stimulation. Preliminary data suggests that the expression of the receptor is not unique to LPS stimulation (i.e. CD14/TLR4 mediated) but rather can be induced by other inflammatory stimuli such as ConA (data not shown). It would be interesting to determine whether other components linked to pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) would trigger similar upregulation of the receptor on myeloid cells. This knowledge may assist in further defining the receptor recognized by VISTA.COMP.

In conclusion, we have demonstrated a redundant cellular pathway where VISTA signals a receptor on myeloid cells to dampen the inflammatory immune response. *In vivo* results from an acute inflammatory model underscore the potential use of exogenous VISTA constructs such as VISTA.COMP as anti-inflammatory therapeutics.

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CRediT authorship contribution statement

Yu-Heng Vivian Ma: Conceptualization, Methodology, Software, Formal analysis, Investigation, Writing – original draft, Visualization. **Amanda Sparkes:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **Shrayasee Saha:** Validation, Investigation. **Jean Gariépy:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2022.104581.

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