Defensin DEFB103 bidirectionally regulates chemokine and cytokine responses to a pro-inflammatory stimulus

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Human β defensin DEFB103 acts as both a stimulant and an attenuator of chemokine and cytokine responses: a dichotomy that is not entirely understood. Our predicted results using an in silico simulation model of dendritic cells and our observed results in human myeloid dendritic cells, show that DEFB103 significantly (p < 0.05) enhanced 6 responses, attenuated 7 responses, and both enhanced/attenuated the CXCL1 and TNF responses to Porphyromonas gingivalis hemagglutinin B (HagB). In murine JAWSII dendritic cells, DEFB103 significantly attenuated, yet rarely enhanced, the Cxcl2, Il6, and Csf3 responses to HagB; and in C57/BL6 mice, DEFB103 significantly enhanced, yet rarely attenuated, the Cxcl1, Csf1, and Csf3 responses. Thus, DEFB103 influences pro-inflammatory activities with the concentration of DEFB103 and order of timing of DEFB103 exposure to dendritic cells, with respect to microbial antigen exposure to cells, being paramount in orchestrating the onset, magnitude, and composition of the chemokine and cytokine response.

Human β defensin 3 (DEFB103) is a small, yet potent antimicrobial host defense peptide with diverse innate immune activities. It is prominent throughout the oronasal cavity and is present in nasal mucus, saliva, and gingival crevicular fluid at concentrations as high as 6.2 μg/ml. Monocytes or dendritic cells treated with DEFB103 alone or with a microbial antigen have both pro-inflammatory and anti-inflammatory activities: a dichotomy that is not entirely well understood, but important given the recent recognition of the role of DEFB103 in the production of cytokines and inflammation associated with mucosal infections, aero-digestive cancers like squamous cell carcinoma of the head and neck, auto-immune and auto-inflammatory diseases, and severe trauma. Furthermore, DEFB103 is overexpressed in individuals with intense inflammation associated with squamous cell carcinoma of the head and neck where it may function as a chemo-attractant to recruit macrophages.

The role of DEFB103 in regulating chemokine and pro-inflammatory cytokine responses to specific pro-inflammatory antigenic exposure is largely unknown, particularly the effects of DEFB103 concentration, like overexpression in squamous cell carcinomas, and order of timing of DEFB103 with respect to antigen exposure to dendritic cells. Compelling evidence clearly suggests that DEFB103 and other α- and β-defensins have pro-inflammatory activities; and chemokines and pro-inflammatory cytokines are upregulated and produced in stimulated cells. Equally compelling evidence suggests that DEFB103 and other α- and β-defensins have opposite and potent anti-inflammatory activities. Human neutrophil peptide α-defensin DEFA1 blocks the release of IL1B, but not TNF, from lipopolysaccharide-activated human monocytes; DEFA1-3 inhibit the secretion of multiple pro-inflammatory cytokines from macrophages; and DEFB103 attenuates the IL6, IL10, CSF2, and TNF responses of human myeloid dendritic cells to Porphyromonas gingivalis hemagglutinin B (HagB).

Influencing events in ‘defensin controlled inflammation’ likely involve extracellular binding of defensins to microbial antigens, defensin-altered binding of antigens to cell surfaces, or defensin-altered signaling.
Here we report the conditions in which DEFB103 bidirectionally regulates the chemokine and cytokine responses of human myeloid dendritic cells to a pro-inflammatory stimulus and demonstrate that the concentration of DEFB103 and order of timing of DEFB103 exposure to cells, with respect to microbial antigen exposure to cells, are paramount in orchestrating the onset, magnitude, and composition of the dendritic cell chemokine and cytokine response.

Results

The predicted influence of DEFB103 on a HagB-induced response in an in silico simulation model of human dendritic cells. HagB stimulates dendritic cells in a TLR4-dependent manner and MyD88 and TRIF are required for their optimal activation and DEFB103 affects the activity of pro-inflammatory pathways associated with MyD88 and TRIF. Therefore to determine the parameters of DEFB103 influencing the HagB-induced responses seen at 16 hours, we utilized a simulation model of dendritic cells that provides a dynamic and transparent view of the dendritic cell physiology in silico. This simulation technology has been used to predict biomarker responses, phenotype responses, and chemokine and cytokine responses in various disease based cell systems including epithelial-based tumor cells, keratinocytes, monocytes, and other immune cells. An approach has enabled design and validation of therapy against rheumatoid arthritis, neuroprotective targets with implications for Parkinson’s therapy, and anti-tumor combination targeted therapy against specific tumor baselines. The predictions of the dendritic cell model for HagB and E. coli 55:B5 endotoxin were tested and prospectively validated by our observed responses with the same triggers. The blinded predicted results and observed responses for HagB and E. coli 55:B5 endotoxin had a high correlation of greater than 80% (Supplemental Table 1). At a 1:10 molar ratio of DEFB103 to HagB, like that used in our prior studies, the dendritic cell model predicted a DEFB103-induced attenuation in expression levels of CCL3, CCL4, CSF2, IL6, IL10, and TNF in HagB-exposed human myeloid dendritic cells compared to that of HagB stimulation alone on the referenced baseline (Figure 1a). This predicted response correlated with the observed attenuated response of chemokines and pro-inflammatory cytokines in our previous work: the attenuated HagB-induced responses after co-DEFB103 treatment in human myeloid dendritic cells (Figure 3, response seen in group 3, Table 1); and our observed results (p < 0.05) for Cxcl2, Il6, and Csf3 with similar trends for Ccl3 and Tnf in murine JAWS II dendritic cells (Figure 4, Table 2).

The model also prospectively predicted that an increase in DEFB103 could account in part for the enhanced DEFB103-induced chemokine and cytokine responses of HagB-exposed human myeloid dendritic cells observed in Figure 3 (e.g., the responses seen in Groups 2, 4, and 5). For example, with a 10,000:1 molar ratio of DEFB103 to HagB, the dendritic cell model predicted that DEFB103 would enhance the expression of CCL3, CCL4, CSF2, IL6, IL10, and TNF in HagB-exposed human myeloid dendritic cells compared to that of HagB stimulation alone on the referenced baseline (Figure 1b). This phenomenon was observed (Table 1) and results using 20 μM DEFB103 resulted in high concentrations of CCL3, CCL4, and TNF.

The observed influence of DEFB103 on a HagB-induced human myeloid dendritic cell response. HagB is a potent pro-inflammatory stimulus. HagB induced the production of chemokines and cytokines comparable to that of E. coli 55:B5 endotoxin (Supplemental Table 2, Figure 2). To establish that HagB was a comprehensive pro-inflammatory stimulus for human myeloid dendritic cells, LGM-3 containing 2.0, 0.2, 0.02, and 0.002 μM HagB; 2.0, 0.2, 0.02, and 0.002 μM heated HagB (ΔHagB); 847.0, 79.0, 8.1, and 0.9 ng/ml endotoxin; and 0.01 M PBS, pH 7.2 were added to adhered human myeloid dendritic cells and incubated for 0, 2, 4, 8, and 16 hours. At 16 hours post treatment, 0.02, 0.2, and 2.0 μM HagB induced a robust chemokine and pro-inflammatory cytokine response in 21 chemokines and cytokines (e.g., >100 pg/ml), a moderate response in 16 chemokines and cytokines (e.g., 11–100 pg/ml); and a negligible response in 13 chemokines and cytokines (e.g., <10 pg/ml).

HagB-induced responses were similar in magnitude to that induced by 847.0, 79.0, 8.1, and 0.9 ng/ml E. coli 55:B5 endotoxin, and not due to any trace amounts of endotoxin that may have carried over from the Ni-NTA purification process and accompanied HagB in the inocula. This was confirmed by heating and denaturing HagB: ΔHagB induced significantly lower chemokine and cytokine responses (p < 0.05; Supplemental Table 2, Figure 2). Furthermore, there were no significant differences (p > 0.05) in the endotoxin content in cell culture inocula and these results are listed at the top of Supplemental Tables 2-4. Therefore, 0.02 μM HagB was selected as the stimulatory dose: the dendritic cell response to 0.02 μM HagB was both high enough and low enough to show chemokine and cytokine fluctuations and the response to 0.02 μM ΔHagB was negligible and significantly lower (Supplemental Table 2).

Influence of DEFB103 on the HagB-induced response. We observed that DEFB103 induced a unique bidirectional modulation of the chemokine and pro-inflammatory cytokine response of HagB-exposed human myeloid dendritic cells. By 2 hours, the DEFB103-induced responses of 42 chemokine and cytokines of HagB-exposed human myeloid dendritic cells start to separate into 3 distinct groups: 18 chemokine and cytokine responses that are low or negligible; 18 responses that are neither enhanced nor attenuated after pre-, co-, or post-DEFB103 treatment (p > 0.05); and 6 responses that are enhanced after pre- or post-DEFB103 treatment (p < 0.05). By 16 hours, the DEFB103-induced chemokine and cytokine responses of HagB-exposed cells separate into 5 distinct groups: 14 chemokine and cytokine responses that are low or negligible; 13 elevated responses that are neither enhanced nor attenuated after pre-, co-, or post-DEFB103 treatment (p > 0.05); 7 responses that are attenuated after co-DEFB103 treatment (p < 0.05), and 2 responses that are bidirectionally pliable and are both enhanced and attenuated after pre-, co-, or post-DEFB103 treatment (p < 0.05) (Supplemental Table 3, Figure 3). This latter group includes CXCL1 and TNF.

In another series of experiments, we assessed the effect of DEFB103 and HagB concentration on the temporal exposure of DEFB103 to HagB-exposed human myeloid dendritic cells. DEFB103 (0.2, 2.0, or 20.0 μM) was given to human myeloid dendritic cells pre- (1 hour before), co-, or post- (1 hour after) HagB treatment (0.02 or 0.2 μM). DEFB103 had a concentration dependent ability to both attenuate and enhance the chemokine and pro-inflammatory cytokine response (Table 1). Timing was still important. CCL3, CCL4, and TNF responses to 0.02 μM HagB were both enhanced and attenuated when 0.2 and 2.0 μM DEFB103 was given pre-/post- or co-HagB exposure, respectively.

DEFB103 attenuated the HagB-induced chemokine and cytokine responses of murine JAWS II dendritic cells. In JAWS II dendritic cells, only one aspect of the bidirectional mechanism was seen. DEFB103 significantly attenuated, yet rarely enhanced, the chemokine and pro-inflammatory cytokine response to HagB (Supplemental Table 4, Figure 4, Table 2). Seven of ten chemokines and pro-inflammatory cytokines were detected 16 hours after treatment to 0.02 μM HagB (Table 2). DEFB103 did not enhance any chemokine or pro-inflammatory cytokine responses to HagB. DEFB103 attenuated the HagB-induced response only after JAWS II dendritic cells were exposed to a mixture of DEFB103 and HagB that was prepared by adding DEFB103 to HagB in tissue culture medium and incubating the mixture at 37°C for 30 minutes (e.g., Group 3,
Figure 1 | The predictive in silico dendritic cell model generated response to Porphyromonas gingivalis hemagglutinin B (HagB). The percentage change mentioned in the figures is with respect to the control reference baseline. (a) The predicted effect of lower over-expression of DEFB103 along with P. gingivalis HagB stimulation on CCL3, CCL4, CSF2, IL6, IL10 and TNF. Compared to HagB stimulation alone on the referenced baseline, the levels of CCL3, CCL4, CSF2, IL6, IL10 and TNF were significantly reduced. (b) The predicted effect of very high over-expression of DEFB103 along with HagB stimulation on CCL3, CCL4, CSF2, IL6, IL10 and TNF. Compared to HagB stimulation alone on the referenced baseline, the levels of CCL3, CCL4, CSF2, IL6, IL10 and TNF had significantly increased. (c) The network schematic of the HagB and DEFB103 regulations in the virtual dendritic cell platform. HagB provides activating signals to CD14 and activin receptors. On binding to CD14 and activin receptors, it stimulates the release of pro-inflammatory cytokines like CCL3, CCL4, CSF2, IL6, IL10 and TNF via activation of MYD88/TRAF6 and SHC1/ERK pathways respectively. Both activin and CD14 signals converge at the activation of MAPK, AKT and NFAT. DEFB103 exerts both an activating and inhibitory effect on dendritic cells. It binds to G-protein coupled receptors CCR2B and CCR6, which stimulate Gi and Gq respectively and activate LYN/SYK/PLC/PKC/DAG pathway converging at the activation of AKT, NFKB and NFAT. DEFB103 also negatively regulates the pro-inflammatory signal by inhibiting CD14, CD40, and IFNG receptors. The dotted red lines in the figure indicate inhibitory links and dotted fluorescent green lines indicate activation links. Downstream effects (activation links) of each transcription factor are shown by unique colored solid lines.
DEFB103 enhanced the HagB-induced chemokine and cytokine responses of mice. In mice, the opposite aspect of the bidirectional mechanisms was seen. DEFB103 significantly enhanced, yet rarely attenuated, the chemokine and pro-inflammatory cytokine response to intranasal administration of HagB (Supplemental Table 5, Figure 5, Table 2). HagB alone induces Ccl2, Ccl3, Ccf3, Cxcl1, Cxcl2, Il6, Tnf, Csf1, Csf2, and Csf3 responses in the nasal wash fluid supernatant (Table 2), all important in the initiation and maintenance of murine oronasal inflammatory responses\(^{30}\). Prior administration of DEFB103 intranasally enhances (\(p < 0.05\)) the HagB-induced Ccl1, Csf3, and Csf1 responses with Ccl3, Ccl4, Cxcl2, Il6, and Tnf showing similar, but not quite significant trends. Furthermore, the DEFB103 enhanced response occurs early (e.g., 2 hours in Cxcl1), becomes robust, and remains sustained throughout the 16 hour response (Figure 5a). At 2 hours post intranasal inoculation, DEFB103 enhances (\(p < 0.05\)) the HagB-induced Cxcl1, Csf3, and Csf1 responses with Ccl3, Ccl4, Cxcl2, Il6, and Tnf showing similar trends (Figure 5a). Negligible chemokine and cytokine responses were induced by administration of 0.01 M PBS or DEFB103 alone.

Discussion
In this study, we sought to identify the conditions in which DEFB103 regulates the chemokine and cytokine responses of human myeloid dendritic cells. Cells were exposed to DEFB103 (0.2, 2.0, or 20.0 \(\mu\)M), HagB (0.02 or 0.2 \(\mu\)M), or 0.01 M PBS, pH 7.2. Cells were also exposed to DEFB103 and then exposed to HagB one hour later (pre-DEFB103 treatment); exposed to a mixture of DEFB103 and HagB (co-DEFB103 treatment); or exposed to HagB and then exposed to DEFB103 one hour later (post-DEFB103 treatment). Overall, the concentration of DEFB103 was important; CCL3, CCL4, and TNF responses to HagB were high at 16 hours. Timing of DEFB103 exposure was also important; CCL3, CCL4, and TNF responses to 0.02 \(\mu\)M HagB were both enhanced and attenuated when 0.2 and 2.0 \(\mu\)M DEFB103 was given pre- and post- or co-HagB exposure, respectively. The mean and standard error (Std Err, \(n = 3\)) of log10 concentrations of CCL3, CCL4, and TNF are listed. Statistical analysis was performed as described in the Methods and means within each chemokine response not connected by the same letter are significantly different (\(p < 0.05\)).

| Table 1 | The concentration and temporal effect of DEFB103 on the HagB-induced CCL3, CCL4, and TNF responses of human myeloid dendritic cells. Cells were exposed to DEFB103 (0.2, 2.0, or 20.0 \(\mu\)M), HagB (0.02 or 0.2 \(\mu\)M), or 0.01 M PBS, pH 7.2. Cells were also exposed to DEFB103 and then exposed to HagB one hour later (pre-DEFB103 treatment); exposed to a mixture of DEFB103 and HagB (co-DEFB103 treatment); or exposed to HagB and then exposed to DEFB103 one hour later (post-DEFB103 treatment). Overall, the concentration of DEFB103 was important; CCL3, CCL4, and TNF responses to HagB were high at 16 hours. Timing of DEFB103 exposure was also important; CCL3, CCL4, and TNF responses to 0.02 \(\mu\)M HagB were both enhanced and attenuated when 0.2 and 2.0 \(\mu\)M DEFB103 was given pre- and post- or co-HagB exposure, respectively. The mean and standard error (Std Err, \(n = 3\)) of log10 concentrations of CCL3, CCL4, and TNF are listed. Statistical analysis was performed as described in the Methods and means within each chemokine response not connected by the same letter are significantly different (\(p < 0.05\)).

| DEFB103 or control treatment | 0.01 M PBS | 0.02 \(\mu\)M HagB | 0.20 \(\mu\)M HagB |
|-------------------------------|------------|----------------|-----------------|
| CCL3 responses                |            |                |
| **0.01 M PBS**                | 1.92 (0.04) g | 2.16 (0.04) e,f | 3.58 (0.04) a   |
| **0.20 \(\mu\)M DEFB103**    | 1.90 (0.04) g | 2.36 (0.04) d,e | 3.60 (0.04) a   |
| Pre-treatment                 |            |                |
| 0.20 \(\mu\)M DEFB103         | 2.31 (0.04) d,e | 2.32 (0.04) d,e | 3.49 (0.04) a,b |
| Co-treatment                  | 1.98 (0.04) f,g | 2.64 (0.04) c   | 3.51 (0.04) a,b |
| Post-treatment                | 2.64 (0.04) c | 3.52 (0.04) a   |
| **2.0 \(\mu\)M DEFB103**     | 3.58 (0.04) a | 3.58 (0.04) a   | 3.62 (0.04) a   |
| Pre-treatment                 |            |                |
| 0.01 M PBS                    | 2.56 (0.03) i | 2.71 (0.03) g,h,i | 3.50 (0.03) a,b |
| Co-treatment                  | 2.81 (0.03) f,g,h | 3.43 (0.03) b,c |
| Post-treatment                | 2.61 (0.03) i | 3.40 (0.03) b,c |
| **2.0 \(\mu\)M DEFB103**     | 2.64 (0.03) i | 2.89 (0.03) e,f | 3.40 (0.03) b,c |
| Pre-treatment                 | 2.80 (0.03) f,g,h | 2.85 (0.03) e,f | 3.34 (0.03) c   |
| 20.0 \(\mu\)M DEFB103         | 3.59 (0.03) a | 3.66 (0.03) a   | 3.08 (0.03) d   |
| Co-treatment                  | 3.66 (0.03) a | 3.66 (0.03) a   | 3.30 (0.03) c   |
| **2.0 \(\mu\)M DEFB103**     | 3.64 (0.03) a | 3.59 (0.03) a   | 3.61 (0.03) a   |
| Pre-treatment                 |            |                |
| 0.01 M PBS                    | 1.49 (0.04) m | 1.81 (0.04) k,l | 3.44 (0.04) a   |
| 20.0 \(\mu\)M DEFB103         | 1.52 (0.04) m | 2.01 (0.04) i,j,k | 3.38 (0.04) a,b |
| Co-treatment                  | 1.59 (0.04) i,m | 2.16 (0.04) i | 3.17 (0.04) b,c,d |
| Post-treatment                | 2.06 (0.04) i,j,k | 2.99 (0.04) d,e,f |
| **2.0 \(\mu\)M DEFB103**     | 1.82 (0.04) j,k | 1.55 (0.04) m | 2.77 (0.04) g   |
| Pre-treatment                 |            |                |
| 2.00 \(\mu\)M DEFB103         | 2.90 (0.04) e,f,g | 2.79 (0.04) f,g | 3.08 (0.04) c,d,e |
| Co-treatment                  | 2.80 (0.04) f,g | 2.80 (0.04) f,g | 2.43 (0.04) h   |
| Post-treatment                | 2.99 (0.04) d,e,f,g | 2.99 (0.04) d,e,f,g | 3.38 (0.04) a,b |

CCL4 responses

TNF responses
dendritic cells. DEFB103 and other α- and β-defensins are known in some experimental designs to enhance the production of chemokines and pro-inflammatory cytokines in stimulated cells8–11, yet in other experimental designs to attenuate the production of chemokines and pro-inflammatory cytokines in stimulated cells12–14. In this study, we developed a simulation model of human dendritic cells to assess both the effects of HagB and DEFB103 on a comprehensive panel of chemokines, cytokines, and other cell products. We used the dendritic cell model to predict the effect of DEFB103 over-expression on cytokine and chemokine expressions in HagB-exposed dendritic cells. In the system, HagB is shown to activate dendritic cells through TLR and activin receptor signaling and DEFB103 is shown to inhibit the activation of dendritic cells by TLR signaling, CD40LG signaling, and IFNG signaling. Along with the inhibitory role of DEFB103, the activation of CCR2B and CCR6 receptors by DEFB103 is also modeled (Figure 1c). The model also prospectively predicted that an increase in DEFB103 could account, in part for the enhanced DEFB103-induced chemokine and cytokine responses of HagB-exposed human myeloid dendritic cells observed in Figure 3 (e.g., responses seen in groups 2, 4, and 5). For example, with a 10,000:1 molar ratio of DEFB103 to HagB, the dendritic cell model predicted that DEFB103 would enhance the expression of CCL3, CCL4, CSF2, IL6, IL10, and TNF in HagB-exposed human myeloid dendritic cells compared to that of HagB stimulation alone on the referenced baseline (Figure 1b). This phenomenon was observed (Table 1) and results using 20 μM DEFB103 resulted in high concentrations of CCL3, CCL4, and TNF.

We validated the model by comparing the predicted and observed responses using HagB and lipopolysaccharide as pro-inflammatory stimuli (Supplemental Table 1). We then used the model to predict the effects of HagB and DEFB103 on the response. Our observed results suggest that DEFB103 can act as both a stimulant and an attenuator of chemokine and pro-inflammatory cytokine responses depending upon the timing of TLR stimulation. Both the concentration of DEFB103 and the temporal treatment of DEFB103 to cells, with respect to microbial antigen treatment of cells, were important in the magnitude of the overall chemokine and cytokine response. Some interesting trends are evident. First, when DEFB103 was mixed with HagB for 30 minutes at 37°C before exposure, co-DEFB103 treatment generally attenuated the HagB-induced chemokine and cytokine responses. When DEFB103 was given one hour before or one hour after exposure, pre-DEFB103 and post-DEFB103 treatment generally enhanced the HagB-induced chemokine and cytokine responses. Second, high 20 μM concentrations of DEFB103 generally enhanced responses of many chemokines and cytokines that typically were attenuated at lower 0.2 or 2.0 μM concentrations of DEFB103. Third, this phenomenon was observed only with select groups of chemokines and cytokines. The responses of many others were not affected. For example, out of 42 chemokines and cytokines assessed in human myeloid dendritic cells (Supplemental Table 3, Figure 3), 0.2 μM DEFB103 only significantly (p < 0.05) enhanced 6 responses, attenuated 7 responses, and both attenuated and enhanced the CXCL1 and TNF responses of human myeloid dendritic cells to 0.02 μM HagB. Fourth, the results in human myeloid dendritic cells, murine JAWSII dendritic cells, and mice had some subtle differences. For example, the bidirectional mechanism was seen in human myeloid dendritic cells but only partially in murine JAWSII dendritic cells and mice. Out of seven chemokines and cytokines assessed in murine JAWSII dendritic cells (Supplemental Table 4, Figure 4), 0.2 μM DEFB103 attenuated the Cxcl2, Il6, and Csf3 responses to 0.02 μM HagB and out of ten chemokines and cytokines assessed in mice (Supplemental Table 5, Figure 5), 0.2 μM DEFB103 significantly enhanced the Cxcl1, Csf3, and Csf1 responses to intranasal administration of 0.2 μM HagB. Thus, DEFB103 significantly alters the onset, magnitude, and composition of the dendritic cell response to a pro-inflammatory stimulus, like HagB, depending upon the concentration and circumstances of exposure.

The exact mechanism for DEFB103 altered chemokine and cytokine responses is not known. The binding of DEFB103 to HagB17,21 may play a partial role by altering the binding of HagB to TLR on the

Figure 2 | Porphyromonas gingivalis hemagglutinin B (HagB) is a potent pro-inflammatory stimulus. Bar graphs show the IL6, IL10, CSF2, TNF, CCL3, and CCL4 responses of human myeloid dendritic cells at 16 hours after exposure to inocula containing 2.0, 0.2, 0.02, and 0.002 μM HagB (Groups 1–4, respectively), 2.0, 0.2, 0.02, and 0.002 μM heated HagB (ΔHagB, Groups 5–8, respectively), 847.0, 79.0, 8.1, and 0.9 ng/ml detected endotoxin (Groups 9–12, respectively), and 0.01 M PBS, pH 7.2 (Group 13). HagB, but not ΔHagB, induced the production of chemokines and cytokines comparable to that of E. coli 55:B5 endotoxin (*, p < 0.05). Statistical analysis was performed as described in the Methods on all log10-transformed chemokine and cytokine concentrations and the significant differences among all groups (p < 0.05) are listed in Supplemental Table 2.
Figure 3 | The influence of DEFB103 on the 0.02 μM Porphyromonas gingivalis hemagglutinin B (HagB)-induced chemokine and cytokine responses of human myeloid dendritic cells. (a) The TNF response of human myeloid dendritic cells exposed for 0, 2, 4, 8, and 16 hours to HagB (Group 1); DEFB103 and then exposed to HagB one hour later (pre-DEFB103 treatment, Group 2); a mixture of DEFB103 and HagB (co-DEFB103 treatment, Group 3); DEFB103 and HagB at the same time but not mixed together like that in Group 3 (Group 4); HagB and then exposed to DEFB103 one hour later (post-DEFB103 treatment, Group 5); DEFB103 (Group 6); or 0.01 M PBS, pH 7.2 (Group 7). (b) The TNF response of human myeloid dendritic cells exposed for 2 hours to Groups 1–7 and (c) the TNF response of human myeloid dendritic cells exposed for 16 hours to Groups 1–7. (d) At 2 hours, 18 of 42 HagB-induced chemokine and cytokine responses were not enhanced or attenuated after pre-, co-, or post-DEFB103 treatment (p > 0.05). At 16 hours, 13 of 42 HagB-induced chemokine and cytokine responses were not enhanced or attenuated after pre-, co-, or post-DEFB103 treatment (p > 0.05). At 16 hours, 13 of 42 HagB-induced chemokine and cytokine responses were not enhanced or attenuated after pre-, co-, or post-DEFB103 treatment (p > 0.05). 6 of 42 responses were enhanced after either pre- or post-DEFB103 treatment (p < 0.05); 7 of 42 responses were attenuated after co-DEFB103 treatment (p < 0.05); and 2 of 42 responses were both enhanced and attenuated after pre-, co-, or post-DEFB103 treatment (p < 0.05). Statistical analysis was performed as described in the Methods on all log10-transformed chemokine and cytokine concentrations and the significant differences among all groups (p < 0.05) are listed in Supplemental Table 3.
The ability of DEFB103 to bidirectionally modulate chemokine and cytokine responses of murine JAWSII dendritic cells. (a) The Cxcl2 response of JAWSII dendritic cells exposed for 0, 2, 4, 8, and 16 hours to HagB (Group 1); DEFB103 and then exposed to HagB one hour later (pre-DEFB103 treatment, Group 2); a mixture of DEFB103 and HagB (co-DEFB103 treatment, Group 3); DEFB103 and HagB at the same time but not mixed together like that in Group 3 (Group 4); HagB and then exposed to DEFB103 one hour later (post-DEFB103 treatment, Group 5); DEFB103 (Group 6); or 0.01 M PBS, pH 7.2 (Group 7). (b) The Cxcl2 response of JAWSII dendritic cells exposed for 2 hours to Groups 1–7 and (c) the Cxcl2 response of JAWSII dendritic cells exposed for 16 hours to Groups 1–7. At 16 hours, 0.2 μM DEFB103 significantly attenuated the 0.02 μM HagB-induced Cxcl2 response (*, p < 0.05, Group 3). Statistical analysis was performed as described in the Methods on all log10-transformed chemokine and cytokine concentrations and the significant differences among all groups (p < 0.05) are listed in Supplemental Table 4.

dendritic cell surface partially retarding the activation of underlying MAPK pathways leading to the attenuated HagB-induced response. However, when administered separately and in differing order to dendritic cells, DEFB103 and HagB may synergistically activate similar pathways and exaggerate a HagB-induced response stronger than that seen by HagB alone. Finally, it is also possible that the ability of DEFB103 to rapidly enter cells also plays a part. The trend in that seen by HagB alone. Finally, it is also possible that the ability of DEFB103 to rapidly enter cells21 also plays a part. The trend in that seen by HagB alone. Finally, it is also possible that the ability of DEFB103 to rapidly enter cells also plays a part. The trend in that seen by HagB alone. Finally, it is also possible that the ability of DEFB103 to rapidly enter cells also plays a part. The trend in that seen by HagB alone. 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other infectious diseases24–29. The dendritic cell model was assembled from published Walkersville, MD) in triplicate using Solutions and inocula of triggers etc. statistical variation in the results of the kinetic based, deterministic, simulation model. biomarkers analyzed include CCL3, CCL4, CSF2, IL6, IL10 and TNF. There is no analyzed compared to HagB stimulation alone on the referenced baseline. The key HagB was introduced with a concentration of 0.001 uM along with very high (10000 referenced baseline. A second study was carried out in the control system wherein here against HagB and lipopolysaccharide (Supplemental Table 1). Simulation retrospectively validated against an extensive set of simulation engine created by Cellworks Group Inc., was used to simulate the dendritic pathways FAS and TNFSF10. The time-dependent changes in the fluxes of the includes different chemokine receptors; d) signaling pathways related to maturation of dendritic cells, which include tumor necrosis factor family members and activation of dendritic cells, which include FC receptors, Toll-like receptors (TLRs) etc.; b) factors associated with the include factors associated with the migration of dendritic cells, which adhesion of dendritic cells including integrin receptors ITGAL, ICAM1, CD44 and CD58, and CD209; c) factors associated with the migration of dendritic cells, which includes different chemokine receptors; d) signaling pathways related to maturation and activation of dendritic cells, which include tumor necrosis factor family members comprising TNF, LTA, TNFSF14, TNFSF18, CD40LG, interleukins IL12, IL4, IL1B, IL2, IFNA1 and IFNG, growth factors EGF and CSF2, and others (CLRC7A, INHBE, etc.); e) inhibitory pathways VEGFA, IL10, IL6 and TGFA; f) kinases and transcription factors associated with dendritic cell physiology; and g) apoptotic pathways FAS and TNFSF10. The time-dependent changes in the fluxes of the constituent signaling pathways were modeled utilizing modified ordinary differential equations (ODEs) and mass action kinetics. A proprietary technology platform and simulation engine created by Cellworks Group Inc., was used to simulate the dendritic cell model with ODEs solved by the Radau method. The model was prospectively and retrospectively validated against an extensive set of in vitro and in vivo studies and here against HagB and lipopolysaccharide (Supplemental Table 1). Simulation protocol: in the dendritic cell model, HagB was introduced with a concentration of 0.001 uM along with a 10-fold over-expression of DEFB103 and the effect on downstream markers were analyzed compared to HagB stimulation alone on the referenced baseline. A second study was carried out in the control system wherein HagB was introduced with a concentration of 0.001 uM along with very high (10000 fold) over-expression of DEFB103 and the effect on downstream markers were analyzed compared to HagB stimulation alone on the referenced baseline. The key biomarkers analyzed include CCL3, CCL4, CSF2, IL6, IL10 and TNF. There is no statistical variation in the results of the kinetic based, deterministic, simulation model. However, there can be variations with varying the simulation protocols — i.e., strength of triggers etc.

## Solutions and inocula.

Weight per volume endotoxin content was determined on all complete solubilization. Weight per volume solutions of 1000.0, 100.0, 10.0, and 0.00007 ng endotoxin/µg (n = 3 tests). E. coli 055:BS endotoxin (Lonza Walkersville, Inc., Walkersville, MD) was suspended in pyrogen-free water and heated at 56°C for 30 minutes to facilitate complete solubilization. Weight per volume solutions of 1000.0, 1000.0, 10.0, and 1.0 mg/ml endotoxin contained 847.0, 79.0, 8.1, and 0.9 mg/ml detected endotoxin (QCL-1000, Lonza Walkersville, Inc., Walkersville, MD). HagB is a 49 kDa pro-inflammatory protein and was prepared by cloning hag of \( P. gingivalis \) (1.4 kb) into the vector pQE31 (QIAGEN Inc., Valencia, CA); expressing HagB in \( E. coli \) M15(DEP4)/pQE-31-TX1; and isolating HagB from \( E. coli \) lysate supernatants with a Ni-NTA spin column (HisPur, Pierce Biotechnology, Rockford, IL)\(^{a,b}\). Bound HagB was eluted with 0.25 M imidazole in 0.01 M Tris, 0.5 M NaCl, \( \text{pH} 7.4 \). The purity of HagB was verified by SDS-PAGE and contained 0.014 ng endotoxin/µg HagB. An aliquot of HagB (called AHBg) was inactivated by heating at 121°C for 15 minutes.

## Cell Lines and media.

**Human myeloid dendritic cells.** Human myeloid dendritic cells were prepared as previously described\(^{30}\) from human peripheral blood mononuclear cells (Lonza Walkersville, Inc., Walkersville, MD). Commercially derived human myeloid dendritic cells were also used (Lonza Walkersville, Inc., Walkersville, MD). Cells were grown in Lymphocyte Growth Media-3 (LGM-3, Lonza Walkersville, Inc., Walkersville, MD), a serum-free, complete medium containing 0.053 ± 0.001 (Std Err, n = 3 tests) ng/ml endotoxin; counted to determine the total and viable cell concentrations, the latter using immunofluorescence in the presence of 25 ng/ml propidium iodide to detect dead cells; and adjusted to contain 1 × 10\(^6\) viable cells per ml. Cells were plated at a density of 2.6 × 10\(^6\) viable cells per cm\(^2\) and allowed to attach

### Table 1 | The influence of DEFB103 on the 0.02 µM Porphyromonas gingivalis hemagglutinin B (HagB)-induced chemokine and cytokine responses of murine JAWSII dendritic cells and mice. In JAWS II dendritic cells, DEFB103 significantly attenuated HagB-induced Cxcl2, Il6, and CsF3 responses (e.g., Group 1 vs. Group 3, p < 0.05); Ccl3 and Tnf responses showed similar attenuated response trends but were not significantly different and Ccl2 and Ccl4 responses were not attenuated. In mice, DEFB103 significantly enhanced the HagB-induced Cxcl1 and CsF3 responses (e.g., Group 1 vs. Group 2, p < 0.05) and HagB-induced CsF2 response (e.g., Group 1 vs. Group 4, p < 0.05). In JAWS II dendritic cells, statistical analysis was performed as described in the Methods on all log\(_{10}\) transformed chemokine and cytokine concentrations and the significant differences among all groups are listed in Supplemental Table 4. In mice, statistical analysis was performed on the mean and standard error (Std Err, n = 9) of log\(_{10}\) concentrations of chemokines and cytokines in nasal wash fluid supernatants as described in the Methods at 0, 2, 4, 8, and 16 hours for Cxcl1, Il6, and CsF3 and on data at 2 and 16 hours for Ccl2, Ccl3, Ccl4, Cxcl2, TNFα, CsF2, and CsF1. The significant differences among all groups are listed in Supplemental Table 5.

| Chemokine or cytokine | JAWS II dendritic cells | Differences in responses at 2 hours (p < 0.05) | Differences in responses at 16 hours (p < 0.05) |
|-----------------------|------------------------|-----------------------------------------------|-----------------------------------------------|
|                       | Range (pg/ml, 3 experiments, n = 6) | Pre-DEFB103 enhancement Grp 1 vs. 2 | Co-DEFB103 attenuation Grp 1 vs. 3 | Post-DEFB103 enhancement Grp 1 vs. 5 | Range (pg/ml, 3 experiments, n = 9) | Pre-DEFB103 enhancement Grp 1 vs. 2 | Co-DEFB103 attenuation Grp 1 vs. 3 | Post-DEFB103 enhancement Grp 1 vs. 5 |
| Ccl2                  | 2–39                   | No                                                      | No                                                      | No                                                      | 2–44                                                | No                                                      | No                                                      | No                                                      |
| Ccl3                  | 27–123                 | No                                                      | No                                                      | No                                                      | 45–264                                              | No                                                      | No                                                      | No                                                      |
| Ccl4                  | 1–66                   | No                                                      | No                                                      | No                                                      | 2–66                                                | No                                                      | No                                                      | No                                                      |
| Cxcl2                 | 376–2,240              | No                                                      | No                                                      | No                                                      | 347–10,500                                          | No                                                      | Yes                                                     | No                                                      |
| Il6                   | 18–386                 | No                                                      | No                                                      | No                                                      | 24–673                                              | No                                                      | No                                                      | Yes                                                     |
| Tnf                   | 4–70                   | No                                                      | No                                                      | No                                                      | 6–69                                                | No                                                      | No                                                      | Yes                                                     |
| CsF3                  | 6–41                   | No                                                      | No                                                      | No                                                      | 10–62                                               | No                                                      | No                                                      | Yes                                                     |

| Mice                  | Range (pg/ml, 3 experiments, n = 9) | Pre-DEFB103 enhancement Grp 1 vs. 2 | Co-DEFB103 attenuation Grp 1 vs. 3 | Post-DEFB103 enhancement Grp 1 vs. 4 | Range (pg/ml, 3 experiments, n = 9) | Pre-DEFB103 enhancement Grp 1 vs. 2 | Co-DEFB103 attenuation Grp 1 vs. 3 | Post-DEFB103 enhancement Grp 1 vs. 4 |
|-----------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Ccl2                  | 2–18                                                 | No                                                      | No                                                      | No                                                      | 15–30                                               | No                                                      | No                                                      | No                                                      |
| Ccl3                  | 2–19                                                 | No                                                      | No                                                      | No                                                      | 20–178                                              | No                                                      | No                                                      | No                                                      |
| Ccl4                  | 2–22                                                 | No                                                      | No                                                      | No                                                      | 14–116                                              | No                                                      | No                                                      | No                                                      |
| Cxcl1                 | 163–5,120                                           | Yes                                                     | No                                                      | No                                                      | 58–4,530                                            | Yes                                                     | No                                                      | No                                                      |
| Cxcl2                 | 15–2,750                                             | No                                                      | No                                                      | No                                                      | 13–1,360                                            | No                                                      | No                                                      | No                                                      |
| Il6                   | 15–12,400                                            | No                                                      | No                                                      | No                                                      | 11–1,240                                            | No                                                      | No                                                      | No                                                      |
| Tnf                   | 6–17                                                 | No                                                      | No                                                      | No                                                      | 7–23                                                | No                                                      | No                                                      | No                                                      |
| CsF3                  | 290–11,000                                           | No                                                      | No                                                      | No                                                      | 161–11,900                                          | No                                                      | No                                                      | No                                                      |
| CsF2                  | 36–77                                                | No                                                      | No                                                      | No                                                      | 2–70                                                | No                                                      | No                                                      | No                                                      |
| CsF1                  | 3–1,640                                              | No                                                      | No                                                      | No                                                      | 13–319                                              | No                                                      | No                                                      | No                                                      |
for 2 hours. Media and non-adherent cells were removed by aspiration just before adding inocula.

Murine JAWSII dendritic cells. Murine JAWSII dendritic cells (CRL-1904, ATCC, Manassas, VA) were also used, similar to that of other temporal studies of this kind. These cells are an immortalized cell line derived from the bone marrow of C57BL/6 mice and have been used to show temporal enhancement and attenuation of synthetic surfactant protein-A derived peptide on lipopolysaccharide-induced release of TNFα. They were grown in Minimum Essential Medium Alpha Medium with 1% L-glutamine, 20% fetal bovine serum, 5 ng/ml Csf2, and 50 μg/ml gentamicin sulfate. This media contained 0.040 ± 0.003 (Std Err, n = 3 tests) ng/ml endotoxin. Cells were counted to determine the total and viable cell concentrations, adjusted to contain 1 × 10⁷ viable cells per ml, and plated at a density of 2.6 × 10⁵ viable cells per cm².

Dendritic cell response to HagB and DEFB103. In the first series of experiments, human myeloid dendritic cells were first exposed to HagB to i) establish an exposure dose capable of inducing a comprehensive chemokine and pro-inflammatory cytokine response, ii) establish that HagB and not endotoxin contamination was responsible for the pro-inflammatory response, and iii) validate the virtual, integrated dendritic cell model. In Groups 1–4, cells were exposed to 2.0, 0.2, 0.02, and 0.002 μM HagB (Supplemental Table 2, Figure 2). In Groups 5–8, cells were exposed to 2.0, 0.2, 0.02, and 0.002 μM AFagB. In Groups 9–12, cells were exposed to 847.0, 79.0, 8.1, and 0.9 ng/ml E. coli 55:B5 endotoxin. In Group 13, cells were exposed to 0.01 M PBS, pH 7.2. At 0, 2, 4, 8, and 16 hour time points, 300 μl culture media was removed for the determination of chemokines and cytokines and 300 μl of LGM-3 was added back to each flask.

In the second series of experiments, human myeloid dendritic cells and murine JAWSII dendritic cells were then exposed to DEFB103, HagB, and 0.01 M PBS, pH 7.2 to assess the effect of DEFB103 on the HagB-induced chemokine and cytokine response (Supplemental Table 3, Figure 3). In Group 1, cells were exposed to HagB. In Group 2, cells were first exposed to DEFB103 and then exposed to HagB one hour later (pre-DEFB103 treatment). In Group 3, cells were exposed to a mixture of DEFB103 and HagB (co-DEFB103 treatment). The mixture was prepared by adding DEFB103 to HagB in LGM-3 and incubating the mixture at 37°C for 30 minutes. In Group 4, cells were exposed to DEFB103 and HagB at the same time but they were not mixed together like that in Group 3. In Group 5, cells were first exposed to HagB and then exposed to DEFB103 one hour later (post-DEFB103 treatment). In Group 6, cells were exposed to DEFB103. In Group 7, cells were exposed to 0.01 M PBS, pH 7.2. At 0, 2, 4, 8, and 16 hours, post-inoculation, 300 μl tissue culture media was removed and 300 μl of tissue culture media was added back to each flask.

Murine response to HagB and DEFB103. Mice are good models to assess the in vivo effects of DEFB103 on HagB-induced responses: HagB induces a chemokine and pro-inflammatory cytokine response and mouse defensin 14 is the orthologue of DEFB103β2. In this study, 90 adult female C57BL/6 mice (5–7 months of age; Charles River Breeding Laboratories, Inc., Willingham, MA) were used and housed in The University of Iowa Medical Laboratory rodent facility. The facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International; is a registered research facility with the United States Department of Agriculture (no. 42-R-0004); and has a Public Health Services Animal Welfare Assurance (A3021-01) on file. All mice were handled humanely according to The University of Iowa Animal Care and Use protocol number 1005104.

For each experiment, 15 mice (3 mice per each of 5 time periods) were sedated by a brief 30 second exposure to an oxygen/carbon dioxide inhalation mixture and 10 μl of inulin containing 0.2 μM HagB, 0.1 μM DEFB103, or 0.01 M PBS, pH 7.2 was dispensed with a micropipette into the nares (5 μl/mouse). All mice were maintained in sternal recumbency until awake. In Group 1, mice were exposed to HagB only. In Group 2, mice were first exposed to DEFB103 and then exposed to HagB one hour later. In Group 3, mice were exposed to a mixture of DEFB103 and HagB. The mixture was prepared by adding DEFB103 to HagB in 0.01 M PBS, pH 7.2 and incubating the mixture at 37°C for 30 minutes. In Group 4, mice were first exposed to HagB and then exposed to DEFB103 one hour later. In Group 5, mice were exposed to DEFB103. In Group 6, mice were exposed to 0.01 M PBS, pH 7.2. At 0, 2, 4, 8, and 16 hours, post-inoculation, mice were euthanized. 200 μl nasal wash fluid was collected and centrifuged to pellet the cells and debris. The nasal wash fluid supernatant was removed and stored at −80°C.

Determination of chemokines and cytokines. The concentrations of chemokines and cytokines in 25 μl dendritic cell culture supernatants or 25 μl mouse nasal wash fluid supernatants were determined with commercial multiplexed fluorescent bead-based immunoassays (EMD Millipore, Billerica, MA) in the Luminex 100 IS Instrument (Luminex, Austin, TX) as previously described in detail for dendritic cell culture supernatants and mouse nasal wash fluid supernatants. Chemokine and cytokine standards ranged from 3.2 to 10,000 pg/ml, and concentrations of chemokines and cytokines in each unknown sample were interpolated from plotted curves of the known standards vs. their median fluorescent intensities. The
HUGO Gene Nomenclature for chemokines, cytokines, and defensins (http://www.genenames.org/) was used throughout the manuscript.

**Statistical analysis.** A log10-transformation was applied to all the chemokine and cytokine concentrations detected in the tissue culture supernatants and mouse nasal wash supernatants. The log transformation attenuates the positive skew in the distributions of the chemokine and cytokine concentrations and makes the normality assumption more defensible. For the mouse nasal wash fluid supernatants, a two-way mixed effects Analysis of Variance (ANOVA) was fit to the log-transformed concentrations, where the two fixed factors were group and time. Pairwise analogus two-way fixed effects ANOVA was fit to the log-transformed concentrations, where again, the two fixed factors were group and time. Pairwise group comparisons were conducted using the method of Tukey’s Honest Significant Differences (HSD). A 0.05 level was used to determine statistically significant differences. All analyses were conducted using JMP (Version 10.0, SAS, Cary, NC).

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**Author contributions**

K.A.B. designed the experiments, analyzed the data, and wrote the manuscript. S.V., S.R., S.A.P. and R.V. developed the virtual integrative dendritic cell model (Supplemental Table 1 and Figures 1a, b, c and d) and S.V., using this model, assessed the activation pathways and virtual outcome of dendritic cells treated with HagB, endotoxin, and DEFB103 (Supplemental Tables 1 and 2 and Figures 1a, 1b, 1c, and 2). L.A.M. and M.R. performed the work with K.A.B. in Supplemental Table 2 and Figure 2. L.E.H. performed the work with human myeloid dendritic cells and JAWSIIdendritic cells (Supplemental Tables 3 and 4 and Figures 3 and 4). K.K. performed the work with mice in Supplemental Table 5 and Figure 5. E.N.R. performed the work with K.A.B. in Table 1. A.F.P. provided the HagB, C. coli MG1655[pREP4QE]-3-TX1 expressing HagB, and helped troubleshoot the HagB purification process. J.E.C. supervised and assisted with the statistical analysis of the study.

**Additional information**

Competing financial interests: The authors declare no competing financial interests.

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