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**Bright expression of CD91 identifies highly activated human dendritic cells that can be expanded by defensins.**

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**Short title:** defensin-induced CD91<sup>bright</sup> activated DCs

**Key words:** dendritic cells, CD91, defensins, Human Neutrophil Peptide-1, Human Beta Defensin-1

**Abbreviations:** Heat Shock Proteins: HSP; Fluorescence minus one: FMO; human immunodeficiency virus type-1: HIV-1; lipopolysaccharide: LPS; monocyte-derived dendritic cells: moDCs; peripheral blood mononuclear cells: PBMCs; recombinant human granulocyte–macrophage colony-stimulating factor: rhGM-CSF; recombinant Human Beta Defensin-1: rHBD-1; recombinant Human Neutrophil Peptide-1: rHNP-1.

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**Abstract**

CD91 is a scavenger receptor expressed by different immune cells and its ligands defensins have been demonstrated to contribute to immune responses against infections and tumors. We previously demonstrated that CD91 is expressed on human monocyte-derived dendritic cells (moDCs) and that human defensins stimulate in vitro the activation of these cells. In this study, we observed that CD91 is expressed at different levels on two distinct moDC subsets: CD91<sup>dim</sup> and CD91<sup>bright</sup> moDCs. Although CD91<sup>bright</sup> moDCs represented a small proportion of total moDCs, this subset showed higher levels of activation and maturation markers compared to CD91<sup>dim</sup> moDCs. The frequency of CD91<sup>bright</sup> moDCs increased by

~50% after *in vitro* stimulation with recombinant Human Neutrophil Peptide-1 (rHNP-1) and recombinant Human Beta Defensin-1 (rHBD-1), while lipopolysaccharide (LPS) stimulation decreased it by ~35%. Both defensins up-regulated moDC expression of CD80, CD40, CD83 and HLA-DR, although to a lower extent compared with LPS. Notably, upon culture with rHNP-1 and rHBD-1, CD91<sup>bright</sup> moDCs maintained their higher activation/maturation status, while this was lost upon culture with LPS. Our findings suggest that defensins promote the differentiation into activated CD91<sup>bright</sup> DCs and may encourage the exploitation of the CD91/defensins axis as a novel therapeutic strategy to potentiate antimicrobial and antitumor immune response.

## Introduction

CD91 is a member of the Low-density lipoprotein receptor family that recognizes more than 40 structurally and functionally distinct ligands.<sup>1</sup> Particularly, CD91 has been demonstrated to bind and internalize  $\alpha_2$ -Macroglobulin,<sup>2</sup> C1q<sup>3</sup> and defensins.<sup>4</sup> Reflecting this diversity of ligands, CD91 displays a wide tissue distribution and participates in a variety of physiological responses, including lipoprotein metabolism, proteinase homeostasis and cell migration. Subsequent studies defined CD91 as an immunologically relevant receptor for Heat Shock Proteins (HSPs) and molecular chaperones.<sup>5,6</sup> It is expressed on different cell types, including dendritic cells, monocytes, macrophages, B and T cells as well as splenocytes and thymocytes.<sup>7</sup> In addition, CD91 is expressed on monocyte-derived dendritic cells (moDCs) and it plays a role in the internalization of CD91-targeted antigens and their presentation to T cells.<sup>8</sup> Moreover, the importance of this molecule in the immune response has been strengthened by the observation that CD91 represents an important route for stimulating CD8<sup>+</sup> T cell responses by MHC class I-restricted presentation.<sup>9</sup>

The scavenger receptor CD91 is involved in wide a variety of diseases and infections. Kebba et al, showed that a significantly higher surface expression of CD91 on monocytes of human immunodeficiency virus type-1 (HIV-1)-infected long-term nonprogressors may contribute to host anti-HIV-1 defence and play a role in protection against HIV-1 infection.<sup>10</sup> Further, the up-regulation of CD91 in patients affected by cancer may represent an additional strategy to improve DC activation and subsequent stimulation of a specific CD8+ T cell response towards tumor cells dying an immunogenic cell death.<sup>11</sup> Of note, CD91 ligands HSPs,  $\alpha_2$ -Macroglobulin and defensins are currently under investigation in pre-clinical and clinical trials exploring new therapeutic strategies for the treatment of infectious diseases and cancers.<sup>12,13,14</sup>

In a previous study, we demonstrated that moDCs express CD91 and that moDC treatment with human defensins up-regulates the surface expression of CD91 as well as the activation/maturation markers on these cells.<sup>15</sup> In this study, we described two subsets of moDCs, characterized by variable level of CD91 expression and different state of activation/maturation. We further investigated the effects of recombinant human alpha and beta defensins, Human Neutrophil Peptide-1 (HNP-1) and Human Beta Defensin-1 (rHBD-1), on these two subsets of moDCs.

## **Materials and Methods**

### ***In vitro* culture of moDCs**

Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation from standard buffy coat preparations of healthy donors. Monocytes were obtained from adherent PBMCs (> 90% pure CD14<sup>+</sup>, as assessed by flow cytometry) and cultured in RPMI-1640 (Euroclone, Wetherby, UK) supplemented with 10% heat-inactivated

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fetal calf serum (Gibco, Invitrogen Co., Carlsbad, CA, USA), in the presence of 800 U/ml recombinant human granulocyte–macrophage colony-stimulating factor (rhGM-CSF; Strathmann Biotech, Hannover, Germany) and 10 ng/ml rhIL-4 (R&D Systems) at 37°C in 5% CO<sub>2</sub> for 5 days. Immature moDCs were characterized by long dendrites and expression of high levels of CD1a (>90%) and low levels of CD14 (<5%). Cell viability was > 90% in all experiments, as assessed by trypan blue exclusion. These immature moDCs were stimulated for 18 hr by adding full-length 100 ng/ml rHNP-1 (30 aa), 500 ng/ml rHBD-1 (36 aa; both from AlphaDiagnostic, San Antonio, TX, USA), 20 µg/ml HSP90 (Enzo Life Sciences, Lausen, Switzerland), or 100ng/ml lipopolysaccharide (LPS) (serotype 055:B5, Sigma Chemicals Co., St. Louis, MO, USA). The concentration and the duration of culture stimulation were established based on preliminary experiments.<sup>15</sup> Endotoxin contamination in culture media and defensins was excluded by Limulus amoebocyte lysate assay (BioWhittaker, Walkersville, MD, USA) following the manufacturer’s protocol.

### **Flow cytometric analysis**

Phenotypic analysis was performed by 5-color flow cytometry. Briefly, 1x10<sup>5</sup> cells were incubated for 20 min at 4°C with different mAbs: anti-CD91 FITC- (Biomac, Leipzig, Germany), -CD80 PE-, -CD86 APC-, -CD1a PE-, -CD83 APC-, -CD14 APC-, -CD40 PE-, -CCR7 PE-, HLA-DR APC-Cy7-conjugated (all from BD Biosciences, San Diego, Ca, USA). To optimize the staining, mAbs were titrated in preliminary experiments. 7-Amino-actinomycin D (7-AAD, Sigma Aldrich) was used to exclude non-viable cells from the analysis. Fluorescence minus one (FMO) samples were used as negative controls.

Cells were collected and analyzed using a FACSCanto II (Becton Dickinson) flow cytometer, and data analysis was performed using FACSDiva (Becton Dickinson) and FlowJo software (TreeStar Inc., Ashland, OR, USA).

## Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0a (GraphPad Software, San Diego, CA). All statistical analyses assumed a two-sided significance level of  $p < 0.05$ . The Student's paired t-test was used for comparisons between groups.

## Results

### Different levels of CD91 identify two moDC subpopulations

Others and we previously showed that CD91 is expressed on moDCs.<sup>9,15-17</sup> However, whether CD91 is expressed differently in moDC subpopulations is still unknown. To investigate the expression of CD91 receptor, we cultured human monocytes with GM-CSF and IL-4 for 5 days to generate immature moDCs that were subsequently characterized by flow cytometry. As expected,<sup>15</sup> we observed that during the differentiation of monocytes into moDCs characterized by down-regulation of CD14 and up-regulation of CD1a (Figure 1A), a partial down-regulation of CD91 occurred in parallel (Figure 1B). Moreover, our results clearly showed that CD91<sup>+</sup> moDCs are segregated into two distinct populations: CD91 is highly expressed in a small proportion of total moDCs (CD91<sup>bright</sup> moDCs) (mean  $\pm$  SE, 1.19%  $\pm$  0.28), while the majority of cells expressed low levels of CD91 receptor (CD91<sup>dim</sup> moDCs) (Figure 2A).

### CD91<sup>bright</sup> moDCs show an activated phenotype

The ability of DCs to activate immune responses depends on their maturation status and the expression of costimulatory molecules. In order to investigate whether CD91<sup>bright</sup> and CD91<sup>dim</sup> subsets differ in term of activation/maturation, we evaluated their surface expression of CD80, CD86, CD40, CD83, HLA-DR molecules. As shown in Figure 2B,

CD91<sup>bright</sup> moDCs expressed significantly higher levels of all the activation and maturation markers compared to CD91<sup>dim</sup> moDCs: CD80 (MFI, mean  $\pm$  SE, 905  $\pm$  116 vs. 647  $\pm$  95,  $P < 0.05$ ), CD86 (MFI, 4864  $\pm$  861 vs. 3093  $\pm$  473,  $P = 0.05$ ), CD40 (MFI, 1287  $\pm$  157 vs. 813  $\pm$  125,  $P < 0.01$ ), CD83 (MFI, 1235  $\pm$  99 vs. 865  $\pm$  120,  $P < 0.01$ ) and HLA-DR (MFI, 32273  $\pm$  6815 vs. 20578  $\pm$  6862,  $P < 0.05$ ). As shown in the same Figure, CD91<sup>bright</sup> moDCs expressed significantly higher levels of CCR7 than CD91<sup>dim</sup> moDCs (MFI, 3814  $\pm$  649 vs. 1520  $\pm$  185,  $P = 0.01$ )

### **rHNP-1 and rHBD-1 increase the proportion of the CD91<sup>bright</sup> moDC subset**

Based on our previous findings indicating that moDC treatment with human defensins up-regulates the surface expression of CD91, we next evaluated whether *in vitro* stimulation with rHNP-1 and rHBD-1 may indeed positively affect the frequency of CD91<sup>bright</sup> moDCs. Interestingly, we observed that the proportion of CD91<sup>bright</sup> moDCs increased after rHNP-1 stimulation by 45.4  $\pm$  15.4 % (mean  $\pm$  SE,  $P < 0.01$ ) and after rHBD-1 stimulation by 47.0  $\pm$  17.4% ( $P < 0.05$ ). On the contrary, the proportion of CD91<sup>bright</sup> moDCs decreased after treatment with the CD91 ligand HSP90 by -32.7  $\pm$  6.2 % ( $P < 0.05$ ) and after LPS stimulation by -34.0  $\pm$  8.9% ( $P < 0.05$ ) (Figures 3A and 3B).

### **rHNP-1 and rHBD-1 increase the activation of CD91<sup>bright</sup> moDC subset**

Next, we performed experiments to test the ability of CD91<sup>bright</sup> moDCs to respond to defensins. First, we confirmed previously published data that both defensins promoted the activation and maturation of total moDCs: rHNP-1 vs. unstimulated CD80 (MFI, mean  $\pm$  SE, 739  $\pm$  37 vs. 611  $\pm$  39,  $P < 0.05$ ), CD86 (3482  $\pm$  1162 vs. 2984  $\pm$  557,  $P = ns$ ), CD40 (1432  $\pm$  101 vs. 1018.16  $\pm$  174,  $P < 0.05$ ), CD83 (1288  $\pm$  252 vs. 738  $\pm$  165,  $P < 0.05$ ) and HLA-DR (24026  $\pm$  3209 vs. 14490  $\pm$  3905,  $P < 0.05$ ); rHBD-1 vs. unstimulated CD80 (830  $\pm$  64 vs. 611

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$\pm 39$ ,  $P<0.05$ ), CD86 ( $5327 \pm 1589$  vs.  $2984 \pm 557$ ,  $P=ns$ ), CD40 ( $1549 \pm 145$  vs.  $1018.16 \pm 174$ ,  $P<0.05$ ), CD83 ( $1229 \pm 253$  vs.  $738 \pm 165$ ,  $P=ns$ ) and HLA-DR ( $26580 \pm 4690$  vs.  $14490 \pm 3905$ ,  $P<0.05$ ) (Figure 4). We further analysed the *in vitro* effects of rHNP-1, rHBD-1 and LPS stimulation on CD91<sup>bright</sup> compared with CD91<sup>dim</sup> moDCs. As shown in the same Figure, rHNP-1 induced significantly higher levels of all the activation markers on CD91<sup>bright</sup> moDCs compared to CD91<sup>dim</sup> moDCs (MFI on CD91<sup>bright</sup> vs. CD91<sup>dim</sup> cells, CD80:  $864 \pm 51$  vs.  $721 \pm 45$ ,  $P<0.05$ ; CD86:  $4373 \pm 1260$  vs.  $3463 \pm 1154$ ,  $P<0.05$ ; CD40:  $2164 \pm 289$  vs.  $1367 \pm 132$ ,  $P<0.05$ ; HLA-DR:  $40483 \pm 5467$  vs.  $23769 \pm 7002$ ,  $P<0.05$ ). Similar results were observed when moDCs were stimulated with rHBD-1 (CD80:  $1216 \pm 264$  vs.  $1012 \pm 190$ ,  $P<0.05$ ; CD40:  $1956 \pm 138$  vs.  $1323 \pm 196$ ,  $P<0.05$ ; CD83:  $1775 \pm 279$  vs.  $1224 \pm 253$ ,  $P<0.05$ ; HLA-DR:  $41818 \pm 5536$  vs.  $26457 \pm 3637$ ,  $P<0.05$ ). Conversely, when moDCs were stimulated with LPS, no significant differences in the expression of any activation marker was observed between CD91<sup>bright</sup> and CD91<sup>dim</sup> cells. Overall, these results indicate that CD91<sup>bright</sup> moDCs undergone stimulation with rHNP-1 and rHBD-1 maintained their higher state of activation/maturation compared to CD91<sup>dim</sup> moDCs while this property was lost upon LPS stimulation.

## Discussion

DCs are potent antigen presenting cells that sense environmental stimuli through a wide repertoire of receptors expressed on their surface.<sup>18</sup> We and others previously described that moDCs express CD91, a polyfunctional receptor that binds several molecules including defensins.<sup>4,9,15-17</sup> In this study we confirmed our previous observations and, by using multiparametric flow cytometry analysis, we showed for the first time to our knowledge the presence of two distinct subsets of moDCs based on their CD91 expression: CD91<sup>bright</sup> and CD91<sup>dim</sup> moDCs. These subsets were characterized by a different phenotype. In fact, although CD91<sup>bright</sup> cells represented a small proportion of moDCs, this subset showed a

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higher expression of activation/maturation markers compared to CD91<sup>dim</sup> moDCs, suggesting that these two subsets could have different roles in the immune response. This hypothesis may be further supported by a previous study that correlated the full maturation status of pDCs with a greater increase of CD91 expression and increased CD91 ligand binding capacity.<sup>19</sup> Interestingly, in our study *in vitro* stimulation with defensins increased the proportion of CD91<sup>bright</sup> moDCs and preserved their higher activation/maturation status compared to CD91<sup>dim</sup> moDCs, suggesting that CD91<sup>bright</sup> moDCs may be more sensitive to CD91 stimulation. Notably, defensins appeared unique in their ability to up-regulate their own receptor, as CD91 up-regulation was not shared by HSP90, another CD91 ligand highly expressed in eukariotic cells. Indeed, in accordance to one of two previous studies addressing the effects of HSPs on the expression of CD91 on different antigen presenting cells,<sup>19,20</sup> we observed that HSP90 decreased the frequency of CD91<sup>bright</sup> moDCs. Not even the ability of defensins to up-regulate CD91 was shared by LPS, a common pro-inflammatory molecule that signals through toll-like receptor 4. Indeed, also LPS decreased the frequency of CD91<sup>bright</sup> moDCs. This finding is in accordance with our previous study,<sup>15</sup> reporting LPS-induced down-regulation of CD91 expression on moDCs, possibly supported by shedding of CD91 from cell surface.<sup>21</sup>

Although there are evidences that CD91-mediated immune activation can represent a powerful mechanism of immune regulation, the pathway/s regulating expression of CD91 have not been fully defined so far. CD91 is up-regulated on monocytes of HIV<sup>+</sup> long term nonprogressor subjects, it may enhance cross-presentation of HIV antigens and may in turn enhance the stimulation of activated anti-HIV CTLs.<sup>22</sup> Moreover, the pivotal role of CD91 regulation on DCs has been demonstrated in the context of primary effusion lymphoma: DC activation was completely inhibited when these cells were pre-treated with a neutralizing antibody directed against CD91,<sup>11</sup> thus suggesting that the up-regulation of CD91 in cancer patients may represent an additional strategy to improve DC activation and subsequent

stimulation of a specific CD8<sup>+</sup> T cell response. Notably, we observed that CD91<sup>bright</sup> cells expressed significantly higher levels of CCR7 than CD91<sup>dim</sup> cells, suggesting that CD91<sup>bright</sup> moDC subset might preferentially migrate to secondary lymphoid nodes, where they may elicit adaptive immune responses.

Our results are intriguing because of the plasticity of dendritic cells in the setting of different immune responses. Thus, we could hypothesize a differential role of CD91<sup>bright</sup> and CD91<sup>dim</sup> moDC subsets in activating the immune response, possibly involving a differential production of pro-inflammatory and regulatory cytokines. These two cell subsets may also differentially privilege MHC-I rather than MHC-II presentation. Due to the low frequency of CD91<sup>bright</sup> moDCs in our cultures, we could not separate these populations in order to compare their antigen presenting ability. Experiments of cell depletion were not undertaken, as they may provide only approximate and indirect functional data. Additional studies will be required in the future to further characterize the role of CD91<sup>bright</sup> and CD91<sup>dim</sup> DCs in the immune response.

Taken together, our results suggest that moDCs preferentially respond to defensins by increasing the frequency of CD91<sup>bright</sup> moDCs and promoting a higher state of activation/maturation of these cells compared to CD91<sup>dim</sup> moDCs. The identification of a subset of moDCs expressing high levels of CD91 and molecules involved in DC-T cell interaction suggests that these CD91<sup>bright</sup> cells may be particularly devoted to the activation of adaptive immune responses against antigens that may be delivered to DCs through CD91 ligands, in particular defensins. These results may provide new insights into the immune potentiating effects of defensins that are currently under intense investigation as possible new therapeutic agents aimed to potentiate antimicrobial and antitumor immunity.

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## Conflicts of interest

The authors declare no conflicting financial interests.

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- Accepted Article
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## Figure legends

**Figure 1. The expression of CD91 is partially down-regulated during differentiation of monocytes into moDCs.** Monocytes obtained by plastic adhesion from freshly isolated PBMCs were stained for surface expression of CD14, CD1a and CD91 before and after their differentiation into moDCs obtained by 5 day-culture with rhGM-CSF and rhIL-4. (A) Dot plots showing the gating strategy and the expression of CD14 and CD1a on monocytes (upper row) and moDCs (lower row). (B) Histogram showing the mean fluorescence intensity (MFI) of CD91 expression on monocytes (gray line), moDCs (black line), and FMO negative control (dotted line). One representative experiment is shown.

**Figure 2. Different levels of CD91 identify two moDC subpopulations.** Immature moDCs were stained for surface expression of CD91 and activation/maturation markers. (A) Representative flow cytometric analysis showing the gating strategy used to define CD91<sup>dim</sup>

and CD91<sup>bright</sup> moDCs. (B) Graphs show the mean fluorescence intensity (MFI) of CD80, CD86, CD40, CD83, CCR7 and HLA-DR molecules in CD91<sup>dim</sup> (white bars) and CD91<sup>bright</sup> (black bars) moDCs from 11 independent experiments. Data shown as mean  $\pm$  SE. \*  $p < 0.05$ , \*\*  $p < 0.01$ .

**Figure 3. rHNP-1 and rHBD-1 increase the proportion of CD91<sup>bright</sup> moDC subset.**

Immature moDCs were incubated with 100 ng/ml rHNP-1, 500 ng/ml rHBD-1, 20  $\mu$ g/ml HSP90 or 100 ng/ml LPS. After 18 hr, the cells were analyzed by flow cytometry to measure cell-surface CD91 expression. (A) Representative flow cytometric analysis showing the increased proportion of CD91<sup>bright</sup> moDC subset after rHNP-1 and rHBD-1 stimulation compared to the decreased proportion after HSP90 and LPS challenge. (B) Change of CD91<sup>bright</sup> moDCs frequency after rHNP-1, rHBD-1, HSP90 and LPS compared to unstimulated conditions. Data shown as mean  $\pm$  SE from 6 independent experiments. \*  $p < 0.05$ , \*\*  $p < 0.01$ .

**Figure 4. HNP-1 and HBD-1 increase the activation of CD91<sup>bright</sup> moDC subset.**

Immature moDCs were stimulated with 100 ng/ml rHNP-1, 500 ng/ml rHBD-1, or 100 ng/ml LPS. After 18 h, the cells were analyzed by flow cytometry to measure cell-surface activation and maturation markers expression on total moDCs (white bars), CD91<sup>dim</sup> moDCs (black bars) and CD91<sup>bright</sup> moDCs (shaded bars). Graphs show the mean fluorescence intensity (MFI) of CD80, CD86, CD40, CD83 and HLA-DR molecules. Data shown as mean  $\pm$  SE from 6 independent experiments and analyzed by the paired t-test). #:  $p < 0.05$  stimulated total moDCs compared to unstimulated (NS) cells; \*  $p < 0.05$ : CD91<sup>dim</sup> moDCs compared with CD91<sup>bright</sup> moDCs.

Figure 1

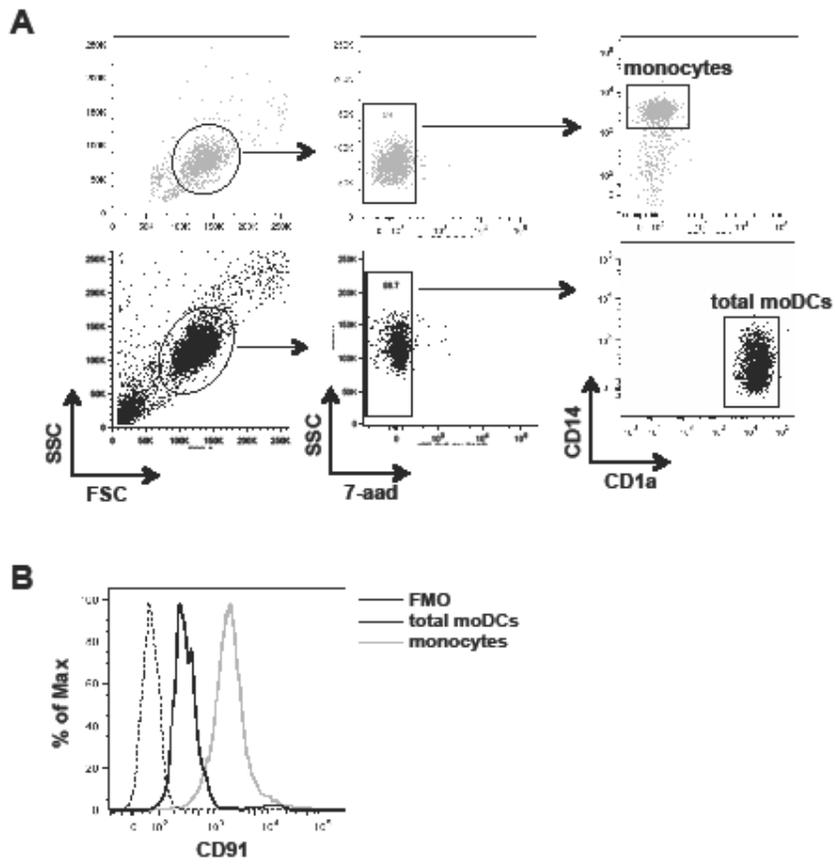
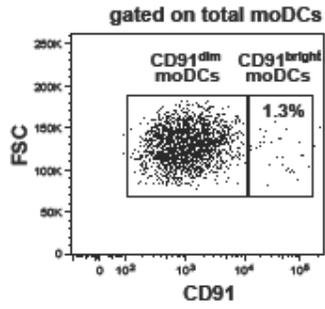


Figure 2

A



B

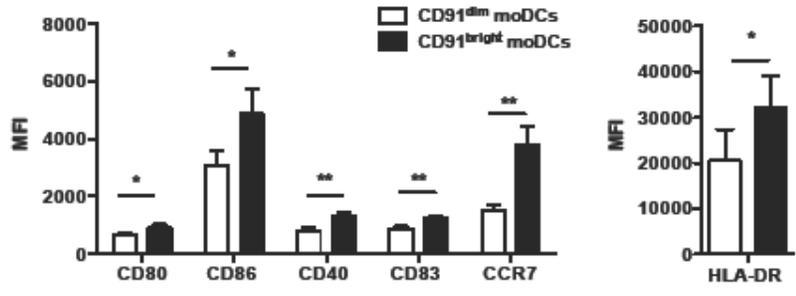


Figure 3

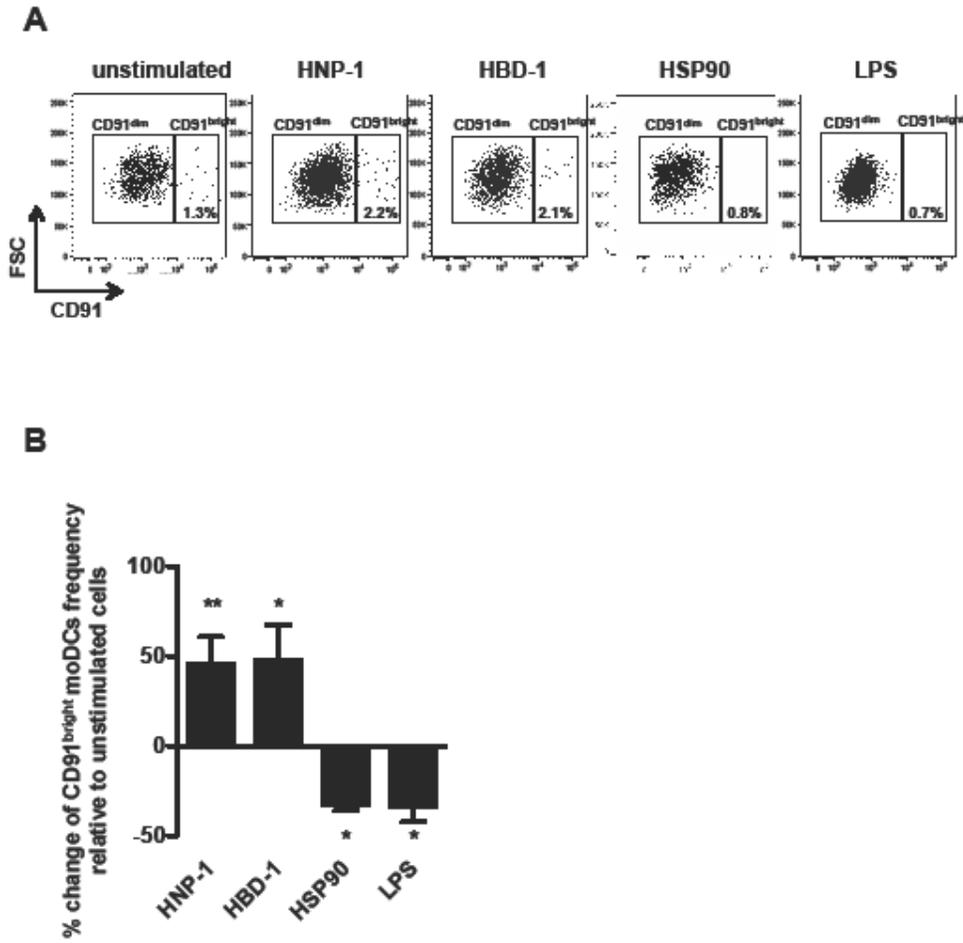


Figure 4

