Mincle polarizes human monocyte and neutrophil responses to *Candida albicans*

Dipti Vijayan[1], Kristen J. Radford[2], Anthony G. Beckhouse[1], Robert B. Ashman[3], Christine A. Wells[1]

1 Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, St Lucia, Brisbane 4072

2 Mater Medical Research Institute, Raymond Terrace, South Brisbane, 4101

3 School of Dentistry, The University of Queensland, Turbot Street, Brisbane 4000

Running title: Dichotomous Mincle expression on human leukocytes

Correspondence: Associate Professor Christine Wells, Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, St Lucia, Brisbane, Australia, 4072. Email- c.wells@uq.edu.au Telephone +61 7 33463853 Facsimile +61 7 33463973

Abstract

The distribution and function of the C-type lectin Mincle has not previously been investigated in human cells, although mouse models have demonstrated a non-redundant role for Mincle in the host response to fungal infections. The current study identified an unusual pattern of reciprocal expression of Mincle on peripheral blood monocytes or neutrophils isolated from the same donor. Expression on monocytes was inversely correlated with phagocytosis and yeast killing, but was necessary for the induction of inflammatory cytokines in response to *ex-vivo Candida* challenge. In contrast, Mincle expression on neutrophils was associated with phagocytic and candidacidal potential of those cells. *Candida* challenge upregulated Mincle expression but only in Mincle+ cells. These data highlight species-specific differences between the regulation of Mincle expression in mouse and man. Reciprocal expression of Mincle modified the candidacidal potential of monocytes or neutrophils, suggesting it may also polarize the type of host response to fungal infection.

Keywords: *Candida* infections, C-type lectin, dichotomous expression, Mincle, monocytes, neutrophils

Mincle (also known as Clec4e) belongs to the C-type lectin family of pattern recognition receptors that are crucial in antifungal immunity. Mincle recognizes ligands from several genera of fungi; Mincle-knockout mice are susceptible to infection with *Candida albicans* [(1)], and *Malassezia spp* [(2)], Mincle protein binds *Saccharomyces cerevesciae* [(3)], and is necessary for immune recognition of *Fonsecaea pedrosoi* [(4)]. These data indicate that Mincle forms an integral part of the first-line of defence against a broad spectrum of fungal pathogens.

Upon ligand recognition, Mincle activates the spleen tyrosine kinase (Syk) and other downstream signalling cascades to induce inflammatory cytokines [(5-7)]. This is a highly conserved signalling cascade shared with related lectins;- Dectin-1 and Dectin-2. While Dectin-1 interacts directly with Syk [(8)], Mincle and Dectin-2 associate with the FcRγ adapter protein to engage the Syk signalling pathway [(5, 9)]. Dectin-1 and Dectin-2 are phagocytic receptors [(10)], and although Mincle is also recruited to the phagocytic cup of wild-type mouse macrophages, mouse macrophages lacking Mincle have normal uptake of pathogens [(1, 5)].

Mincle was first identified as an LPS-inducible protein in mouse macrophages [(11)], and its inducible regulation across myeloid and lymphoid cells has been demonstrated using rodent models [(1, 5, 11-14)]. While mouse experiments signify a role for Mincle in innate immune function, the expression or function of Mincle on human leukocytes has not been previously addressed. This study assessed the distribution of Mincle on human peripheral blood cells and identified a reciprocal pattern of expression between monocytes and neutrophils that was accompanied by a functional polarization of cellular responses to *Candida albicans*.

RESULTS

Human Mincle is predominantly a myeloid receptor

Mincle expression was consistent with its role as an innate immune pattern-recognition receptor. Mincle was readily detected on myeloid cells including monocytes, neutrophils, myeloid DCs (mDCs) as well as B cells, but was absent on plasmacytoid DCs (pDCs), T cell subsets, NK and NKT cells (Figure 1a). In addition, surface expression was retained when CD14+ monocytes were differentiated *ex-vivo* to form monocyte-derived macrophages (MDMs) and monocyte-derived dendritic cells (MDDCs) (Figure 1b).

Reciprocal pattern of Mincle expression on monocytes or neutrophils

Examination of Mincle expression on leucocytes from forty-nine donors revealed an unusual reciprocity between CD14+ monocytes and neutrophils isolated from the same donor (Figure 2a and Supplementary file 1). The most common pattern of expression was monocyte+ /neutrophil-, which was observed in more than 52% of donors screened. A further 17% of donors exhibited the reciprocal neutrophil high expression (monocyte- /neutrophil+) pattern. Mincle was detectable on both monocytes and neutrophils in less than 20% of donors and a further 11% of donors failed to express Mincle on either cell type. This pattern of expression was not influenced by age, sex or the percentage distribution of blood cells in this cohort (Table 1).

Neutrophils are sensitive phagocytes that are notorious for activation during isolation. We confirmed that the bias against Mincle+ neutrophils was not a consequence of neutrophil isolation or handling. We assessed Mincle expression on whole blood and Ficoll-isolated cells, and demonstrated that the pattern of expression of neutrophil activation markers CD62L and CD11b was identical between the two isolation techniques (Figure 2b). The dichotomy of Mincle expression could also be seen on whole blood monocytes or neutrophils (Figure 2c). Mincle expression on PMA treated neutrophils was equivalent to that of non-activated cells, indicating that the pattern of expression was independent of the cell activation/maturation processes (Supplementary file 2a).

We also confirmed that monocyte maturation did not correlate with Mincle expression. Detection of Mincle on CD14+CD16- and CD14+CD16+ monocyte subsets indicated that receptor expression was not restricted to inflammatory or activated cells (Supplementary File 2b-c). Mincle expression on monocytes was highly correlated with expression on circulating myeloid DCs (Pearson's correlation r=0.990). It was also determined that Mincle expression on monocytes was predictive of the pattern of expression on monocyte-derived macrophages (MDMs) (Supplementary File 2d-e).

We next examined the stability of this pattern of reciprocal expression by re-bleeding donors. The patterns of Mincle expression on monocytes and neutrophils were consistently reproducible if donors were re-bled within hours (Supplementary file 3). However on re-bleeding donors (n=15) over several weeks, we observed that Mincle expression could switch between monocytes and neutrophils within the same donor, although the reciprocity of expression between these cell types was faithfully maintained (Figure 3a and Supplementary file 4). The covariance of Mincle expression between monocytes and neutrophils on independent donors, or across donors who had been bled multiple times, indicated that Mincle expression on monocytes and neutrophils was likely to be reciprocally regulated.

As Dectin-1 belongs to the same family of pattern recognition receptors as Mincle and has an increasing importance in human candidiasis [15], we next examined whether it also exhibited reciprocity of expression in monocytes and neutrophils derived from the same donor. No covariance of Dectin-1 was observed between these cell compartments (Figure 3b).

Surface Mincle is inducible on challenge with fungal pathogen, *Candida albicans*

As Mincle is an inducible protein in mouse macrophages, the regulation of the receptor in response to extrinsic stimuli or environmental cues was investigated [11]. Surprisingly, no increase in surface protein could be detected on isolated monocytes or neutrophils challenged by lipopolysaccharide (LPS) (Figure 4a and Supplementary file 5). Given that Mincle is primarily an antifungal receptor, we investigated whether its expression could be upregulated in response to heat-killed *Candida albicans*. An increase in Mincle expression on both monocytes and neutrophils was evident at two hours (Figure 4a); however this increase was observed only when Mincle was already present on unstimulated cells, and no induction was seen on Mincle- cells (Figure 4b). This suggests that *in vivo* priming may play a critical role in mediating Mincle expression and subsequent upregulation during an infection.

Mincle is differentially associated with *Candida* uptake and killing in monocytes and neutrophils

To our knowledge, this pattern of reciprocal expression has not been previously reported, so we examined whether it was associated with differences in cellular responses to *Candida.* We identified a strong negative correlation between the level of Mincle expression (ΔMFI) and candidacidal activity by Mincle+ monocytes (Pearson r= -0.75, n=30, Figure 5a) that was associated with poorer phagocytosis (r= -0.733, n=7 Figure 5c). In contrast, Mincle expression on neutrophils positively correlated with fungicidal activity of the cells (r=0.7, n=22 Figure 5b), and this correlated with yeast uptake (r=0.561, n= 13 Figure 5d). Thus a pattern of Mincle+ neutrophils and Mincle- monocytes appears most favourable for clearance of *Candida* by human phagocytes.

Mincle is an essential regulator of inflammatory cytokines in human monocytes

Given that Mincle is a key modulator of inflammatory responses in mouse macrophages, we investigated the role of Mincle in the inflammatory response of human monocytes to *Candida albicans.* Using an activating polyclonal Ab, MAB2, TNF production was assessed in human monocytes. Only monocytes expressing Mincle produced TNF in response to MAB2 or *Candida* (Figure 6a, n=6, t-test p=0.0028), and no additive effect was seen in monocytes co-incubated with MAB2 and yeast (Figure 6a). All donors tested were capable of producing TNF in response to unrelated stimuli such as LPS (Figure 6a), indicating that Mincle has a central role in TNF production by human monocytes responding to *Candida*.

Monocytes expressing Mincle also had significantly increased levels of IL-1β when activated with the MAB2 (Figure 6b, n=6, t-test p=0.013) compared to Mincle- monocytes, but we observed much greater donor variability in the expression of this cytokine when challenged with *Candida*. Synergistic activation of IL-1β was observed in Mincle+ monocytes stimulated with *Candida* and MAB2, and this was more pronounced in 2 of the 4 donors. These data suggest that Mincle collaborates with additional receptors/inflammatory pathways in triggering IL-1β. Thus Mincle expression on monocytes appears most favourable for the induction of an inflammatory cytokine response to *Candida*.

Discussion

This study demonstrated that the C-type lectin, Mincle, has an unusual pattern of reciprocal expression on human monocytes or neutrophils. Mincle was most commonly observed on monocytes, with the Mincle+ neutrophils observed much less frequently. Mincle was previously identified as an inducible protein on mouse macrophages [(11)], but in the current study we were unable to alter Mincle expression on human leukocytes over a 24 hour time-course of LPS stimulation, which highlights an important difference in the regulation of this innate immune receptor in mice and humans.

The reciprocal patterns of Mincle expression on monocytes and neutrophils have functional implications. Mincle expression on neutrophils appeared to favour early cellular responses, leading towards clearance of the organisms, whereas Mincle expression on monocytes may polarise cellular responses away from clearance, and favour inflammatory cytokine production. This functional dichotomy in responses could offer a mechanism for the orchestration and fine-tuning of early responses to infection. Indeed, the pattern of receptor expression is likely to be a predictor of the course of an infection, as regulation of pattern-recognition receptors on neutrophils, monocytes, macrophages and DCs demonstrably impacted on the type of cellular response elicited.

These observations may help to explain the different immune responses required for resolution of systemic versus mucosal *Candida* infections. Systemic infection is likely to elicit an immediate response from the neutrophil population [(16)], with less probability of contact with monocytes/macrophages, whereas the response to oral infection may be slower, offering more opportunity for interaction with macrophages/DCs [(17)], and thus elicitation of inflammatory cytokines and adaptive immunity. The switch that directs immune responses to the same pathogen into different pathways has not been identified, and the current study suggests that switching Mincle expression between myeloid compartments may, at least in part, help to direct these events.

Understanding the mechanism driving this dichotomous pattern of expression may provide new ways to understand the influence of environmental events on immune function, and this may be particularly important in pathogens such as *Candida*, which are distributed broadly in the community, and whose significance as an opportunistic pathogen is now well-recognised.

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Methods

Blood collection

Peripheral blood was obtained with informed consent from healthy volunteers following approval from local ethics committees (Australian Red Cross Blood Service, Griffith University ethics HREC DOH/03/09, UQ ethics expedited protocol number 2010001327and Mater Medical Research Institute MMRI#45) in EDTA tubes before processing.

Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

PBMCs were isolated from whole blood using Ficoll hypaque (GE Healthcare) as previously described [(18)]. Human CD14 and CD16 microbeads (Miltenyi Biotec) were used for isolation of CD14+CD16- monocytes, CD16+CD14+ monocytes and CD14- lymphocytes by positive selection or negative depletion protocols respectively. The resulting fractions were confirmed by flow cytometry using appropriate cell surface markers.

Monocyte differentiation to macrophages (MDMs) and dendritic cells (MDDCs)

CD14+ monocytes were cultured for 6 days in complete RPMI media supplemented 100ng/ml of human M-CSF (Peprotech) for differentiation to MDMs.

MDDC were generated by culture for 5–6 days in complete AB medium supplemented with 800 U/ ml GM-CSF (Invitrogen) and 1000 U/ ml IL-4 (Invitrogen).

Isolation of neutrophils

The bottom most layer of the Ficoll preparation containing RBCs and neutrophils was incubated at 4°C with hypotonic solution (8.3g NH_4Cl, 1g $KHCO_3$ and 18ml 0.5M EDTA) for 10 mins. Cells were thereafter washed twice and resuspended in complete media. Cells were assessed for spontaneous activation during isolation by flow cytometry using neutrophil activation markers CD11b and CD62L. PMA (50ng/ml, Sigma) neutrophils, incubated for 30 mins were used as a positive control for examining neutrophil activation.

Monoclonal antibodies

These included markers for monocytes - CD14-FITC (Miltenyi Biotec) and CD16-PE (eBiosciences); neutrophils- CD11b-FITC, CD62L-PE (BD Biosciences); T-cells- CD3-FITC, CD4-PE, CD8-PerCp; B cells - CD19-FITC; NK cells- (CD56-PE); DCs were defined as being Lineage-HLA-DR+ CD11c+ for mDCs and Lineage-HLA-DR+ CD11c- for pDCs - Lineage markers (CD3-FITC, CD14-FITC, CD19-FITC, CD20-FITC, CD34-FITC , CD56-FITC), HLA-DR-APC Cy7, CD11c-PE (all from BD Biosciences), Mincle (Abnova, clone 2D12) and Dectin-1 (RnD Systems, clone 259931) .

Flow Cytometry

Cells were blocked (human Fc-block, Miltenyi Biotec), stained with an optimal dilution of primary/isotype Ab for 30mins at 4°C. After washes in FACs buffer, they were stained with secondary Ab (Alexafluor-647, Invitrogen) in the dark for another 30mins at 4°C. Cells were labelled with appropriate cell markers for 15mins, 4°C. Whole blood was stained similarly and the cells were lysed with RBC lysis buffer before analysis. Antibody staining was analysed by BD FACS Aria, Beckman Coulter (Cyan) and Accuri Cytometers

Yeast strain and culture medium

Candida albicans isolate 3630 was a clinical isolate obtained from the Australian Medical Mycology Reference Laboratory (AMMRL), Sydney. Yeast was grown in Sabouraud's broth, with continuous agitation, overnight at 28°C. Yeasts were killed at 95°C for 10 mins.

Mincle stimulation studies

Monocytes and neutrophils were stimulated with LPS (100ng/ml) and heat killed *Candida* (MOI: 2) for two hours at 37°C, 5%CO_2.

Preparation of Mincle activating Ab

An affinity-purified polyclonal Ab against an extracellular domain of Mincle, conserved between mouse and humans, was raised in rabbits by a commercial supplier (Bio Synthesis) using the hydrophilic peptide TQEEQEFLFRTKPKRKEF. The Ab was supplied at a stock concentration of 0.43 mg/ml.

Mincle-Ab activation studies

Monocytes and neutrophils were incubated with 2ug activating polyclonal anti-mouse Mincle Ab for 30 mins at 4°C. The cells were then incubated with yeasts and cytokines-TNF and IL-1β after 2 hrs of incubation were measured.

Phagocytosis assay

Candida albicans were labelled with FITC for 1hr in dark. These labelled yeasts were thoroughly washed in saline before incubating with the effectors. Phagocytosis was initiated at 37°, 5% CO_2. Uptake was stopped after 30 min with ice-cold PBS. The distinction between internalized yeast cells and those attached to monocyte/neutrophil surface was achieved *via* quenching the FITC-fluorescence with trypan blue and analysing the fluorescence by Accuri cytometers.

MTS killing

Monocytes and neutrophils were incubated with *Candida* (MOI: 1) in a 96 well plate at 37°C, 5%CO_2 for 90 mins. Monocytes and neutrophils were lysed in 0.1% TritonX-100 for 5 mins, followed by centrifugation and washes with saline. After two washed, MTT/PMS solution (Promega) was added as per manufacturer's instructions and incubated overnight at 37°C, 5% CO_2. Optical Density (OD) was determined using the ELISA plate reader (BioTek) at 490nm. The absorbance directly correlates with the cell number.

% killing = [1-({OD of treated effectors-OD of effector cells only}/ OD of yeast only)] x 100

ELISA

Monocytes and neutrophils were incubated with heat killed *C. albicans* (MOI:15) in 96-well plates for 2 hours. Supernatants were collected and assessed for TNF and IL-1β (BD Biosciences) according to the manufacturer's instructions.

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Figure legends

Figure 1. Characterization of surface Mincle on human peripheral blood cells by flow cytometry. White histogram= isotype control, shaded histogram= Mincle staining

(a) Mincle expression was detected above isotype staining on primary cells including monocytes, neutrophils, myeloid DCs, B cells, but not plasmacytoid DCs, T cell subsets, NK or NKT cells. (b) Mincle was detected on ex vivo differentiated CD14+cells including macrophages (MDMs) and dendritic cells (MDDCs).

Figure 2. Dichotomous pattern of Mincle expression on monocytes or neutrophils. X axis shows the ΔMFI levels of Mincle expression on monocytes or neutrophils, determined by median fluorescence intensity corrected for non-specific binding by subtraction of MFI values corresponding to isotype matched controls. (**a**) Each bar represents one peripheral blood sample (n=75). Expression levels on monocytes shown to the left, on neutrophils shown to the right. Samples were ranked by Mincle expression on monocytes. (**b**) Scatter plot of CD26L and CD11b expression in neutrophils gated from whole blood (left) or Ficoll-isolated (right) was used to determine the impact of isolation method on activation status. Lower panels phorbol 12-myristate 13-acetate (PMA) treatment (50ng/ml, 30min) on neutrophils gated from whole blood (left) or Ficoll-isolated neutrophils (right) caused an identical shift in CD62L expression. **(c)** White histogram= isotype control, shaded histogram= Mincle staining Flow cytometry histograms demonstrating that the pattern of Mincle expression was not altered by isolation methods. Left: whole-blood gated monocytes or neutrophils, right: Ficoll-isolated monocytes or neutrophils.

Figure 3. Pattern of C-type lectin expression on human monocytes and neutrophils in donors re-bled after several weeks (n=15)**.** White histogram= isotype control, shaded histogram= Mincle staining. Three distinct patterns were observed: Donor 1 exemplified Mincle expression swap from monocytes to neutrophils. Donor 2 exemplified the switch from neutrophils to monocytes upon re-bleeding. Donor 3 exemplified a switch from a non-expressor to a Mincle+ monocyte profile. **(b)** Dectin-1 distribution on monocytes or neutrophils. X axis shows the ΔMFI levels of Dectin-1 expression on monocytes (left) or neutrophils (right), determined by median fluorescence intensity corrected for non-specific binding by subtraction of MFI values corresponding to isotype matched controls. Each bar represents one peripheral blood sample (n=17). Samples were ranked by Dectin-1 expression on monocytes.

Figure 4. Expression of Mincle on monocytes or neutrophils exposed to *ex vivo* inflammatory stimuli. White histogram= isotype control, shaded histogram= Mincle staining Panel A illustrates a donor expressing Mincle on both monocytes and neutrophils. Panel B illustrates a non-Mincle expressing

donor. Top panels: LPS (100ng/ml) for 2 hrs (representative donor shown from 49 donors). Bottom panels: *Candida albicans* MOI 2, 2 hours (representative donor shown from 7 donors).

Figure 5 Phagocytosis and killing of *Candida albicans* by human monocytes or neutrophils. Mincle MFI values of monocytes and neutrophils from each donor were plotted against % *Candida* killing or uptake. X axis shows the ΔMFI levels of Mincle expression on monocytes (top and bottom left) or neutrophils (top and bottom right).Y axis represents % *Candida* killing (upper panel) or % *Candida* uptake (lower panel). (**a**) Mincle expression on monocytes is negatively correlated with candicidal activity of the cells (Pearson r= -0.75, n=30). **(b)** Mincle expression on neutrophils is correlated with candicidal activity (Pearson r= 0.7, n=22) (**c**) Uptake of *Candida* is impaired in Mincle+ monocytes (Pearson r= -0.733, n=7) **(d)** Uptake of *Candida* is correlated with Mincle expression on neutrophils (Pearson r= 0.561, n=13).

Figure 6. Role of Mincle in the induction of inflammatory cytokines. Y axis shows the concentration of (a) TNF or (b) IL-1β. X-axis conditions (2hours) (Left-right) Monocyte control, isotype control, MAB2 (2ug/ml), *C. albicans*, isotype + *C. albicans*, MAB2 + *C. albicans*, LPS (100ng/ml). Each symbol indicates an individual donor: Closed symbols= Mincle + monocytes, Open symbols= Mincle- monocytes. P values determined using student t-test (n=4 per cytokine).

Tables

Table 1. Mincle expression on monocytes or neutrophils in each donor (n=7) and their correlation to their age, gender and percentage distribution of white blood cells in the peripheral blood namely monocytes (M), lymphocytes (L) and neutrophils (N). (***High Mincle expression, **Moderate expression of Mincle, *Low expression and – no expression)

Mincle expression on monocytes	Mincle expression on neutrophils	Age	Gender	% of WBC in the human blood		
				N	M	L
***	-	40-50	F	64.46	6.24	29.3
***	-	40-50	M	64.7	10.6	25.2
***	**	40-50	M	69.2	4.05	22.3
***	**	40-50	M	60.3	9	32.96
*	**	40-50	F	69.03	5.77	22.2
-	***	40-50	F	60.6	5.94	31.9
-	***	40-50	M	66.56	9.30	21.5

Supplementary Files:

Supplementary information is available at Immunology and Cell Biology's website- http://www.nature.com/icb/index.html

File 1. Characterization of Mincle expression on monocytes , neutrophils and B cells by fluorescence microscopy

File 2: Mincle expression on activated cells and other myeloid cell populations

File 3: Pattern of Mincle expression on monocytes and neutrophils in a donor within hours of initial bleeding.

File 4: Summary of median fluorescent intensity (MFI) of Mincle expression in all donors

File 5: Time course of LPS induction to determine Mincle expression on monocytes and neutrophils

Figure 1

(a) Primary cells

CD14+ Monocytes

Neutrophils

Myeloid DCs

B cells

Plasmacytoid DCs

CD4+Tcells

CD8+Tcells

NK cells

NKT cells

10^0 10^1 10^2 10^3 10^4

(b) Cultured cells

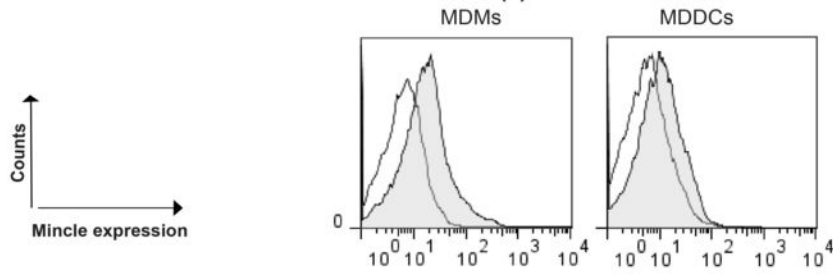
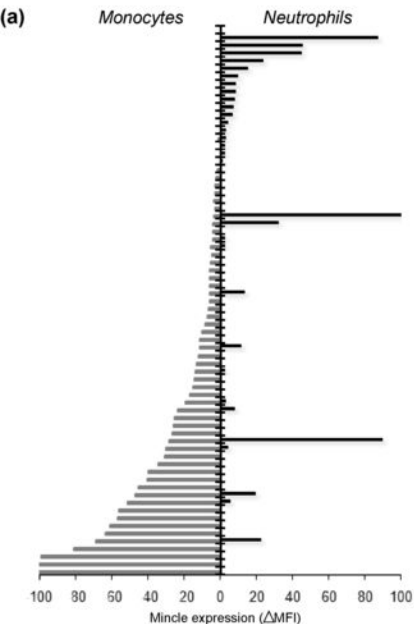
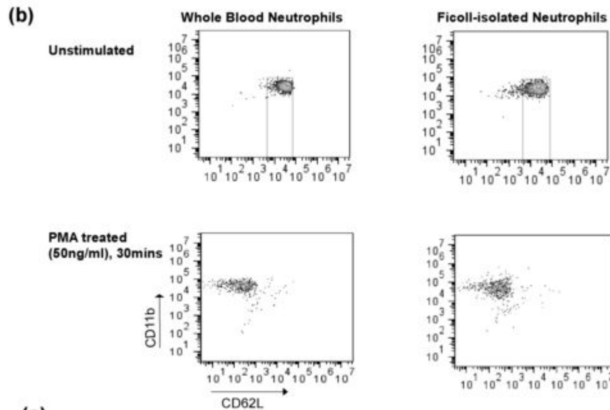


Figure 2





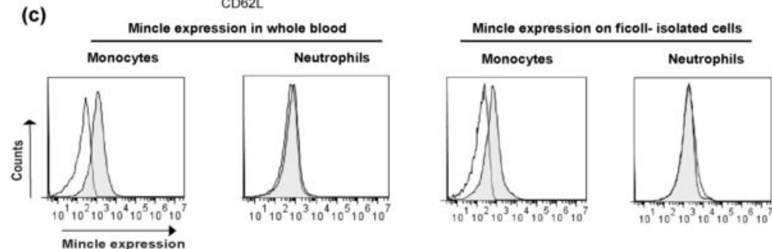


Figure 3

(a)

First Bleed — Monocytes, Neutrophils

Re-bleeding — Monocytes, Neutrophils

Donor 1

Donor 2

Donor 3

Counts

Mincle expression

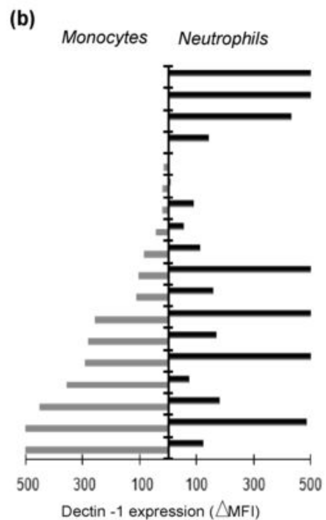
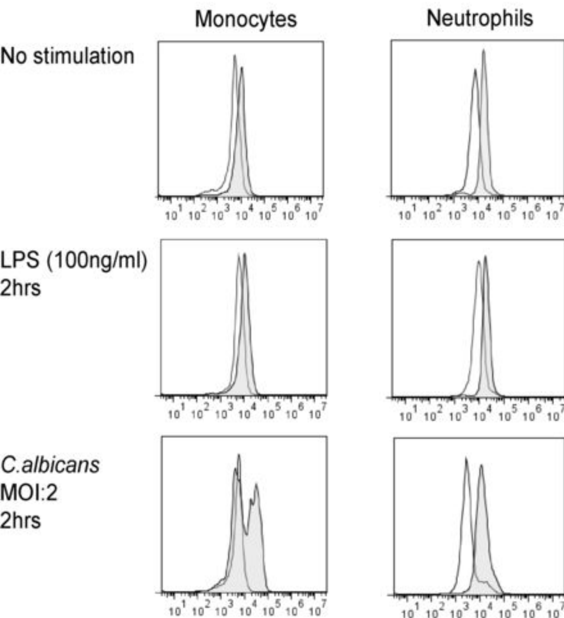


Figure 4



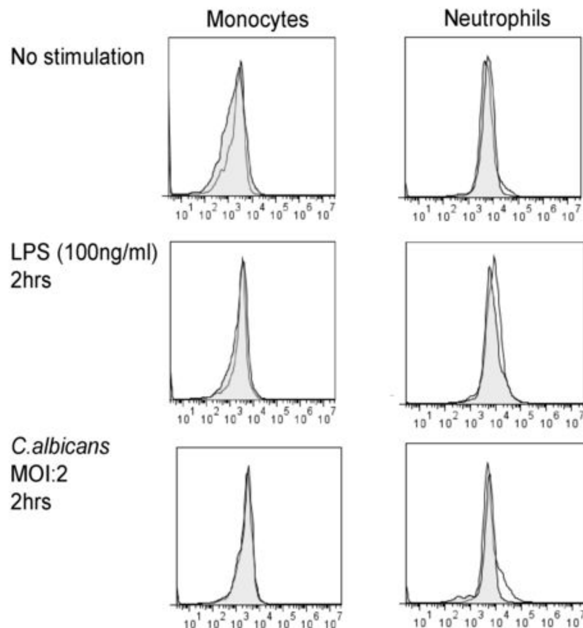
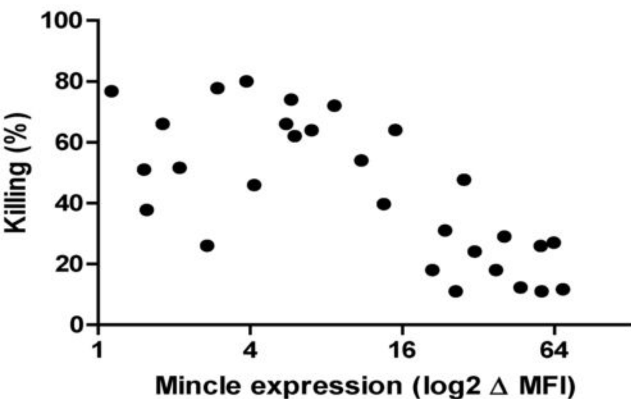
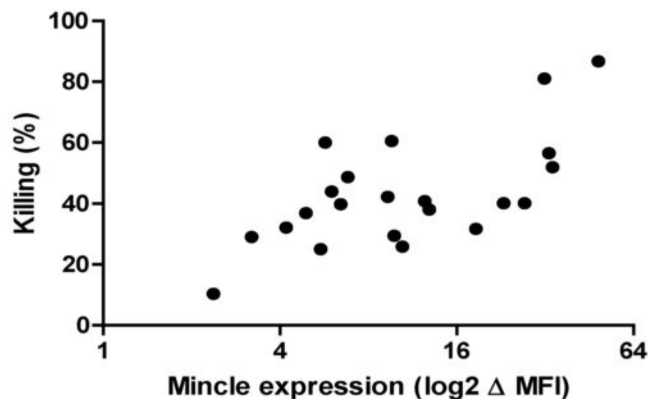


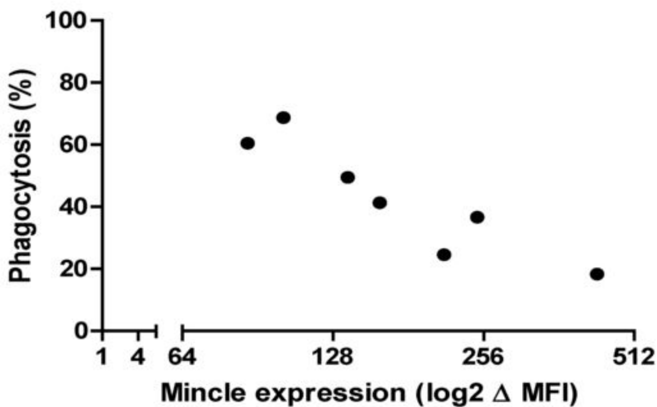
Figure 5

Killing





Phagocytosis



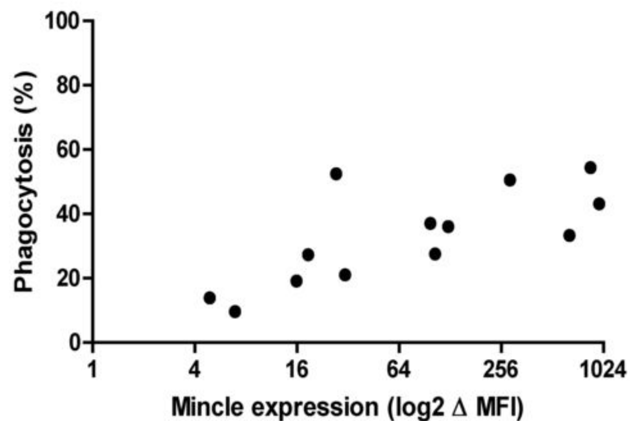


Figure 6