IFN-γ enhances both the anti-bacterial and the pro-inflammatory response of human mast cells to *S. aureus*

Emily J. Swindle[1*], Jared M. Brown[2], Madeleine Rådinger[3], Frank R. DeLeo[4] and Dean D. Metcalfe[5]

Running title: IFN-γ primes huMCs for enhanced responses to *S. aureus*

[1]Academic Unit of Clinical and Experimental Sciences, Faculty of Medicine, University of Southampton, University Hospital Southampton, Southampton SO16 6YD, UK, [2]Department of Pharmaceutical Sciences, Skaggs School of Pharmacy and Pharmaceutical Sciences, The University of Colorado Anschutz Medical Campus, Aurora, CO, USA, [3]Krefting Research Centre, Department of Internal Medicine and Clinical Nutrition, University of Gothenburg, Göteborg, Sweden, [4]Laboratory of Human Bacterial Pathogenesis, Rocky Mountain Laboratories and National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MA, USA and [5]Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA.

*Corresponding Author: Emily J. Swindle, Academic Unit of Clinical and Experimental Sciences, Faculty of Medicine, University of Southampton, University Hospital Southampton, Southampton SO16 6YD, UK. Tel. +44 (0)2381 208975; Fax. +44(0)2380 511761. E-Mail: e.j.swindle@soton.ac.uk

This is the accepted version of the following article: Swindle EJ, Brown J, Radinger M, DeLeo FR, Metcalfe DE. IFN-gamma enhances the anti-bacterial and pro-inflammatory response of human mast cells to S. aureus. Immunology. 2015; 146(3):470-485 which has been published in final form at [http://onlinelibrary.wiley.com/doi/10.1111/imm.12524/full]

Keywords: Interferon-γ, mast cells, bacteria, innate immunity, integrin-β1

Abbreviations: AUC, area under the curve; DCF, dichlorodifluorescein; DPI, diphenyleneiodonium; huMC, human mast cells; ROS, reactive oxygen species.

Summary

Human mast cells (huMCs) are involved in both innate and adaptive immune responses where they release mediators including amines, reactive oxygen species (ROS), eicosanoids and cytokines. We have reported that IFN-γ enhances FcγR-dependent ROS production. The aim of this study was to extend these observations by investigating the effect of IFN-γ on the biological responses of huMCs to *Staphylococcus aureus*. We found that exposure of huMCs to *S. aureus* generated intra- and extra-cellular ROS, which were enhanced in the presence of IFN-γ. IFN-γ also promoted bacteria killing, β-hexosaminidase release and eicosanoid production. IFN-γ similarly increased expression of mRNAs encoding CCL1-CCL4, GM-CSF, TNF-α and CXCL8 in *S. aureus*-stimulated huMCs. The ability of IFN-γ to increase CXCL8 and GM-CSF protein levels was confirmed by ELISA. Fibronectin or an integrin-β_1 blocking Ab completely abrogated IFN-γ-dependent *S. aureus* binding and reduced *S. aureus*-dependent CXCL8 secretion. These data demonstrate that IFN-γ primes huMCs for enhanced anti-bacterial and pro-inflammatory responses to *S. aureus,* partially mediated by integrin-β_1.

Introduction

Mast cells (MCs) are tissue resident cells that while classically associated with allergic inflammation [1], have been reported to participate in the innate immune response to infectious organisms including bacteria [2]. *In vitro* challenge of mouse MCs with *S. aureus* or its cell wall component peptidoglycan (PGN) generates cytokines including IL-6 and TNFα [3, 4] and induces MC degranulation [5]. Furthermore, studies in mice lacking MCs due to defective c-Kit signalling (WBB6F$_1$-*Kit*$^{W/W\text{-}v}$ and C57BL/6-*Kit*$^{w\text{-}sh/w\text{-}sh}$), reveal a protective role for MCs against *S. aureus* infection and bacterial cell wall components [6-8], although when MC deficiency was independent of KIT signalling (Mcpt5-Cre^{+} x R-DTA), MCs were not essential in protection [3].

There is less known concerning the response of human MCs (huMCs) to bacteria. What has been reported is that huMCs phagocytose and kill FimH+ *E. coli* and *S. aureus*, and release TNFα and chemokines [9-12], in part via a TLR2- and CD48-dependent mechanism [13, 14]. HuMCs are also reported to release extracellular traps (MCETs) for bacteria [15, 16]. Following the observation that mouse MCs generate ROS that contribute to bacterial killing [17], we reported that MCs generate ROS from the enzymes 5LO and COX following FcεRI and FcγR aggregation [18-20]. This is of interest as it is known that huMCs respond to IFNγ by upregulating the surface expression of FcγRI [21-24] and TLR4 [25].

Based both on the observations that huMC are able to engulf bacteria and that IFN-γ up-regulates the expression of some surface receptors on huMCs, we were interested in determining whether IFN-γ, as would be produced by infiltrating inflammatory cells, would

This is the accepted version of the following article: Swindle EJ, Brown J, Radinger M, DeLeo FR, Metcalfe DE. IFN-gamma enhances the anti-bacterial and pro-inflammatory response of human mast cells to S. aureus. Immunology. 2015; 146(3):470-485 which has been published in final form at [http://onlinelibrary.wiley.com/doi/10.1111/imm.12524/full]

further enhance the response of huMCs to a bacteria (*S. aureus*). As will be shown, IFN-γ substantially increases the ability of huMCs to respond to *S. aureus*. by enhancing ROS production, MC degranulation and eicosanoid, cytokine and chemokine production. We also found that these responses are mediated, in part, through a β_1 integrin receptor-mediated process. These observations are consistent with the conclusion that the involvement of huMCs in innate defence mechanisms against bacteria is further enhanced by IFN-γ, providing a mechanism to increase the efficiency of these tissue resident effector cells in control of infection as inflammation progresses.

Materials and Methods

Mice and Materials

Wild type (WT) C57BL6 mice (< 6 months old, 20 g), *Tlr2*-deficient and *Scarb1*-deficient mice were from Jackson Laboratories (Bar Harbor, ME). Aged-matched WT and *Msr1*-deficient mice were a kind gift from Dr. Andrij Holian (Dept. Biomedical and Pharmaceutical Sciences, Univ. Montana, MT). Mice were maintained and sacrificed in accordance with the National Institutes of Health guidelines on animal care and use, which was reviewed and approved by the NIAID Animal Use Committee.

BSA, diphenyleneiodonium (DPI), saponin, superoxide dismutase (SOD) and trypan blue (St. Louis, MI); dichlorofluorescein (DCF) diacetate (Calbiochem; EMD Biosciences, La Jolla, CA), FR122047 and zileuton (Cayman Chemicals, Ann Arbor, MI); diogenes (National Diagnostics, Atlanta, GA) and cell culture media and supplements (Invitrogen (Carlsbad, CA; or Biosource International Camarillo, CA), human recombinant

This is the accepted version of the following article: Swindle EJ, Brown J, Radinger M, DeLeo FR, Metcalfe DE. IFN-gamma enhances the anti-bacterial and pro-inflammatory response of human mast cells to S. aureus. Immunology. 2015; 146(3):470-485 which has been published in final form at [http://onlinelibrary.wiley.com/doi/10.1111/imm.12524/full]

GM-CSF, IFN-γ, IL-3, IL-4, IL-6, SCF and TNFα (Peprotech, Rocky Hill, NJ); dextran (T-500) and Ficoll-Hypaque (Amersham Biosciences, Piscataway, NJ) and Luria Bertani (LB) broth and agar (KD Medical, Columbia, MD).

Cell cultures

HuMCs were derived from CD34+ peripheral blood mononuclear cells obtained following informed consent from normal volunteers on a protocol approved by the NIAID Institutional Review Board (IRB). CD34+ cells were cultured in IL-3 (wk 1 only, 30 ng/ml), SCF (100 ng/ml) and IL-6 (100 ng/ml) as described [19]. HuMC were >99% pure by toluidine blue staining of cytospin preparations and were used after 7-10 wks of culture. Polymorphonuclear leukocytes (PMN) were isolated from human peripheral blood of normal volunteers [20] using a protocol approved by the NIAID IRB. PMN were kept on ice in PBS containing glucose (10 mM) until studied.

Mouse bone marrow-derived MCs (mBMMC) were cultured from femoral marrow cells in RPMI medium supplemented with FBS (10%), penicillin (100 U/ml), streptomycin (100 μg/ml), HEPES (25 mM), sodium pyruvate (1 mM), non-essential amino acids (1 mM), 2-ME (0.0035%) and mouse IL-3 (30 ng/ml) as described [20]. mBMMC were >99% pure by toluidine blue staining of cytospin preparations and were used after 4-6 wks of culture.

Staphylococcus aureus culture

A 10 μl frozen stock of *Staphylococcus aureus* (ATCC 27217; strain 502A) was added to Luria-Bertani (LB) broth (6 ml) and cultured overnight at 37˚C with shaking (200 rpm).

This is the accepted version of the following article: Swindle EJ, Brown J, Radinger M, DeLeo FR, Metcalfe DE. IFN-gamma enhances the anti-bacterial and pro-inflammatory response of human mast cells to S. aureus. Immunology. 2015; 146(3):470-485 which has been published in final form at [http://onlinelibrary.wiley.com/doi/10.1111/imm.12524/full]

Then, a 100-fold dilution of the culture was incubated for a further 2.5 h with shaking (200 rpm) to obtain bacteria in late logarithmic stage growth (OD_{600}<1). Viable bacteria/ml was determined by measuring OD_{600} and comparing this value to known OD_{600} values for CFU/ml from a pre-determined standard curve. Bacteria were then centrifuged at 10,000 × *g* for 4 min and washed 3X with appropriate media prior to use.

Intracellular ROS detection by microfluorimetry

Intracellular ROS production was measured in a 96-well microplate assay employing DCF (the intracellular product of DCF-diacetate (DCF-DA) that fluoresces in the presence of ROS). MCs or PMN ($1x10^6$/ml) were incubated with DCF-DA (20 μM, 20 min) at 4ºC with rotation. Cells were then washed (HEPES buffer) and added (2×10^5/well) to a black opaque 96-well microplate containing enzyme inhibitors and bacteria on ice. The activation of huMCs by *S. aureus* was then synchronized by centrifugation of the microplate at 170 × g for 8 min at 4ºC. DCF fluorescence was monitored at 30-s intervals for 1 h at 37ºC using a GENios fluorescent plate reader (ReTirSoft Inc, Toronto, Ontario, Canada) with excitation and emission wavelengths of 492nm and 535nm, respectively. Fluorescence was expressed as relative fluorescent units (RFU) and the kinetic data were collected using an XFlour4 macro within Microsoft Excel.

Extracellular ROS detection by microflourimetry

Extracellular ROS production was measured in a 96-well plate assay employing the chemiluminescent probe Diogenes which is used to detect extracellular superoxide. Briefly,

huMC or PMN were washed (HEPES buffer) and added (4×10^5/well) to a white opaque 96-well microplate containing enzyme inhibitors, stimulants (bacteria) and Diogenes reagent. The assay was then synchronized by centrifugation (170 × g, 8 min at 4ºC) prior to monitoring chemiluminescence at 120-s intervals for 1 h at 37ºC using a GENios fluorescent plate reader (ReTirSoft Inc, Toronto, Ontario, Canada) and expressed as relative luminescent units (RLU). The kinetic data were collected using an XFlour4 macro within Microsoft Excel.

Bacterial killing assay

HuMC or PMN (4×10^5/well) were added to a 48-well plate containing bacteria, activation by *S. aureus* was synchronized by centrifugation (170 × g, 8 min at 4ºC), and assay plates were then incubated at 37˚C for 0.5-3 h. Bacteria in the absence of huMC or PMN were used as controls for bacterial viability. At each time point, samples were placed on ice, lysed (0.1% saponin, 15 min) and serial dilutions prepared before 50 µl aliquots were spread on LB agar (1.5%) plates, inverted and incubated overnight at 37˚C. The number of CFU/plate were counted and the percentage of bacteria killed by huMC or PMN was determined using the following equation; [CFU (samples containing host cells) ÷ CFU (samples without host cells)] × 100. In experiments where enzyme inhibitors were used, these were added 20 min prior to the start of the assay.

C*ell degranulation assay*

IgE-sensitized MCs (1×10^4/well) in HEPES buffer were added to a 96-well microplate containing bacteria (ratio of 2:1, 20:1, 100:1 MC) or Ag (streptavidin; 100 ng/ml) on ice.

Activation of huMCs by *S. aureus* was then synchronized by centrifugation (170 × g, 8 min at 4ºC) prior to incubation at 37˚C for 1 h. Degranulation was measured as percent release of β-hexosaminidase (β-hex) [20].

LTC_4 and PGD_2 measurements

IgE-sensitized MCs (2×10^5/well) in HEPES buffer were added to a 96-well microplate containing bacteria (ratio of 2:1, 20:1, 100:1 MC) or Ag (streptavidin; 100ng/ml) on ice and assays were synchronized by centrifugation (170 × g, 8 min at 4ºC). Following a 1 h incubation at 37˚C, cell-free supernatants (supts) were removed and analyzed for LTC_4 and PGD_2 by enzyme immunoassay (EIA) (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer's instructions. Supernatants were diluted 1/1000 prior to analysis of PGD_2 release.

Chemokine and cytokine focused SuperArrays

Total RNA was isolated (Superarray, Frederick, MD), 6 μg treated for genomic DNA (gDNA) contamination and reverse transcribed (Superarray). For each quantitative PCR (qPCR) assay, cDNA (50 ng/25 μl of PCR mastermix) was added to a Superarray plate containing primer sets for chemokines (*CCL1-19*, *CXCL1-13*; a total of 84 genes), cytokines (*IL1-25*, *CSF1-2*, *IFNs*, *TGFs*, *TNF*, *TNFSF10-14*, *BMPs*, *GDFs*; a total of 84 genes) and housekeeping genes (HKGs) (*B2M*, *HPRT1*, *RPL13A*, *GAPDH*, *ACTB*) as designed by the manufacturer (Superarray). As a control, DNA and RNA preparations were run on a quality control plate prior to running samples on the pathway-focused Superarrays. All reactions were performed on single samples for 40 cycles as per the manufacturer's

instructions and gene expression was analyzed using the Real-Time PCR cycler ABI PRISM 7700 (Applied Biosystems, Foster City, CA).

Real-time PCR analysis

Total RNA was isolated (RNeasy, Qiagen Inc. Valencia, CA), 2 μg treated for gDNA contamination and reverse transcribed (Qiagen Inc. Valencia, CA). For each quantitative PCR assay, cDNA (50 ng) was mixed with PCR mastermix containing primer sets for CD14, CD36, TLR2, TLR6, MSR1, SCARB1 and SCARB2 or the HKG ACTB as designed by the manufacturer (Qiagen Inc Valencia, CA). As a control, RNA that had not been reverse transcribed to cDNA was used in some PCR reactions. All reactions were performed in triplicate for 40 cycles and gene expression analyzed using the Real-Time PCR cycler ABI PRISM 7700 (Applied Biosystems, Foster City, CA).

Cytokine measurements

HuMC (4×10^5/well) were added to a 48-well plate containing bacteria at a ratio of 20:1 huMC and cultures were synchronized by centrifugation (170 × g, 8 min at 4ºC). Cultures were then placed at 37˚C for 1 h to 24 h and cell-free supernatants analyzed for CXCL8 and GM-CSF by ELISA according to manufacturer's instructions (R&D Systems, Minneapolis, MN).

Receptor expression by flow cytometric analysis

HuMC were washed 3X (PBS+0.1%BSA) and 100 μl (1×10^6/ml) aliquots were incubated with the following Abs for 20 min at 20ºC: APC-conjugated mouse anti-human CD14

(clone 61D3, subclass $IgG_{1\kappa}$), PE-conjugated mouse anti-human TLR2 (clone TL2.1, subclass IgG_{2a}), FITC-conjugated mouse anti-human CD36 (clone NL07, subclass IgM), rat anti-human TLR6 (clone hPer6, subclass $IgG_{2a\kappa}$) (eBiosciences) followed by PE-conjugated goat anti-rat IgGγ (Southern Biotech), mouse anti-human SR-AI/MSRI (clone 351615, subtype IgG_{2b}) then APC-conjugated goat anti-mouse IgG_{2b} (Southern Biotech) or rabbit polyclonal anti-human SCARB1 (Lifespan Biosciences) followed by PE-conjugated goat anti-rabbit IgG (Southern Biotech), PE-conjugated mouse anti-human CR3 (CD11b) (subclass $IgG_{1\kappa}$), PE-conjugated mouse anti-human CR4 (CD11c) (clone 3.9, subclass $IgG_{1\kappa}$), PE-conjugated anti-human or PE-labelled mouse anti-human CD49d (integrin α_4) (clone 9F10, subclass $IgG_{1\kappa}$) PE-conjugated mouse anti-human CD49e (integrin α_5) (clone SAM-1, subclass IgG_{2b}), FITC-conjugated mouse anti-human Integrin CD51/61 ($\alpha_V\beta_3$) (clone 23C6, subclass $IgG_{1\kappa}$) or PE-conjugated mouse anti-human CD29 (β_1) (clone MEM-101A, subclass IgG_1) (eBioscience), PE-labelled mouse anti-human dectin-1 (clone 259931, subclass IgG_{2b}) (R&D systems) or PE-conjugated mouse anti-human CD205 (clone MG38, subclass IgG_{2b}) or APC-labelled mouse anti-human CD206 (clone 19.2, subclass $IgG_{1\kappa}$) (BD). Cells were then washed, resuspended in PBS + 0.1%BSA and analysed using a FACScalibur (Becton Dickinson, San Jose, CA) and CellQuest software (BD) of 10,000 events in a CD117/FcεRI double positive gated area.

Binding of S. aureus to huMC by flow cytometric analysis

S. aureus was grown to mid-log phase (OD_{600} <1), heat killed (60ºC for 0.5 h (1×10^9/ml in PBS)), washed 2X in PBS and resuspended in a FITC (0.1 mg/ml in $NaHCO_3$ [0.1M]) solution and incubated for 40 min with rotation. Following 3X washes, the FITC-labelled

bacteria were resuspended in appropriate media and stored on ice until required. The binding of *S. aureus* to huMC was determined by incubating huMC with increasing ratios of FITC-labelled bacteria (20:1, 100:1; bacteria:huMC) for 1 h at 4ºC. After washing 2X (PBS + 0.1% BSA), huMCs were resuspended in PBS + 0.1% BSA (100 μl) and analysed for FL1 using a FACScalibur (Becton Dickinson, San Jose, CA) and CellQuest software (BD).

Data presentation and statistical analyses

Real-time kinetic readings and grouped data are from a minimum of three separate experiments performed in duplicate using 1 - 3 donors to obtain CD34+ derived huMCs. The area under the curve (AUC) data for total ROS production were determined after baseline subtraction by Prism (GraphPad Software, San Diego, CA). Differences between groups were tested for statistical significance by SigmaPlot. Where data were normally distributed, two-way repeated measures ANOVA with Holm-Sidak correction for multiple comparisons was used. For non-parametric data, a Kruskal-Wallis one way analysis of variance on ranks with Bonferroni correction for multiple comparisons or Wilcoxon signed rank test was used.

Results

IFN-γ enhances ROS generation and killing of *S. aureus* by huMCs

We initially investigated the ability of huMCs to generate ROS and kill *S. aureus* in response to Th1 and Th2 cytokines as might be present as inflammation progresses. As shown in Figure 1a, pre-incubation of huMCs with IFN-γ, TNFα or GM-CSF, but not IL-4, resulted in *S. aureus*-dependent ROS generation. ROS production in huMCs exposed to *S. aureus* and IFN-γ was greater than that in cells exposed to *S. aureus* combined with GM-CSF or TNFα (Fig. 1a). Killing by untreated huMC was 2% compared to 25% in cells treated with IFN-γ ($p < 0.001$). By comparison, TNFα, GM-CSF or IL-4 had a minimal effect on the ability of huMC to kill *S. aureus* (Fig. 1b). These data demonstrate that of the cytokines tested, IFN-γ was by far the most effective at enhancing ROS production and promoting killing of *S. aureus* by huMC.

Since we have demonstrated that FcεRI- and FcγR-dependent intracellular ROS production in huMCs are 5LO- and COX-dependent [20], we next examined the mechanisms by which IFN-γ alters the production of intracellular and extracellular ROS by huMCs exposed to *S. aureus*. As shown in Figure 2a, there was little or no production of ROS by huMCs exposed to *S. aureus* alone. However, huMCs pre-exposed to IFN-γ produced ROS in response to *S. aureus*, and the rate of ROS production was maximal 10-20 min after stimulation (Fig. 2a).This *S. aureus*-dependent ROS generation was ratio dependent, and similar ROS levels were achieved with heat-killed bacteria. In contrast, coating of bacteria by serum opsonisation or with *S. aureus*-specific IgG elicited lower levels of ROS from huMCs (Fig. S1a-c). Also, *S. aureus*-derived PGN or LTA did not induce ROS production (Fig. S1d). The ability of *S. aureus* to trigger extracellular

This is the accepted version of the following article: Swindle EJ, Brown J, Radinger M, DeLeo FR, Metcalfe DE. IFN-gamma enhances the anti-bacterial and pro-inflammatory response of human mast cells to S. aureus. Immunology. 2015; 146(3):470-485 which has been published in final form at [http://onlinelibrary.wiley.com/doi/10.1111/imm.12524/full]

production of ROS in huMCs following treatment with IFNγ was verified by a Diogenes chemiluminescence assay (Fig. 2b).

We next explored the enzymes responsible for ROS production. Incubation of huMCs with the COX inhibitor FR122047, the 5LO inhibitor zileuton (ZT) or a combination of both caused a significant reduction in *S. aureus*-dependent DCF fluorescence (Fig. 2c & 2g) while the flavoenzyme inhibitor DPI and the NOS inhibitor L-NMMA had no inhibitory effect (Fig. 2e & 2g). ZT or FR122047 alone or in combination also had no effect (Fig. 2d & 2h) whilst DPI completely abrogated *S. aureus*-dependent Diogenes chemiluminescence in huMCs (Fig. 2f & 2h). Thus, 5LO and COX were the enzymes responsible for *S. aureus*-dependent intracellular ROS production, while extracellular ROS production was primarily NADPH oxidase-dependent in IFN-γ-treated huMCs.

We then compared IFN-γ-primed huMCs with PMNs for their ability to generate ROS and kill *S. aureus*. As shown in Figure 3a, PMNs, as expected, generated high levels of ROS following exposure to *S. aureus*. Generation of ROS by PMNs was approximately 50-fold greater than that of huMCs (Fig. 3c). Killing of *S. aureus* by PMNs was more rapid than that by huMCs (1.5 h for PMNs versus 3 h for huMCs) (Fig. S2a-b), where by 1.5 h there was a significant killing of *S. aureus* by PMNs, but little killing of the organism by huMCs (Fig 3b & d). These data are consistent with the conclusion that the reduced ability of huMC to generate ROS (compared with PMNs) limits the capacity to kill *S. aureus*.

We next investigated whether 5LO and COX inhibition would affect the killing of *S. aureus* by huMCs. IFNγ-primed huMCs were incubated with ZT, FR122047 or a combination of both inhibitors prior to *S. aureus* exposure for 3 h. As shown in Figure 3f,

pre-incubation of huMCs with 5LO or COX inhibitors had no effect on the killing of *S. aureus*. These findings were confirmed with PMNs (Fig. 3e). Assessment of the role of NADPH oxidase in the killing of *S. aureus* by huMCs and PMNs was hindered by the fact that DPI was toxic to *S. aureus*. These data demonstrate that IFN-γ-primed huMCs are capable of killing *S. aureus*, albeit less efficiently than PMNs and that intracellular ROS derived from 5LO and COX are not involved. These data are consistent with the conclusion that extracellular ROS-derived from NAPDH oxidase is involved in killing of bacteria by IFN-γ-primed huMCs.

IFN-γ enhances the immediate secretory responses of huMCs to S. aureus

Because IFN-γ is known to enhance mediator release following FcγRI aggregation of IFN-γ-treated huMCs[22], we next examined mediator release from huMCs treated with IFN-γ and exposed to *S. aureus*. HuMCs exposed to *S. aureus* in the absence of IFNγ released negligible β-hex (Fig. 4a). However, incubation of huMC with IFN-γ prior to *S. aureus* stimulation elicited a significant increase in percent β-hex release (from 4.36 ± 0.78 % to 14.40 ± 2.14 % at 20:1 ($p \leq 0.001$); from 5.78 ± 1.17 % to 26.90 ± 2.52 % at 100:1 ($p \leq 0.001$)), which was MOI-dependent (Fig. 4a). Similar results were obtained with histamine release (data not shown). Moreover, IFN-γ induced LTC_4 secretion by huMCs following exposure to *S. aureus* (at a ratio of 100 bacteria per huMC, LTC_4 secretion by huMCs increased from 2.35 ± 1.88 ng/ml to 13.68 ± 6.89 ng/ml, $p \leq 0.05$) (Fig. 4b). Similarly, IFN-γ caused a significant increase in *S. aureus*-dependent PGD_2 secretion (Fig. 4c). These data

demonstrate that IFN-γ induces a *S. aureus*-dependent release of β-hex, LTC_4 and PGD_2 by huMCs.

IFN-γ enhances production of chemokines and cytokines by huMCs exposed to *S. aureus*

Since IFN-γ enhanced the immediate secretory responses of huMCs to *S. aureus*, we next determined whether IFN-γ altered late secretory events. HuMCs were incubated with IFN-γ for 48 h prior to exposure to *S. aureus* (20:1) for 2 h, and changes in gene expression were analysed using cytokine and chemokine superarrays. The cytokine array revealed that IFN-γ treated huMCs had a decrease in expression of *CSF2* (0.18±0.06 fold change), *IL18* (0.16±0.08 fold change) and *IL1B* (0.19±0.07 fold change) while there were no alterations in baseline chemokine expression (data not shown). In response to *S. aureus*, huMCs showed a trend for increased expression of the chemokines *CCL1-4*, *CXCL8*, and the cytokines *CSF1-2*, *IL5*, *IL13*, *INHBA*, *TNF* and *TNFSF14* which were further enhanced by IFN-γ (Fig. 5a). The enhancing effect of IFN-γ on *S.aureus*-dependent CXCL8 mRNA expression was confirmed by quantitative real-time PCR (Fig. 5b). To determine whether increases in mRNA correlated with increased levels of protein, we measured CXCL8 and GM-CSF secretion in cell-free supernatants of *S. aureus*-stimulated huMCs cultured with IFN-γ. As shown in Figure 5c-d, exposure of huMCs to *S. aureus* induced CXCL8 and GM-CSF protein release as early as 2 h post stimulation and IFN-γ pre-treatment significantly enhanced CXCL8 secretion at 2-4 h and GM-CSF secretion at 2 h post *S. aureus* stimulation. These data are consistent with the conclusion that IFN-γ enhances the ability of huMCs to release cytokines and chemokines following *S. aureus* exposure.

This is the accepted version of the following article: Swindle EJ, Brown J, Radinger M, DeLeo FR, Metcalfe DE. IFN-gamma enhances the anti-bacterial and pro-inflammatory response of human mast cells to S. aureus. Immunology. 2015; 146(3):470-485 which has been published in final form at [http://onlinelibrary.wiley.com/doi/10.1111/imm.12524/full]

IFN-γ-treated huMCs have increased surface binding of *S. aureus* and have increased expression of receptors associated with bacterial recognition

Since it has been demonstrated that IFN-γ increases FcγRI surface expression, [21] we determined whether IFN-γ also upregulates receptors involved in bacterial recognition. To assess this, we initially investigated the binding of the bacterium to IFN-γ-treated huMCs using FITC-labelled *S. aureus* (FITC-SA) and flow cytometry and demonstrated that IFN-γ significantly increased the binding of *S. aureus* to huMCs (Figure 6a). We next investigated the transcript levels of receptors associated with bacterial recognition of *S. aureus* on huMCs by real-time PCR and flow cytometry including TLR2, TLR6, CD14 and CD36 and additional receptors that could potentially interact with *S. aureus*, including the scavenger receptors (mRNA/protein) (*MSR1*/SR-AI, *SCARB1*/SR-BI, *SCARB2*/SR-BII) [26], lectin receptors (*LY75*/CD205(DEC-205), *MRC1*/CD206(mannose), *CLEC7A*/dectin-1), complement receptors (I*TAGM*/CR3(CD11b), *ITAGX*/CR4(CD11c)) and integrin receptors (*ITAG4*/CD49d(α_4), *ITAG5*/CD49e(α_5), *ITAV*/CD51(α_V), *ITGB1*/CD29(β_1), *ITGB3*/CD61(β_3)) [27, 28]. HuMCs expressed mRNA for the majority of receptors tested with the exception of *TLR4* and *ITAG4* and IFNγ upregulated (fold change) the expression levels of *MSR1* (1.56±0.15), *SCARB2* (2.28±0.49), *TLR2* (2.59±0.38), *ITAG5* (2.56±0.24) and *LY75* (3.39±0.57) (Fig. S3).

To confirm whether changes in mRNA correlated with increased protein expression, we incubated huMCs with Abs specific for these receptors. HuMCs did not express CD14, CD36, TLR6, the alpha subunits of the complement receptors (CR3, CR4), lectin receptors

This is the accepted version of the following article: Swindle EJ, Brown J, Radinger M, DeLeo FR, Metcalfe DE. IFN-gamma enhances the anti-bacterial and pro-inflammatory response of human mast cells to S. aureus. Immunology. 2015; 146(3):470-485 which has been published in final form at [http://onlinelibrary.wiley.com/doi/10.1111/imm.12524/full]

(CD205, CD206, dectin-1) or CD49e, the integrin alpha chain. Further, IFNγ pre-treatment did not induce their expression (Fig. S4). In contrast, treatment of huMCs with IFN-γ caused a significant up regulation of ~~genes encoding~~ TLR2 and scavenger receptors (SR-AI, SR-BI/II), but not integrin receptors (CD49d, CD51/61 or CD29) (Fig. 6b-c). Hence, huMCs treated with IFN-γ have increased gene expression and protein for ~~levels of transcripts encoding~~ TLR2 and scavenger receptors.

Integrin receptors and not TLR2 or SR-AI mediate binding of *S. aureus* to huMCs and the subsequent pro-inflammatory response.

The contribution of bacterial recognition receptors to binding of S. aureus was next investigated by incubating huMCs with blocking Abs to TLR2 or SR-AI. However, the ability of MCs to bind *S. aureus* was unaltered by pre-incubation of huMCs with blocking Abs to TLR2 or SR-AI. Furthermore, binding of FITC-SA to tlr2-, msr1- or scarb1-deficient mBMMCs was also unchanged (Fig. 7a-c). These data indicate that TLR2, SR-AI and SR-BI are not involved in the recognition of S. aureus by MCs. However, binding of FITC-SA to huMCs was blocked completely by pre-incubation with either the isotype (rabbit serum) or blocking Ab for SR-BI/II, (Fig. 7c, 2nd panel). To further investigate the contribution of scavenger receptors, we pre-incubated huMCs with increasing concentrations of Poly I, Poly C or fucoidin (5-500 μg/ml) prior to FITC-SA, but no reduction in FITC-SA binding was observed (data not shown).

Since the scarb1-deficient mBMMC lacked altered FITC-SA binding, we speculated that a component of rabbit serum was inhibiting FITC-SA binding to huMC. We therefore tested the effect of vitronectin, fibronectin (Fn) and BSA (as a control) on SA-binding to

This is the accepted version of the following article: Swindle EJ, Brown J, Radinger M, DeLeo FR, Metcalfe DE. IFN-gamma enhances the anti-bacterial and pro-inflammatory response of human mast cells to S. aureus. Immunology. 2015; 146(3):470-485 which has been published in final form at [http://onlinelibrary.wiley.com/doi/10.1111/imm.12524/full]

huMCs in the presence or absence of IFN-γ. As shown in Figure 8a, Fn reduced both the IFN-γ-enhanced binding of FITC-SA to huMCs as well as in untreated huMCs, while BSA had no effect. Vitronectin had no effect on FITC-SA binding in control or IFN-γ-treated huMCs (data not shown). Furthermore, blocking integrin-β_1 but not -α_4 resulted in complete blockade of FITC-SA binding to huMCs (Fig. 8b). These data are consistent with the involvement of integrin receptors, specifically β_1, in the binding of *S. aureus* to huMC in the presence and absence of IFN-γ.

We next determined whether the recognition of *S. aureus* by huMCs was indeed mediated via integrin receptors and if such an interaction could influence subsequent secretory responses. Pre-incubation of both control and IFN-γ treated huMCs with Fn resulted in a concentration-dependent inhibition of FITC-SA binding, with complete blockade at 100 μg/ml (Fig. 9). Pre-incubation of huMCs with an integrin-β_1 blocking Ab did not alter FITC-SA binding to control cells but did resulted in > 80% reduction of such binding to IFN-γ-treated huMCs (Fig. 9a). These data suggest that binding of *S. aureus* to huMCs is mediated via integrin-β_1 under certain conditions including exposure to IFN-γ.

Next, we investigated the involvement of integrin receptors in *S. aureus*-dependent CXCL8 secretion by huMCs. Following pre-incubation with Fn, there was a concentration-dependent inhibition of CXCL8 secretion in control and IFN-γ-treated huMCs (Fig. 9b). A blocking Ab specific for integrin-β_1 partially inhibited *S. aureus*-dependent CXCL8 secretion in IFN-γ-treated huMCs (Fig. 9b). These data together suggest a major role for integrin-β_1 in the recognition of *S. aureus* by huMC and a partial role for integrin-β_1 in the secretory responses to the pathogen.

Discussion

In this study we demonstrated that IFN-γ enhances the antibacterial response of huMCs to *S. aureus* ~~through~~ and increased ROS production. IFN-γ pre-treatment also induced huMC degranulation and enhanced eicosanoid and pro-inflammatory chemokine and cytokine secretion by *S. aureus*. IFN-γ increased binding of *S. aureus* to huMCs and upregulated receptors associated with bacterial recognition. Blockade of β_1 integrin completely abrogated binding of *S. aureus* with huMCs and partially inhibited cytokine secretion by these cells.

We investigated the ability of Th1 and Th2 cytokines to alter/enhance generation of extracellular ROS by huMCs, and in turn, their ability to kill *S. aureus*. We determined that while the Th2 cytokine IL-4 was capable of eliciting ROS production by huMCs, there was no effective killing of *S. aureus*. Of the Th1 cytokines tested, only IFN-γ caused significant generation of extracellular ROS by huMCs, and promoted killing of *S. aureus*. These data are similar to what has been reported for PMNs, where GM-CSF, TNFα and IFN-γ enhance fMLP-dependent ROS production [29, 30]. The small amount of ROS generated by pre-incubation of huMCs with IL-4 is in contrast to PMNs, which do not generate ROS in response to IL-4 [31].

We have reported that IFN-γ-treated huMCs generate enhanced intracellular ROS following stimulation with IgG-coated latex beads and that the enzymes responsible are 5LO and COX1. This is in contrast to neutrophils, which primarily use NADPH oxidase for ROS production [20]. In the present study, we demonstrate that IFN-γ enhances

intracellular ROS production induced by *S. aureus* and that this is also was 5LO- and COX1-dependent. Extracellular ROS production by huMCs, in contrast, was mediated by NADPH oxidase, since the NADPH oxidase inhibitor DPI and the antioxidant SOD completely abrogated the extracellular ROS production. These findings support the conclusion that different enzymes are responsible for the generation of intracellular vs extracellular ROS in *S. aureus*-stimulated huMCs. We next investigated the role of 5LO and COX1 in mediating killing of *S. aureus* by huMCs. Using inhibitors of these enzymes, we demonstrated 5LO and COX1 were not involved, indicating that extracellular and not intracellular ROS is involved in killing of the bacterium. The investigation of NADPH oxidase was hampered by the fact that the enzyme inhibitor DPI had direct antibacterial activity in the absence of host cells. We did not employ other ROS scavengers/inhibitors such as L-NAC or apocynin, as these may have non-specific effects.

We also investigated inflammatory mediator secretion by huMCs in response to *S. aureus* ± IFN-γ. HuMCs did not degranulate in response to *S. aureus* but generated low levels of PGD_2. In the presence of IFN-γ, degranulation occurred and production of LTC_4 and PGD_2 release was enhanced. This is consistent with a report that rat peritoneal MCs (RPMCs) degranulate in response to *E. coli* [32] and that rats challenged with *S. aureus* have a reduction in granular MCs [33]. Additionally, mice challenged with PGN show increased histamine levels [7]. MC degranulation has also been reported to occur in response to exotoxins from *S. aureus* including δ-toxin [5] and in response to superantigens [34]. Our data demonstrate that MC degranulation can occur in direct response to *S. aureus* following pre-treatment of huMCs with IFN-γ. This suggests that the ability of huMCs to

degranulate in response to bacterial stimuli is enhanced in the context of inflammatory lesions with a Th1 profile such as occurs in chronic lesions of atopic dermatitis (AD) [35].

IFN-γ also enhanced LTC_4 and PGD_2 production induced by *S. aureus* in huMCs. There is little prior evidence of eicosanoid generation induced by *S. aureus*. RPMCs show LTC_4 generation but no degranulation in response to *S. aureus*-derived PGN [36] while in humans Protein A (enterotoxins from *S. aureus*) induced histamine release, leukotriene and prostaglandin production from nasal polyps [37].

Since IFN-γ is known to upregulate FcγRI on huMCs [21], we hypothesised it would also upregulate receptors for bacterial recognition on huMCs. We used qPCR and flow cytometry to investigate expression of a range of receptors both at baseline and induced by IFN-γ. We determined that huMCs increased expression of TLR2 and scavenger receptors following IFN-γ exposure and had high baseline levels of integrin receptors. However, using blocking Abs to these receptors or mBMMCs deficient in these receptors, there was no change in SA-FITC binding, suggesting that these receptors are not involved in the recognition and binding of *S. aureus* by huMCs. This is in agreement with a report demonstrating that *S. aureus* binding to CBMCs is not dependent on TLR2 [13]. There has been no investigation to date regarding the role of scavenger receptors in the huMC response to *S. aureus* but these receptors are known to interact with *S. aureus* in certain cells types [26]. We determined that the integrin β_1 was critical for *S. aureus* binding to IFN-γ-treated huMCs using blocking Abs to this receptor or by blocking integrin receptors via Fn. IFNγ did not increase the mRNA or protein levels of integrin β_1, but it is possible that IFN-γ promotes a conformational change of integrin β_1 or increases clustering

of integrin β_1 receptors resulting in increased binding of bacteria or activation intracellularly. *S. aureus* has been reported to be internalised in mammalian cells by a 'sandwich mechanism' whereby Fn-binding proteins on the bacterium bind to Fn and the Fn-coated bacteria are then recognised by Fn-binding integrins on the cell surface, allowing internalisation [27]. Mouse fibroblasts with mutations in the integrin β_1 receptor have a reduced capacity to internalise *S. aureus* [38] and the epithelial cell line A549 using blocking Abs or siRNA for β_1 inhibited internalisation of *S. aureus* [39] Furthermore, $\alpha_5\beta_1$ integrin has also been demonstrated in epithelial cells to be the receptor involved in mediating cell death, and TNFα release by *S. aureus* α-toxin and pre-incubation with Fn reversed these effects [40]. These studies support our data demonstrating that in huMCs the integrin β_1 receptor is critical for the recognition and internalisation of *S. aureus*.

We also investigated the role of the integrin β_1 in *S. aureus*-mediated cytokine secretion in IFN-γ-treated huMCs and demonstrated a partial role in CXCL8 production. This is in contrast to the report by Rocha-de-Souza *et al* [13] who concluded that a combination of TLR2 and CD48 were involved in *S.aureus*-induced cytokine production. This difference may relate to the source of the huMC cultures where Rocha-de-Souza *et al* used cord blood-derived huMCs while we used peripheral blood-derived huMCs. We did use a TLR2 blocking Ab to investigate the potential role of TLR2 in CXCL8 production but the Ab was without effect (data not shown). We also used blocking Abs to scavenger receptors (*msr1* (SR-AI), *scarb1* (SR-BI/II)) as well as inhibitors of SR-BI (Block lipid transport-1). However, *tlr2*-deficient mBMMCs did have a 50% reduction in *S.aureus*-dependent CCL3 production and *scarb1*-deficient mBMMC had complete abrogation of

S.aureus-dependent CCL3 production (data not shown). Again, we observed no altered binding of *S. aureus* in these mBMMCs. These data appear to demonstrate a difference between mouse and human MCs in the receptors used to recognize *S. aureus* and those involved in mounting a cytokine response. Furthermore, it may not be a single receptor that is involved in recognition and cytokine secretion by mBMMCs but a combination of multiple receptors. Scavenger receptors do not mediated intracellular signalling and have to rely on co-receptors to induce their signals. In mBMMC, it may be that the combination of scavenger receptors with TLR2 and others are involved in the response to *S. aureus*. We were unable to investigate the response in integrin β_1-deficient mBMMCs as these mice are embryonically lethal [41]. Overall, our data demonstrates that integrin receptors are involved in the binding of *S. aureus* and the subsequent cytokine response in huMCs.

In summary, this study demonstrates that huMCs exposed to IFN-γ have an enhanced intracellular and extracellular ROS production in response to the bacterium *S. aureus* and relates to bacterial killing. IFN-γ increases the sensitivity of huMCs to *S. aureus* by inducing degranulation and upregulating eicosanoid, cytokine and chemokine secretion. *S. aureus* binding is increased to IFN-γ-treated huMCs and is mediated by integrin receptors. The secretory response of huMCs to *S. aureus* is partially mediated by integrin receptors. These data show the complexity of the MC response in relationship to the cytokine environment and help explain the role of MCs in innate immunity and in inflammatory processes such as those involved in AD.

Acknowledgements

EJS designed and performed experiments, analyzed data and composed the manuscript. JB and MR provided some of the mast cell cultures and helped with PCRs and ELISAs and edited the manuscript. FRD and DD critically reviewed data and assisted in writing the manuscript. This work was supported by the Division of Intramural Research, NIAID/NIH.

Conflict of Interest Disclosure

The authors declare no conflict of interest

References

1. Metcalfe DD, Baram D, Mekori YA. Mast cells. Physiol Rev 1997; 77(4):1033-79.
2. Chan CY, St John AL, Abraham SN. Plasticity in mast cell responses during bacterial infections. Curr Opin Microbiol 2012; 15(1):78-84.
3. Ronnberg E, Johnzon CF, Calounova G, *et al.* Mast cells are activated by Staphylococcus aureus in vitro but do not influence the outcome of intraperitoneal Staphylococcus aureus infection in vivo. Immunology 2014; 143:155-63.
4. McCurdy JD, Lin TJ, Marshall JS. Toll-like receptor 4-mediated activation of murine mast cells. J Leukoc Biol 2001; 70(6):977-84.
5. Nakamura Y, Oscherwitz J, Cease KB, *et al.* Staphylococcus delta-toxin induces allergic skin disease by activating mast cells. Nature 2013; 503(7476):397-401.
6. Mullaly SC, Kubes P. The role of TLR2 in vivo following challenge with Staphylococcus aureus and prototypic ligands. J Immunol 2006; 177(11):8154-63.
7. Feng BS, He SH, Zheng PY, Wu L, Yang PC. Mast cells play a crucial role in Staphylococcus aureus peptidoglycan-induced diarrhea. Am J Pathol 2007; 171(2):537-47.
8. Jawdat DM, Rowden G, Marshall JS. Mast cells have a pivotal role in TNF-independent lymph node hypertrophy and the mobilization of Langerhans cells in response to bacterial peptidoglycan. J Immunol 2006; 177(3):1755-62.
9. Malaviya R, Gao Z, Thankavel K, van der Merwe PA, Abraham SN. The mast cell tumor necrosis factor alpha response to FimH-expressing Escherichia coli is mediated by the glycosylphosphatidylinositol-anchored molecule CD48. Proc Natl Acad Sci U S A 1999; 96(14):8110-5.
10. Arock M, Ross E, Lai-Kuen R, Averlant G, Gao Z, Abraham SN. Phagocytic and tumor necrosis factor alpha response of human mast cells following exposure to gram-negative and gram-positive bacteria. Infect Immun 1998; 66(12):6030-4.

11. Bischoff SC, Lorentz A, Schwengberg S, Weier G, Raab R, Manns MP. Mast cells are an important cellular source of tumour necrosis factor alpha in human intestinal tissue. Gut 1999; 44(5):643-52.
12. Kulka M, Fukuishi N, Rottem M, Mekori YA, Metcalfe DD. Mast cells, which interact with Escherichia coli, up-regulate genes associated with innate immunity and become less responsive to Fc(epsilon)RI-mediated activation. J Leukoc Biol 2006; 79(2):339-50.
13. Rocha-de-Souza CM, Berent-Maoz B, Mankuta D, Moses AE, Levi-Schaffer F. Human mast cell activation by Staphylococcus aureus: interleukin-8 and tumor necrosis factor alpha release and the role of Toll-like receptor 2 and CD48 molecules. Infect Immun 2008; 76(10):4489-97.
14. Abel J, Goldmann O, Ziegler C, *et al.* Staphylococcus aureus evades the extracellular antimicrobial activity of mast cells by promoting its own uptake. J Innate Immun 2011; 3(5):495-507.
15. von Kockritz-Blickwede M, Goldmann O, Thulin P, Heinemann K, Norrby-Teglund A, Rohde M, Medina E. Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation. Blood 2008; 111(6):3070-80.
16. Branitzki-Heinemann K, Okumura CY, Vollger L, Kawakami Y, Kawakami T, Naim HY, Nizet V, Von Kockritz-Blickwede M. A novel role for the transcription factor HIF-1alpha in the formation of mast cell extracellular traps. Biochem J 2012; 446(1):159-63.
17. Malaviya R, Ross EA, MacGregor JI, Ikeda T, Little JR, Jakschik BA, Abraham SN. Mast cell phagocytosis of FimH-expressing enterobacteria. J Immunol 1994; 152(4):1907-14.
18. Swindle EJ, Hunt JA, Coleman JW. A comparison of reactive oxygen species generation by rat peritoneal macrophages and mast cells using the highly sensitive real-time chemiluminescent probe pholasin: inhibition of antigen-induced mast cell degranulation by macrophage-derived hydrogen peroxide. J Immunol 2002; 169(10):5866-73.
19. Swindle EJ, Metcalfe DD, Coleman JW. Rodent and human mast cells produce functionally significant intracellular reactive oxygen species but not nitric oxide. J Biol Chem 2004; 279(47):48751-9.
20. Swindle EJ, Coleman JW, DeLeo FR, Metcalfe DD. FcepsilonRI- and Fcgamma receptor-mediated production of reactive oxygen species by mast cells is lipoxygenase- and cyclooxygenase-dependent and NADPH oxidase-independent. J Immunol 2007; 179(10):7059-71.
21. Okayama Y, Kirshenbaum AS, Metcalfe DD. Expression of a functional high-affinity IgG receptor, Fc gamma RI, on human mast cells: Up-regulation by IFN-gamma. J Immunol 2000; 164(8):4332-9.
22. Okayama Y, Hagaman DD, Metcalfe DD. A comparison of mediators released or generated by IFN-gamma-treated human mast cells following aggregation of Fc gamma RI or Fc epsilon RI. J Immunol 2001; 166(7):4705-12.
23. Woolhiser MR, Okayama Y, Gilfillan AM, Metcalfe DD. IgG-dependent activation of human mast cells following up-regulation of FcgammaRI by IFN-gamma. Eur J Immunol 2001; 31(11):3298-307.

24. Okayama Y, Tkaczyk C, Metcalfe DD, Gilfillan AM. Comparison of Fc epsilon RI- and Fc gamma RI-mediated degranulation and TNF-alpha synthesis in human mast cells: selective utilization of phosphatidylinositol-3-kinase for Fc gamma RI-induced degranulation. Eur J Immunol 2003; 33(5):1450-9.
25. Okumura S, Kashiwakura J, Tomita H, Matsumoto K, Nakajima T, Saito H, Okayama Y. Identification of specific gene expression profiles in human mast cells mediated by Toll-like receptor 4 and FcepsilonRI. Blood 2003; 102(7):2547-54.
26. Peiser L, Mukhopadhyay S, Gordon S. Scavenger receptors in innate immunity. Curr Opin Immunol 2002; 14(1):123-8.
27. Hauck CR, Ohlsen K. Sticky connections: extracellular matrix protein recognition and integrin-mediated cellular invasion by Staphylococcus aureus. Curr Opin Microbiol 2006; 9(1):5-11.
28. Takada Y, Ye X, Simon S. The integrins. Genome biology 2007; 8(5):215.
29. Dewas C, Dang PM, Gougerot-Pocidalo MA, El-Benna J. TNF-alpha induces phosphorylation of p47(phox) in human neutrophils: partial phosphorylation of p47phox is a common event of priming of human neutrophils by TNF-alpha and granulocyte-macrophage colony-stimulating factor. J Immunol 2003; 171(8):4392-8.
30. Tennenberg SD, Fey DE, Lieser MJ. Oxidative priming of neutrophils by interferon-gamma. J Leukoc Biol 1993; 53(3):301-8.
31. Reglier-Poupet H, Hakim J, Gougerot-Pocidalo MA, Elbim C. Absence of regulation of human polymorphonuclear oxidative burst by interleukin-10, interleukin-4, interleukin-13 and transforming growth factor-beta in whole blood. European cytokine network 1998; 9(4):633-8.
32. Malaviya R, Ross E, Jakschik BA, Abraham SN. Mast cell degranulation induced by type 1 fimbriated Escherichia coli in mice. J Clin Invest 1994; 93(4):1645-53.
33. Sudo LS, Betti F, Hanada S, Sertie JA, Zelante F. Lymphatic mast cell response and effect of compound 48/80 on popliteal lymph node reaction in rats following intracutaneous injection of Staphylococcus aureus. Agents and actions 1994; 42(3-4):135-40.
34. Zhang N, Holtappels G, Gevaert P, Patou J, Dhaliwal B, Gould H, Bachert C. Mucosal tissue polyclonal IgE is functional in response to allergen and SEB. Allergy 2011; 66(1):141-8.
35. Gittler JK, Shemer A, Suarez-Farinas M, *et al.* Progressive activation of T(H)2/T(H)22 cytokines and selective epidermal proteins characterizes acute and chronic atopic dermatitis. J Allergy Clin Immunol 2012; 130(6):1344-54.
36. Wierzbicki M, Brzezinska-Blaszczyk E. Diverse effects of bacterial cell wall components on mast cell degranulation, cysteinyl leukotriene generation and migration. Microbiol Immunol 2009; 53(12):694-703.
37. Patou J, Gevaert P, Van Zele T, Holtappels G, van Cauwenberge P, Bachert C. Staphylococcus aureus enterotoxin B, protein A, and lipoteichoic acid stimulations in nasal polyps. J Allergy Clin Immunol 2008; 121(1):110-5.
38. Fowler T, Johansson S, Wary KK, Hook M. Src kinase has a central role in in vitro cellular internalization of Staphylococcus aureus. Cell Microbiol 2003; 5(6):417-26.

39. Wang JH, Zhang K, Wang N, Qiu XM, Wang YB, He P. Involvement of phosphatidylinositol 3-Kinase/Akt signaling pathway in beta1 integrin-mediated internalization of Staphylococcus aureus by alveolar epithelial cells. J Microbiol 2013; 51(5):644-50.
40. Liang X, Ji Y. Involvement of alpha5beta1-integrin and TNF-alpha in Staphylococcus aureus alpha-toxin-induced death of epithelial cells. Cell Microbiol 2007; 9(7):1809-21.
41. Stephens LE, Sutherland AE, Klimanskaya IV, Andrieux A, Meneses J, Pedersen RA, Damsky CH. Deletion of beta 1 integrins in mice results in inner cell mass failure and peri-implantation lethality. Genes Dev 1995; 9(15):1883-95.

Figure Legends

Figure 1. IFNγ and not IL-4, TNFα or GM-CSF primes huMCs for enhanced production of ROS and bacterial killing of *S. aureus*. huMCs were pre-treated with IFNγ (20 ng/ml), IL-4 (20 ng/ml), TNFα (20 ng/ml) or GM-CSF (20 ng/ml) for 48 h prior to the addition of *S. aureus* (20 bacteria:1 huMC). Extracellular ROS production was determined by Diogenes chemiluminescence for 1 h at 30-s intervals at 37°C (a) and bacterial killing determined after 3h incubation by counting CFUs of cell lysates on agar plates after 24h culture at 37°C (b). Results are shown as either kinetic data of single experiments of cells in the absence (thin lines) or presence of bacteria (bold lines) (a) or means ± S.E. (b) performed in triplicate and n = 3 independent experiments for 1 huMC donor. Differences between individual groups was tested for statistical significance by Kruskal-Wallis one way analysis of variance on ranks with Bonferroni correction for multiple comparisons (***, $p \leq 0.001$ for comparison with control cells not treated with cytokines).

Figure 2. IFNγ primes huMCs for enhanced ROS generation following *S. aureus* exposure. HuMCs were incubated with or without IFNγ (20 ng/ml) for 48 h then exposed

This is the accepted version of the following article: Swindle EJ, Brown J, Radinger M, DeLeo FR, Metcalfe DE. IFN-gamma enhances the anti-bacterial and pro-inflammatory response of human mast cells to S. aureus. Immunology. 2015; 146(3):470-485 which has been published in final form at [http://onlinelibrary.wiley.com/doi/10.1111/imm.12524/full]

to DCF-DA (20 μM) or medium for 20 min. After washing huMCs were incubated in the absence (a&b) or presence of FR122047 (300nM) (c&d), zileuton (20 μM) (c&d), FR122047 (300 nM) + zileuton (20 μM) (c&d), DPI (5 μM) (e&f) or L-NMMA (100 μM) (e) for 10 min prior to the addition of *S. aureus* (20:1) or HEPES buffer (negative control). Intracellular ROS production was determined by DCF fluorescence (a, c, e, g) while extracellular ROS production was determined by Diogenes chemiluminescence (b, d, f, h) for 1 h at 30-s intervals at 37°C. AUC data were calculated for each kinetic curve and averaged (g&h). Results are shown as either kinetic data of single experiments performed in duplicate, n = 4 (a, c, e) or 2 (b, d, f) independent experiments from 2 donors (a-f) or means ± S.E. for AUC data calculated for each kinetic curve, n = 4 independent experiments from 2 donors in duplicate (g) or n = 2, 2 donors in duplicate (h). Results are for huMCs treated with or without IFNγ (a&b) or IFNγ only (c-h). AUC data was calculated for inhibitor curves (g&h) and statistical significance was determined by Kruskal-Wallis one way analysis of variance on ranks with Bonferroni correction for multiple comparisons ($^{+++}$, $p \leq 0.001$ for comparison with control cells and *, $p \leq 0.05$ and ***, $p \leq 0.001$ for comparison with *S. aureus* treated cells).

Figure 3. IFNγ-primed huMCs generate less ROS and are less efficient at killing *S. aureus* than PMNs. HuMCs were pre-treated with IFNγ (20 ng/ml) for 48 h (c, d, e) or PMNs (a, b, f) were stimulated with *S. aureus* (20:1) or HEPES buffer (negative control) in the absence or presence of zileuton (20 μM), FR122047 (300 nM) or a combination of both (e&f). Diogenes chemiluminescence was then monitored for 60 min at 30-s intervals at 37°C (a&c) or bacterial killing was determined after 1.5h (c-d) or 3h (e-f) by counting

CFUs of cell lysates on agar plates after 24h culture at 37°C (c-f). Results are shown as either kinetic data of single experiments performed in duplicate, n = 3 separate huMC donors (a&b) or means ± S.E. of independent experiments performed in duplicate for 2 (e) or 4 (c) separate PMN donors or 3 independent experiments performed in duplicate on a single huMC donor *(*d&f). Differences between individual groups was tested for statistical significance by Wilcoxon signed rank test (*, $p \leq 0.05$ and **, $p \leq 0.01$ for comparison with *S. aureus* alone)

Figure 4. IFNγ enhances the immediate secretory responses of huMCs to *S. aureus*. HuMCs were pre-treated with or without IFNγ (20 ng/ml) for 48 h prior to the addition of *S. aureus* at a ratio of 2:1, 20:1 or 200:1 bacteria:huMC or HEPES buffer (negative control). Cell-free supts were collected after 1 h and quantified for net β-hexosaminidase (a) or LTC_4 (b) and PGD_2 (c) by competitive EIA. Results are means ± S.E. performed in duplicate, n =4 independent experiments for 2 separate donors. Differences between individual groups were tested for statistical significance by two way repeated measures ANOVA with Holm-Sidak correction for multiple comparisons ($^{+++}$, $p<0.01$; $^{+++}$, $p \leq 0.001$ for comparison of IFN-treated cells exposed to increasing ratios of *S. aureus* and *, $p>0.05$; **, $p>0.01$; ***, $p>0.001$ for comparison between untreated and IFNγ treated cells).

Figure 5. IFNγ enhances *S.aureus*-dependent CXCL8 and GM-CSF secretion by huMCs. HuMCs were pre-treated with IFNγ (20 ng/ml) for 48 h prior to the addition of *S. aureus* (20:1). After 2 h, mRNA was isolated, cDNA transcribed and qPCR performed for analysis of cytokine and chemokine induction by S. *aureus* using a SuperArray platform (a)

or real-time PCR (b). For quantification of CXCL8 and GM-CSF protein release, Cell-free supts were collected after 1-8 h and quantified for CXCL8 (c) and GM-CSF (d) by ELISA. Results are average fold change in mRNA levels relative to huMCs without bacterial exposure (a-b) or means ± S.E. performed in duplicate (c-d), n = 3-4 separate huMC donors. Differences between individual groups was tested for statistical significance by two way repeated measures ANOVA with Holm-Sidak correction for multiple comparisons (*, $p \leq 0.05$; p>0.01; ***, p>0.001 for comparison between control and IFNγ stimulated cells exposed to *S. aureus*).

Figure 6. IFNγ enhances *S. aureus* binding and increases TLR and scavenger receptors on huMCs. HuMCs were pre-treated with IFNγ (20 ng/ml) for 48 h prior to addition of FITC-labelled S. aureus (20:1) (a) or Abs to surface receptors (b) and analysed by flow cytometry. Results are representative flow cytometric traces or means ± S.E. performed in duplicate, n = 3 huMC donors. Differences between individual groups was tested for statistical significance by paired Student's t test (*, $p \leq 0.05$, **, $p \leq 0.01$ and ***, $p \leq 0.001$ for comparison between control and IFNγ stimulated cells).

Figure 7. The binding of *S. aureus* to MCs is not mediated via TLR2 or scavenger receptors. HuMCs were pre-treated with IFNγ for 48 h and incubated with blocking Abs to TLR2, SR-AI or SR-BI/II (a-c) for 1 h before either huMC or mBMMC from *tlr2*-deficeint mice (a), *msr1*-deficient mice (b) or *scarb1*-deficient mice (c) were incubated with FITC-SA for a further 1 h. Afterwhich MCs were analysed by flow cytometry for FL1

fluorescence. Results are representative flow cytometric traces or means ± S.E. performed in duplicate, n = 3 separate huMC or mBMMC donor.

Figure 8. Binding of *S. aureus* to huMCs is mediated by integrin receptors. HuMCs were incubated with IFNγ for 48h prior to a 1hr exposure to Fn (100 μg/ml) or BSA (100 μg/ml) (a) or blocking Abs to Integrin-β_1 or Integrin-α_4 (b). After which huMC were exposed to FITC-SA for a further 1hr. *S. aureus* binding to huMCs was then analzyed by flow cytometry for FL1 fluorescence. Results are representative flow cytometric traces or means ± S.E. performed in duplicate, n = 3-4 separate donors of huMCs. Differences between individual groups was tested for statistical significance by two way repeated measures ANOVA with Holm-Sidak correction for multiple comparisons (**, $p<0.01$; ***, $p<0.001$ for comparison of Fibronectin or anti-CD29 Ab-treated cells with control or isotype antibody respectively)

Figure 9. Blockade of integrin receptors on huMCs reduces *S. aureus*-dependent binding and CXCL8 secretion. HuMCs were incubated with IFNγ for 48h prior to a 1 h exposure to increasing concentrations of Fn (1-100 μg/ml) or Integrin-β_1 blocking Ab. Binding of FITC-SA was analysed by flow cytometry after 1h at 4°C (a) or *S. aureus*-induced CXCL8 secretion in cell-free supts after 2h at 37°C (b) was then determined. Results are means ± S.E. performed in duplicate, n = 3-4 huMC donors. Differences between individual groups were tested for statistical significance by two way repeated measures ANOVA with Holm-Sidak correction for multiple comparisons (+, $p<0.05$; ++, p

≤ 0.01 +++, p ≤ 0.001 for comparison between control and IFNγ stimulated cells and *, p<0.05; **, p ≤ 0.01 and ***, p ≤ 0.001 for comparison with *S. aureus* treated cells).

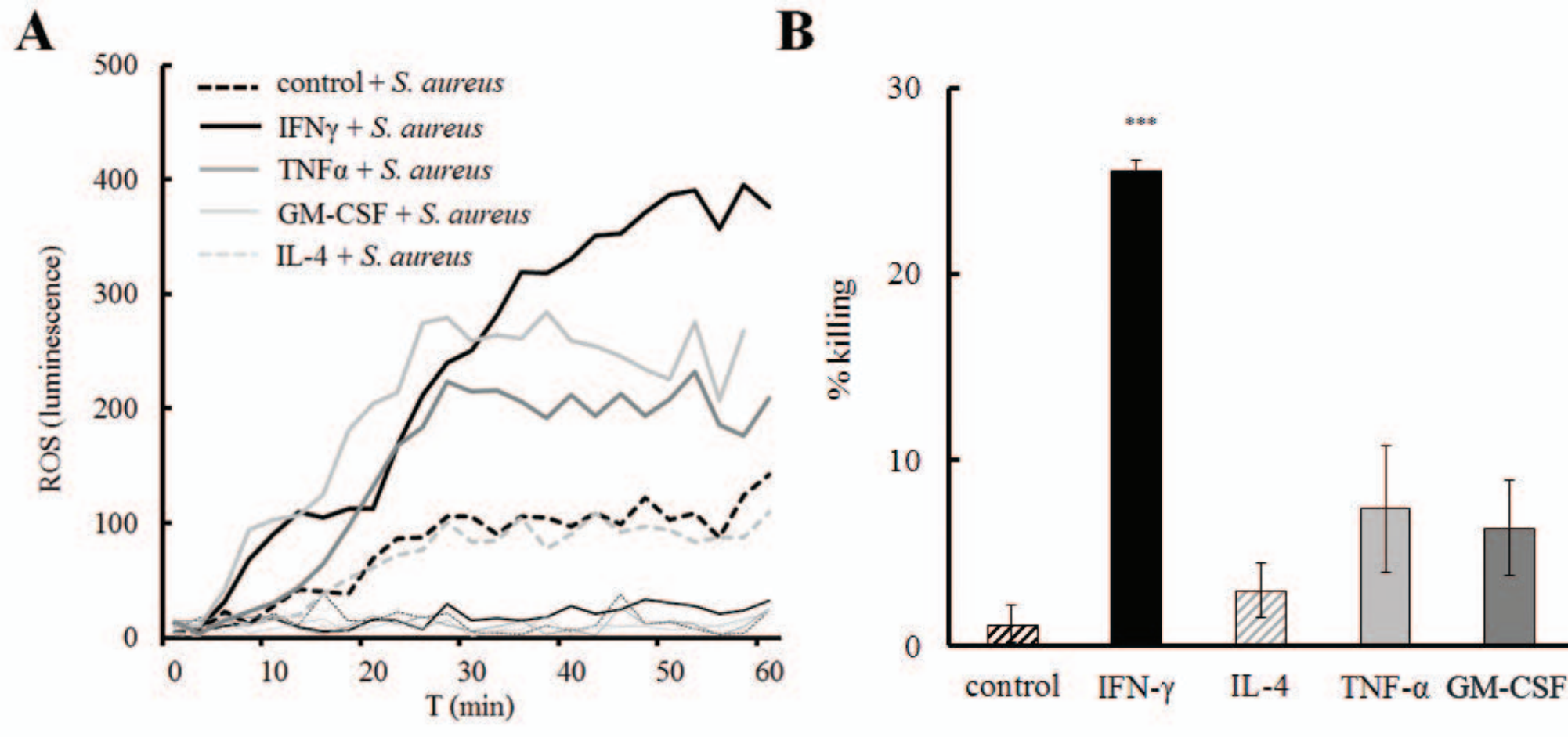
Supplementary Figure S1. huMCs incubated with pre-coated bacteria or cell wall components of *S. aureus* have reduced production of ROS. HuMCs were incubated with or without IFNγ (20 ng/ml) for 48 h then exposed to DCF-DA (20 μM) or medium for 20 min. After washing huMCs were incubated with increasing ratios of 2:1, 20:1 or 100:1 (*S. aureus*:huMC) (a), heat-killed *S. aureus* (b), opsonised or IgG-coated *S. aureus* (c) or the *S. aureus*-derived cell wall components PGN (100 ng/ml) or LTA (100 μg/ml) (d) or HEPES buffer (negative control). Intracellular ROS production was determined by DCF fluorescence for 1 h at 30-s intervals at 37°C. Results are shown as kinetic data of single experiments performed in duplicate and are representative of n=3 (a-c) or n=1 (d) from a single huMC donor.

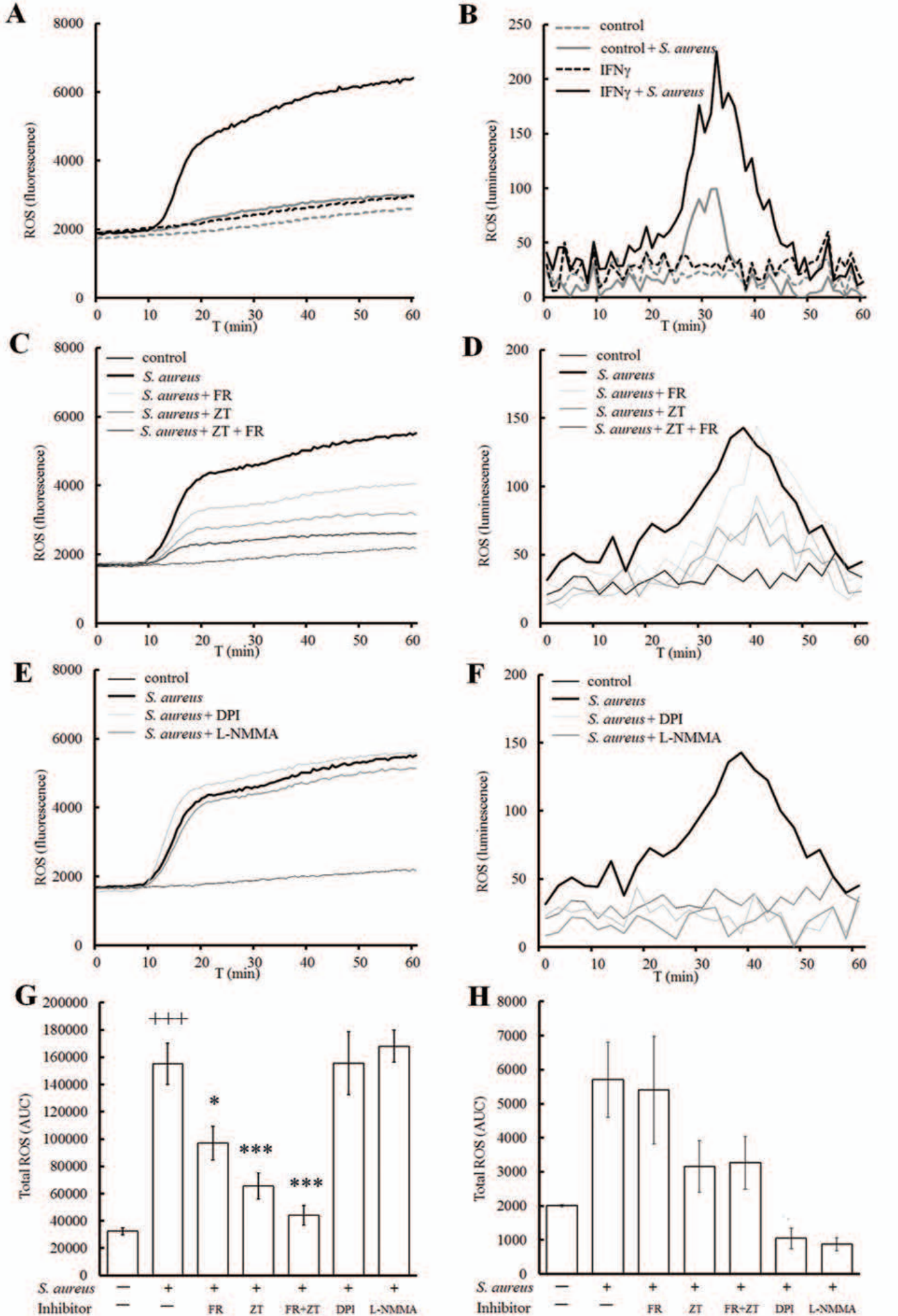
Supplementary Figure S2. A comparison of the time-dependent killing of *S. aureus* by PMNs or huMCs. PMNs (a) or huMCs pre-treated with IFNγ (20 ng/ml) for 48 h (b) were incubated with *S. aureus* (20:1) and bacterial killing was determined after 0 - 3h by counting CFUs of cell lysates on agar plates after 24h culture at 37°C. Results are shown as means ± S.E., n=2 donors of PMNs or huMCs.

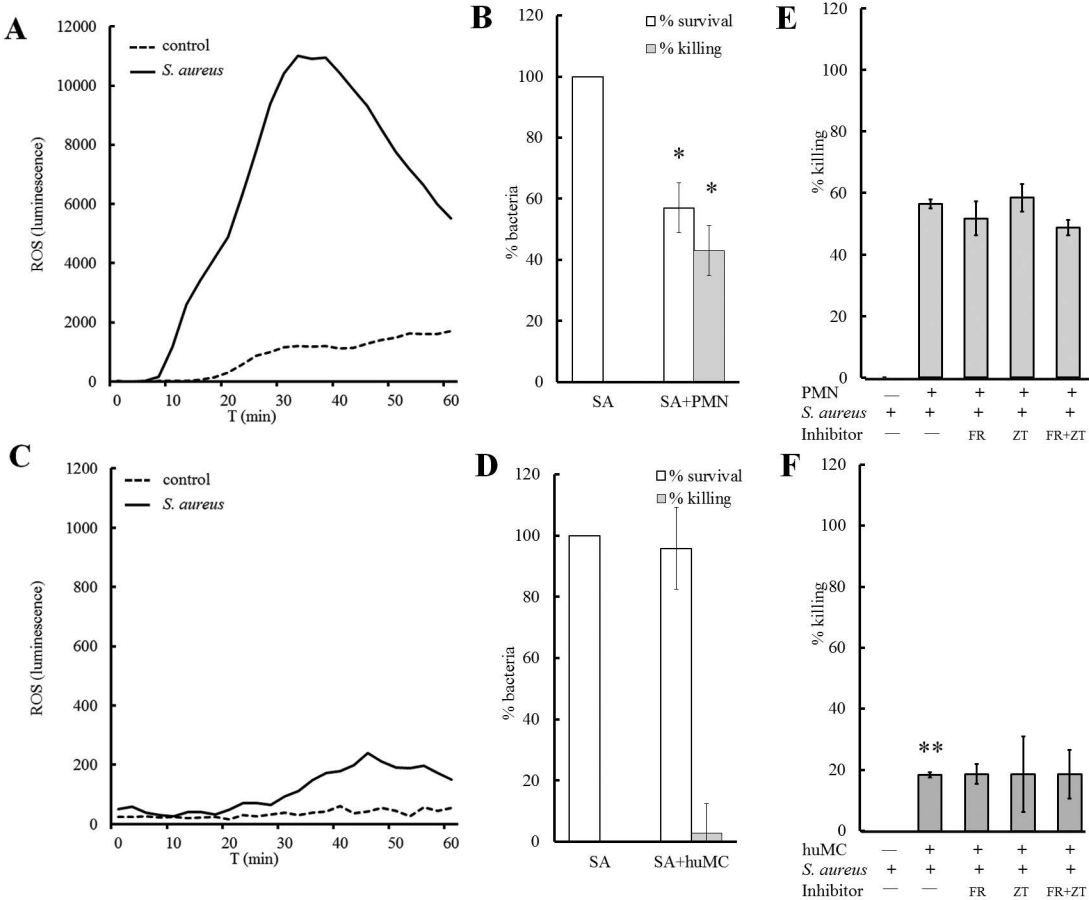
Supplementary Figure S3. IFNγ enhances gene expression of receptors associated with cell recognition of *S. aureus*. HuMCs were pre-treated with IFNγ (20 ng/ml) for 50 h prior to mRNA isolation and transcription of cDNA. qPCR was then performed for detection of

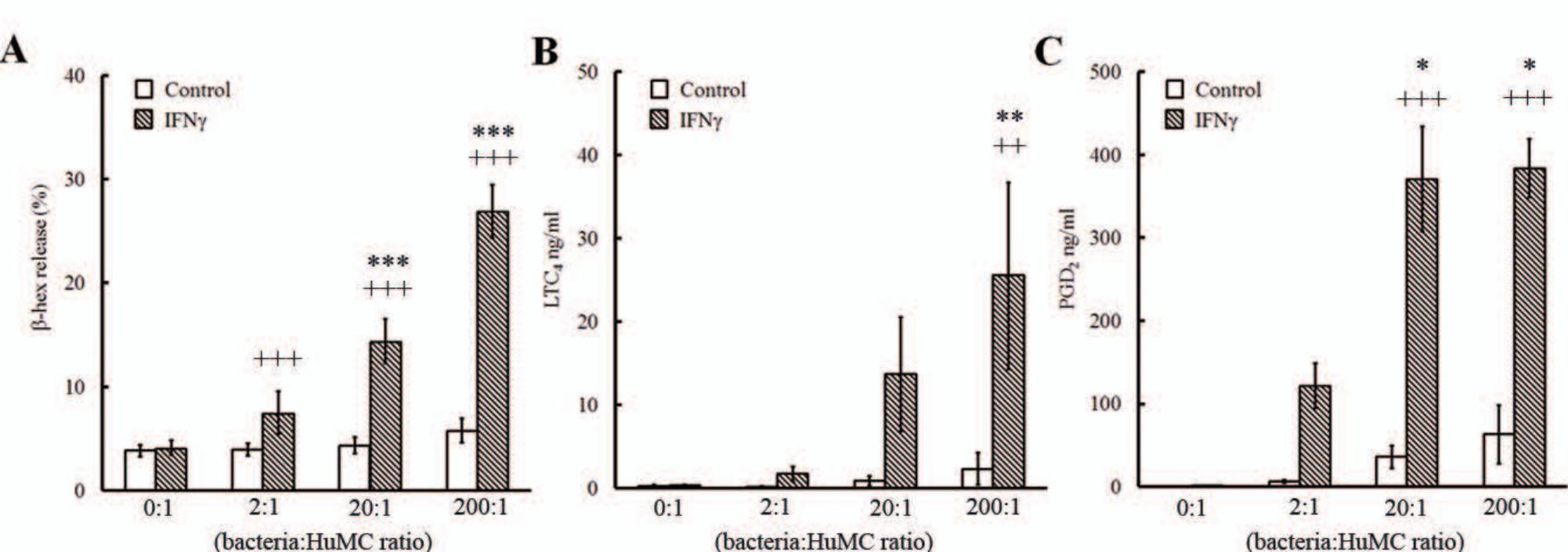
receptors associated with bacterial recognition including scavenger and TLRs (a) and integrin, complement and lectin receptors (b). Results are fold change compared to control cells and are means ± S.E. performed in triplicate on 4 donors of huMCs.

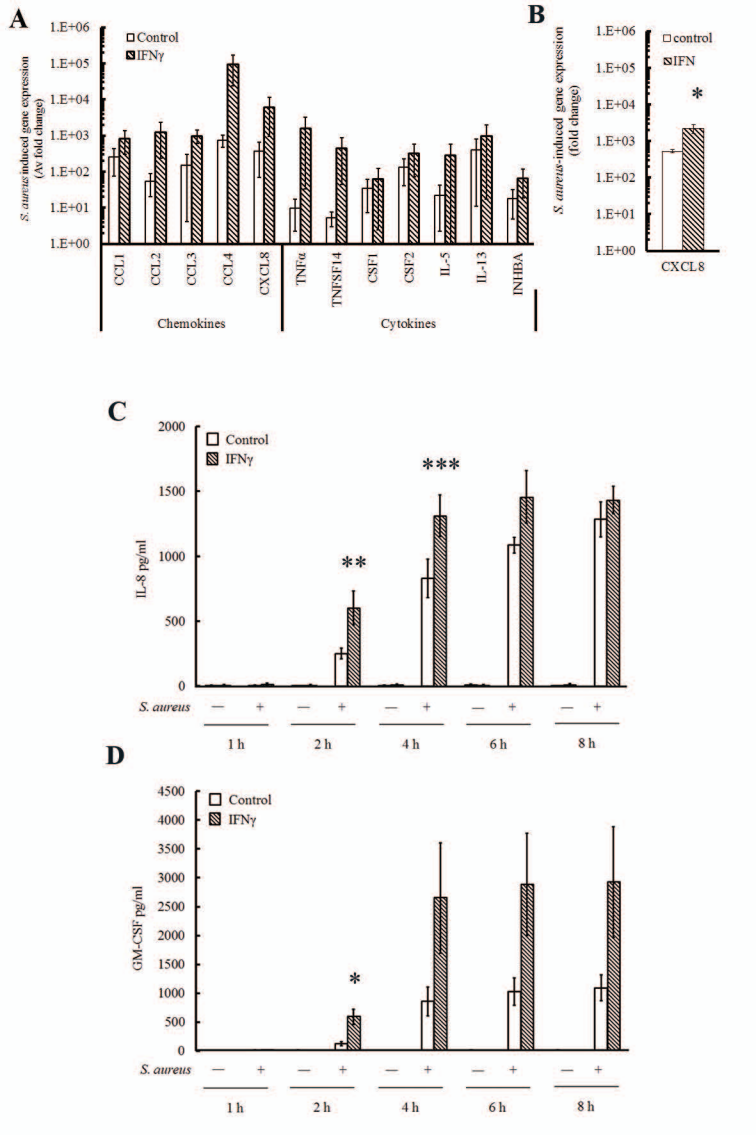
Supplementary Figure S4. IFNγ does not induce expression of CD markers on huMCs associated with *S. aureus* binding. HuMCs were pre-treated with IFNγ (20 ng/ml) for 48 h prior to addition of Abs to surface receptors and analysed by flow cytometry. Results are representative flow cytometric traces (a) or means ± S.E. (b) performed in duplicate, n = 3 huMC separate donors.

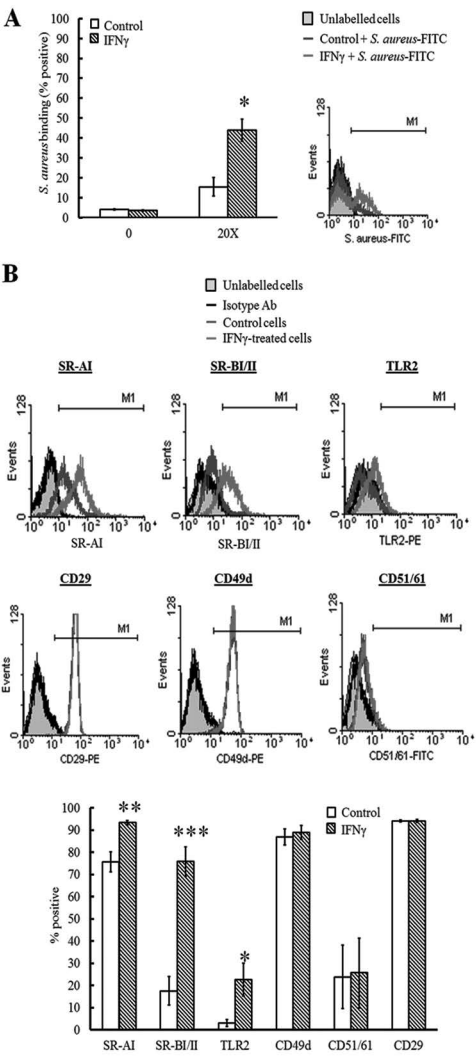

A
500
400
300
200
100
0
ROS (luminescence)
control + S. aureus
IFNγ + S. aureus
TNFα + S. aureus
GM-CSF + S. aureus
IL-4 + S. aureus
0
10
20
30
40
50
60
T (min)
B
30
20
10
0
% killing
***
control
IFN-γ
IL-4
TNF-α
GM-CSF

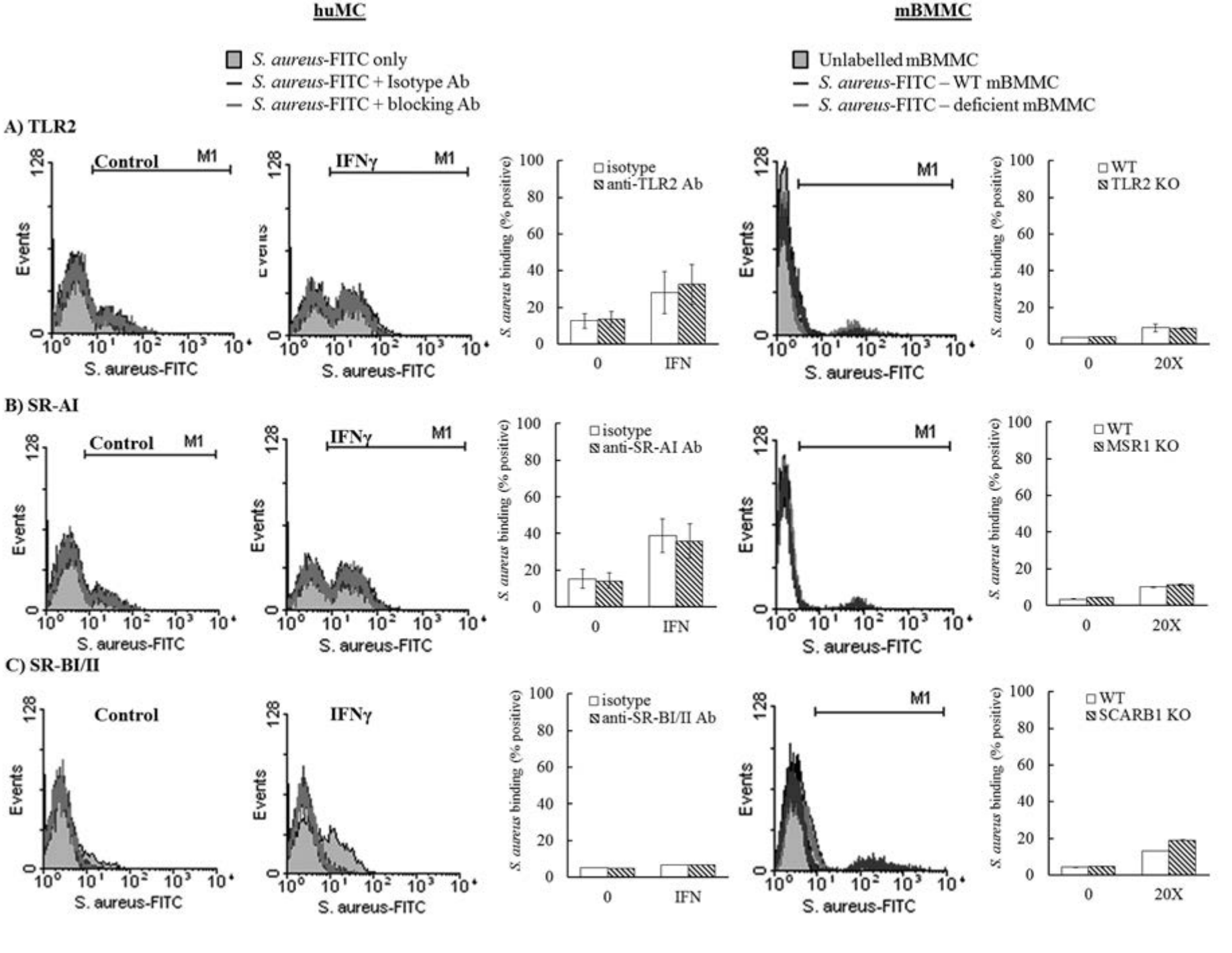

A
ROS (fluorescence)
T (min)
B
control
control + S. aureus
IFNγ
IFNγ + S. aureus
ROS (luminescence)
T (min)
C
control
S. aureus
S. aureus + FR
S. aureus + ZT
S. aureus + ZT + FR
ROS (fluorescence)
T (min)
D
control
S. aureus
S. aureus + FR
S. aureus + ZT
S. aureus + ZT + FR
ROS (luminescence)
T (min)
E
control
S. aureus
S. aureus + DPI
S. aureus + L-NMMA
ROS (fluorescence)
T (min)
F
control
S. aureus
S. aureus + DPI
S. aureus + L-NMMA
ROS (luminescence)
T (min)
G
Total ROS (AUC)
+++
*
***
***
S. aureus
Inhibitor
FR
ZT
FR+ZT
DPI
L-NMMA
H
Total ROS (AUC)
S. aureus
Inhibitor
FR
ZT
FR+ZT
DPI
L-NMMA

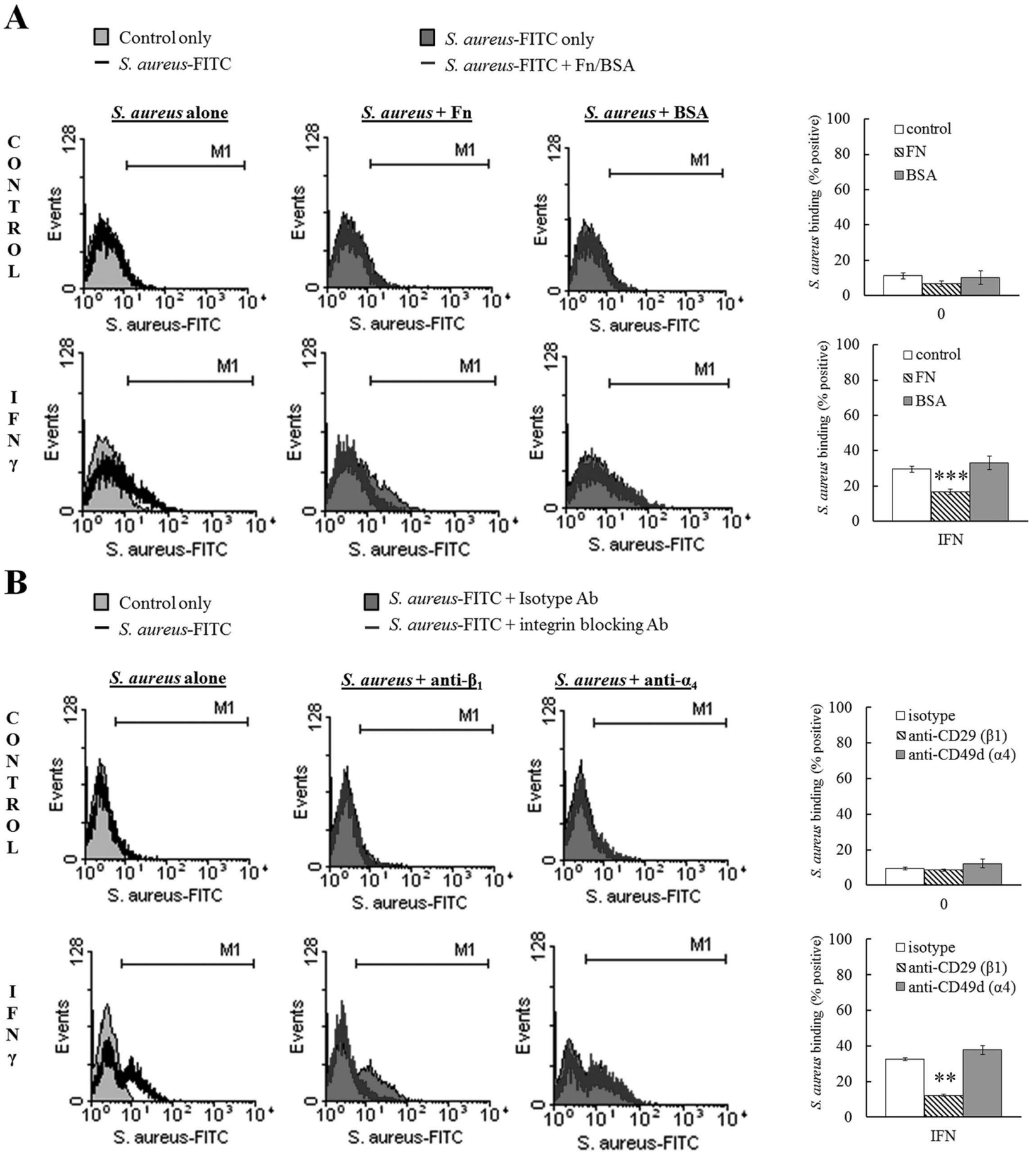

A
control
S. aureus
ROS (luminescence)
T (min)
B
% survival
% killing
% bacteria
SA
SA+PMN
E
% killing
PMN
S. aureus
Inhibitor
FR
ZT
FR+ZT
C
control
S. aureus
ROS (luminescence)
T (min)
D
% survival
% killing
% bacteria
SA
SA+huMC
F
% killing
huMC
S. aureus
Inhibitor
FR
ZT
FR+ZT

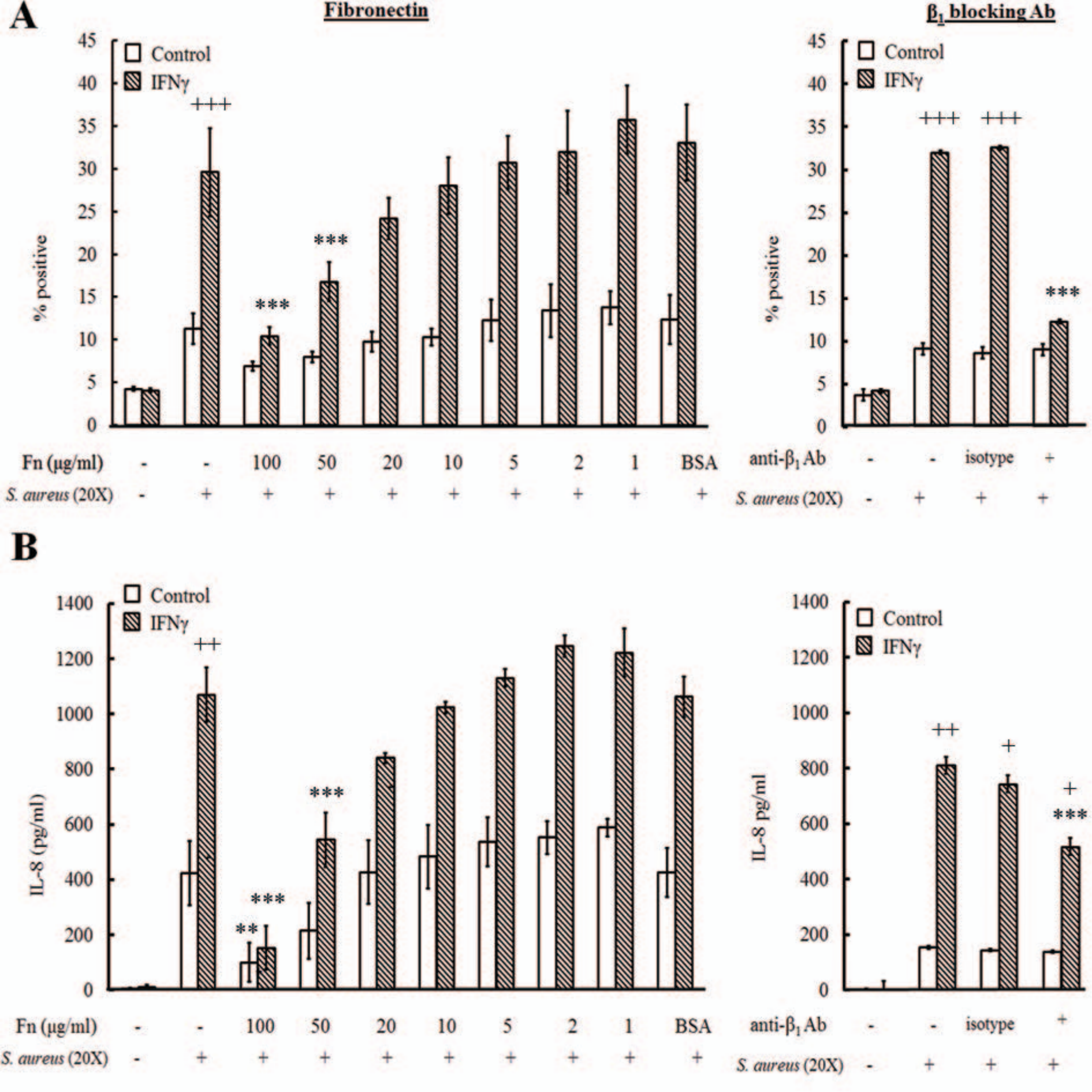

A
B
C
Control
IFNγ
β-hex release (%)
LTC4 ng/ml
PGD2 ng/ml
0:1
2:1
20:1
200:1
(bacteria:HuMC ratio)
***
+++
**
++
*

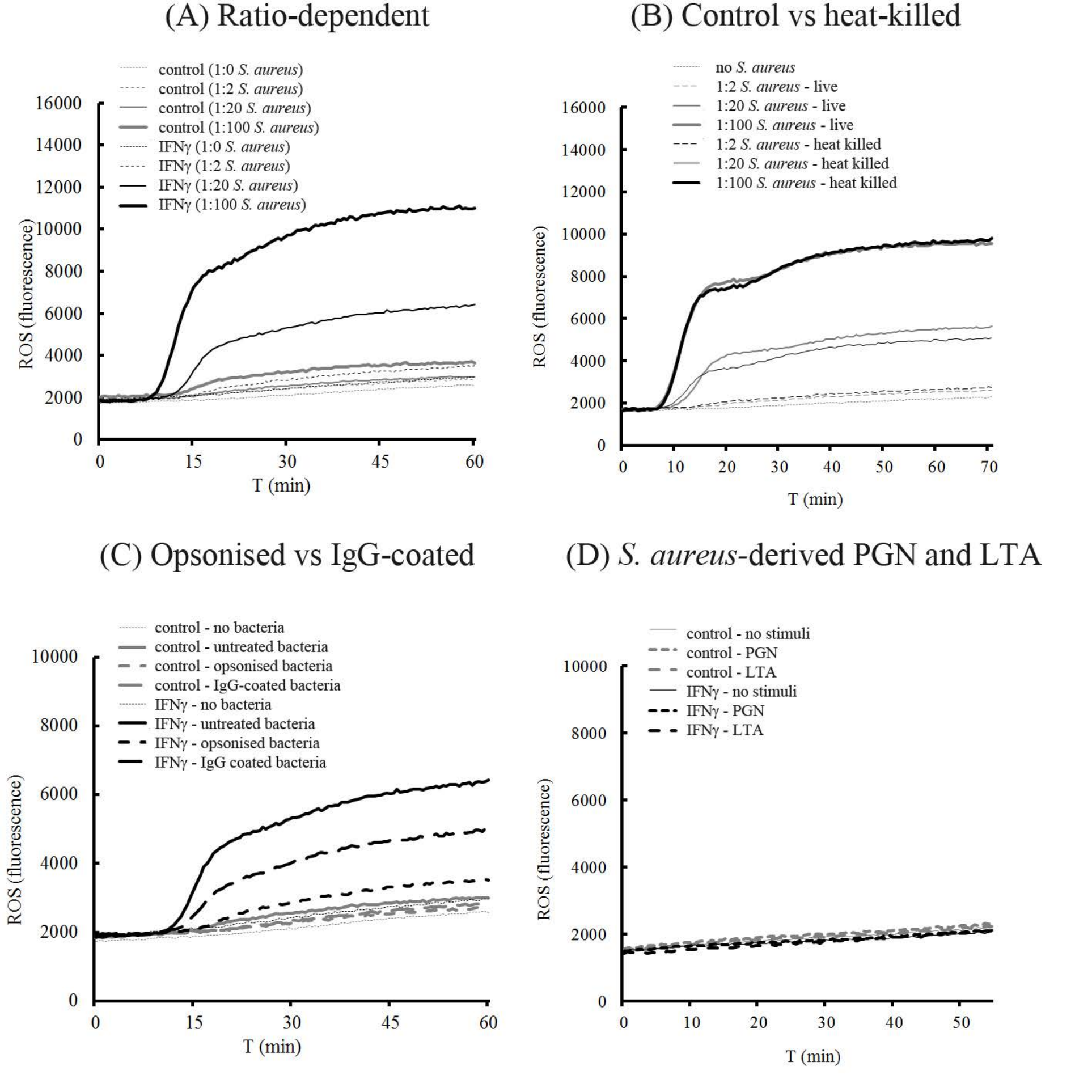

A
S. aureus-induced gene expression (Av fold change)
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
Control
IFNγ
CCL1
CCL2
CCL3
CCL4
CXCL8
TNFα
TNFSF14
CSF1
CSF2
IL-5
IL-13
INHBA
Chemokines
Cytokines
B
S. aureus-induced gene expression (fold change)
control
IFN
*
CXCL8
C
IL-8 pg/ml
2000
1500
1000
500
0
Control
IFNγ
**
***
S. aureus
—
+
1 h
2 h
4 h
6 h
8 h
D
GM-CSF pg/ml
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Control
IFNγ
*
S. aureus
—
+
1 h
2 h
4 h
6 h
8 h

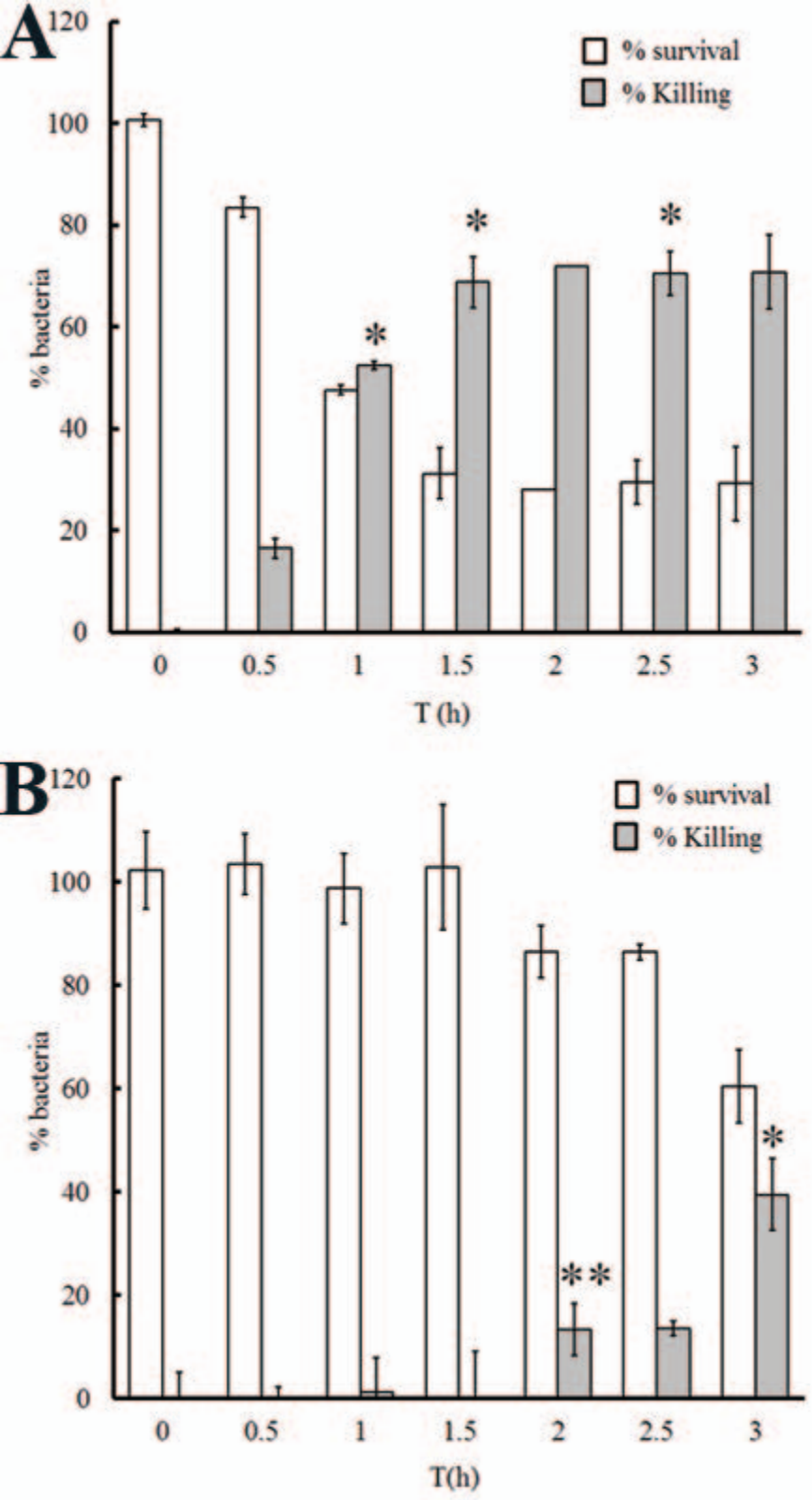

A
Control
IFNγ
S. aureus binding (% positive)
100
90
80
70
60
50
40
30
20
10
0
*
0
20X
Unlabelled cells
Control + S. aureus-FITC
IFNγ + S. aureus-FITC
128
M1
Events
0
10⁰ 10¹ 10² 10³ 10⁴
S. aureus-FITC
B
Unlabelled cells
Isotype Ab
Control cells
IFNγ-treated cells
SR-AI
SR-BI/II
TLR2
SR-AI
SR-BI/II
TLR2-PE
CD29
CD49d
CD51/61
CD29-PE
CD49d-PE
CD51/61-FITC
% positive
**
***
*
Control
IFNγ
SR-AI
SR-BI/II
TLR2
CD49d
CD51/61
CD29


huMC
mBMMC
S. aureus-FITC only
S. aureus-FITC + Isotype Ab
S. aureus-FITC + blocking Ab
Unlabelled mBMMC
S. aureus-FITC – WT mBMMC
S. aureus-FITC – deficient mBMMC
A) TLR2
Control
IFNγ
M1
Events
S. aureus-FITC
S. aureus binding (% positive)
isotype
anti-TLR2 Ab
WT
TLR2 KO
0
IFN
20X
B) SR-AI
isotype
anti-SR-AI Ab
WT
MSR1 KO
C) SR-BI/II
isotype
anti-SR-BI/II Ab
WT
SCARB1 KO

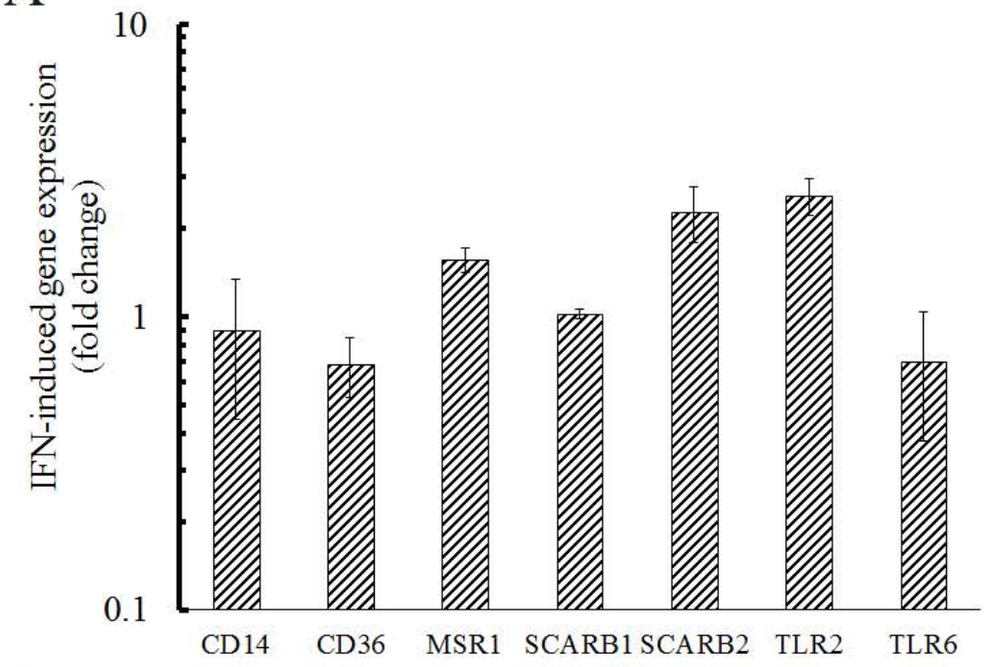

A
Control only
S. aureus-FITC
S. aureus-FITC only
S. aureus-FITC + Fn/BSA
S. aureus alone
S. aureus + Fn
S. aureus + BSA
CONTROL
IFNγ
M1
Events
S. aureus-FITC
S. aureus binding (% positive)
control
FN
BSA
0
IFN
***
B
Control only
S. aureus-FITC
S. aureus-FITC + Isotype Ab
S. aureus-FITC + integrin blocking Ab
S. aureus alone
S. aureus + anti-β1
S. aureus + anti-α4
isotype
anti-CD29 (β1)
anti-CD49d (α4)
**


A
Fibronectin
β1 blocking Ab
Control
IFNγ
% positive
Fn (μg/ml)
S. aureus (20X)
anti-β1 Ab
isotype
B
IL-8 (pg/ml)
IL-8 pg/ml
BSA

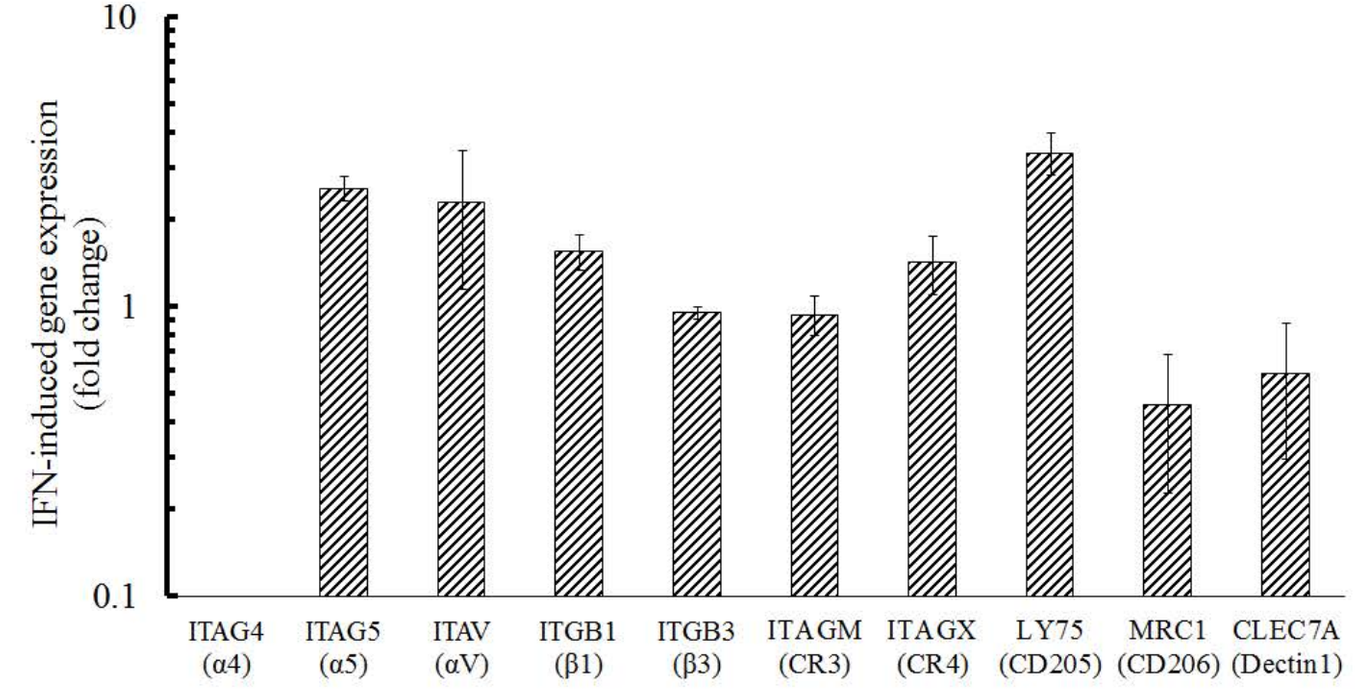

(A) Ratio-dependent
control (1:0 S. aureus)
control (1:2 S. aureus)
control (1:20 S. aureus)
control (1:100 S. aureus)
IFNγ (1:0 S. aureus)
IFNγ (1:2 S. aureus)
IFNγ (1:20 S. aureus)
IFNγ (1:100 S. aureus)
ROS (fluorescence)
T (min)
(B) Control vs heat-killed
no S. aureus
1:2 S. aureus - live
1:20 S. aureus - live
1:100 S. aureus - live
1:2 S. aureus - heat killed
1:20 S. aureus - heat killed
1:100 S. aureus - heat killed
ROS (fluorescence)
T (min)
(C) Opsonised vs IgG-coated
control - no bacteria
control - untreated bacteria
control - opsonised bacteria
control - IgG-coated bacteria
IFNγ - no bacteria
IFNγ - untreated bacteria
IFNγ - opsonised bacteria
IFNγ - IgG coated bacteria
ROS (fluorescence)
T (min)
(D) S. aureus-derived PGN and LTA
control - no stimuli
control - PGN
control - LTA
IFNγ - no stimuli
IFNγ - PGN
IFNγ - LTA
ROS (fluorescence)
T (min)


A
% survival
% Killing
% bacteria
120
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3
T (h)
B
% survival
% Killing
% bacteria
120
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3
T(h)

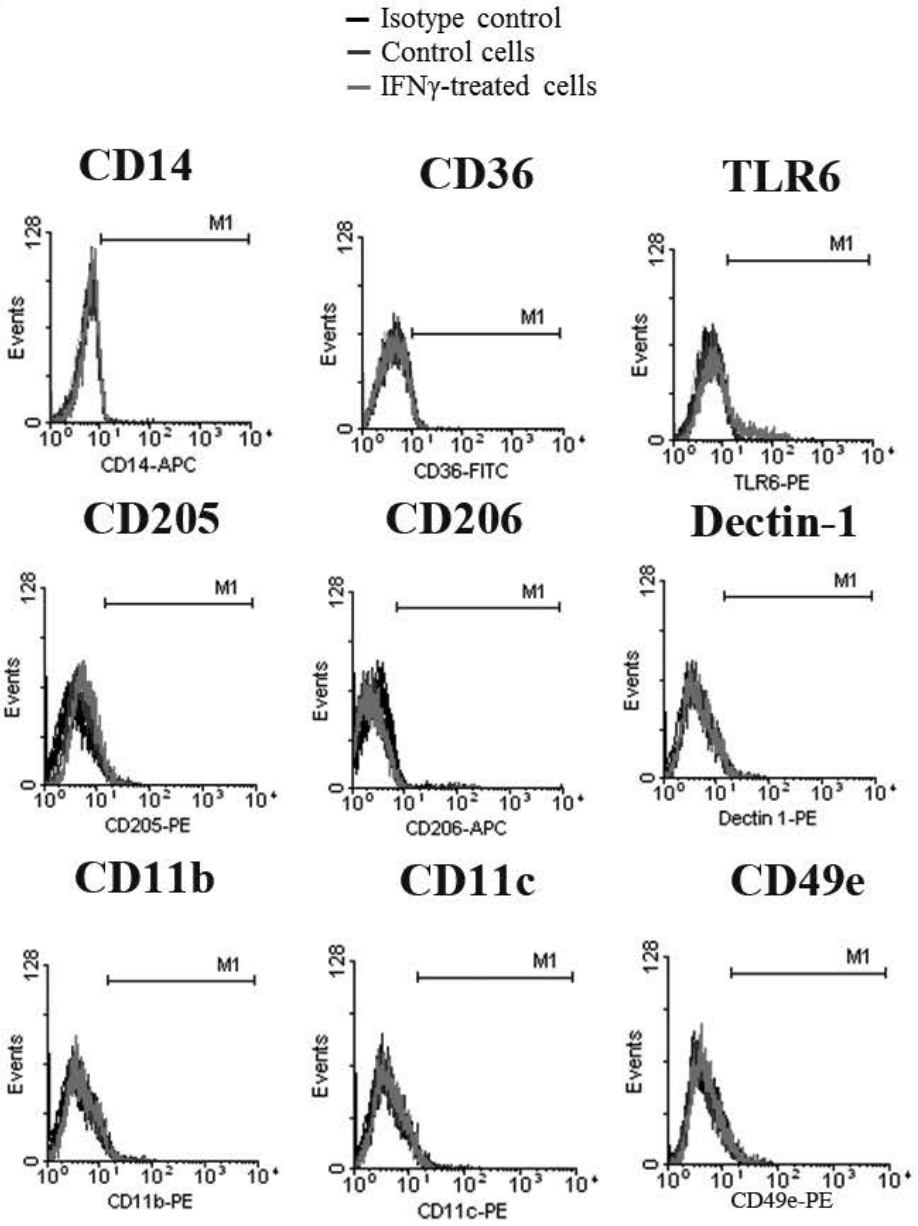
A


10
1
0.1
IFN-induced gene expression
(fold change)
CD14
CD36
MSR1
SCARB1
SCARB2
TLR2
TLR6

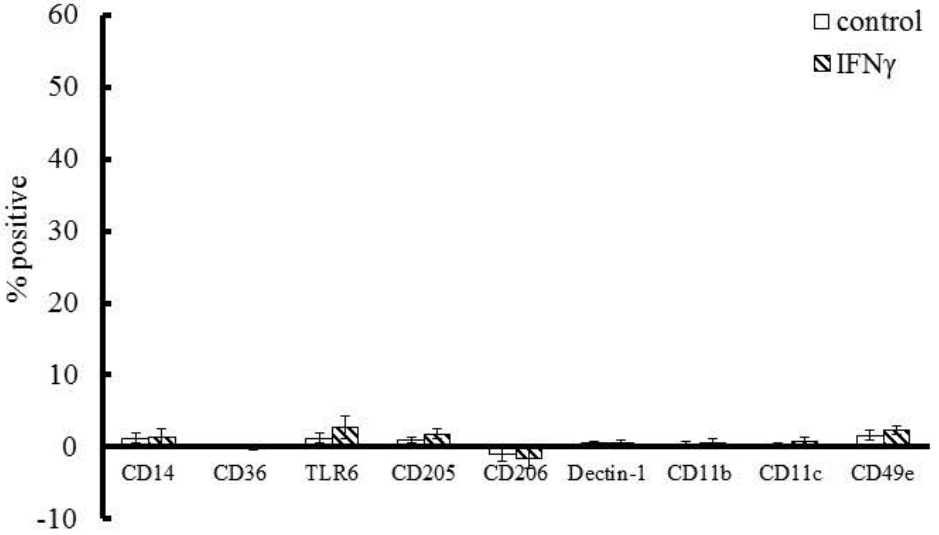
B

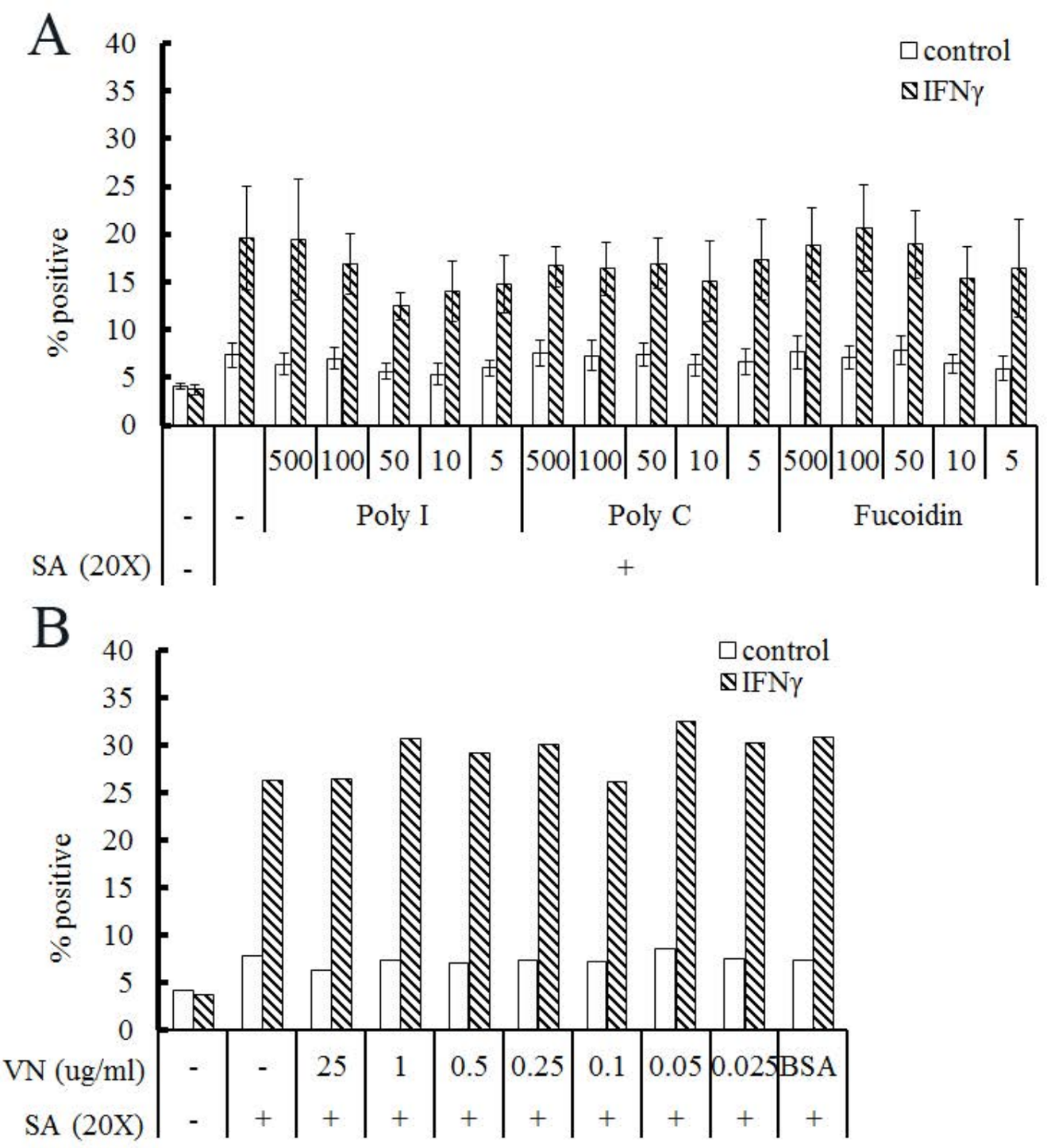

10
1
0.1
IFN-induced gene expression
(fold change)
ITAG4 (α4)
ITAG5 (α5)
ITAV (αV)
ITGB1 (β1)
ITGB3 (β3)
ITAGM (CR3)
ITAGX (CR4)
LY75 (CD205)
MRC1 (CD206)
CLEC7A (Dectin1)

A


Isotype control
Control cells
IFNγ-treated cells
CD14
CD36
TLR6
CD205
CD206
Dectin-1
CD11b
CD11c
CD49e
M1
Events
CD14-APC
CD36-FITC
TLR6-PE
CD205-PE
CD206-APC
Dectin 1-PE
CD11b-PE
CD11c-PE
CD49e-PE

B


control
IFNγ
% positive
60
50
40
30
20
10
0
-10
CD14
CD36
TLR6
CD205
CD206
Dectin-1
CD11b
CD11c
CD49e


A
40
35
30
25
20
15
10
5
0
% positive
control
IFNγ
500 100 50 10 5 500 100 50 10 5 500 100 50 10 5
- - Poly I Poly C Fucoidin
SA (20X) - +

B
40
35
30
25
20
15
10
5
0
% positive
control
IFNγ
VN (ug/ml) - - 25 1 0.5 0.25 0.1 0.05 0.025 BSA
SA (20X) - + + + + + + + + +