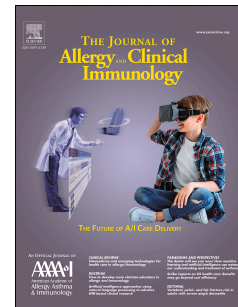


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Abstract

Background: The concept of innate and adaptive effector cells that are repleted by maturing inert progenitor cell populations is changing. Mast cells develop from rare mast cell progenitors populating peripheral tissues at homeostatic conditions, or as a result of induced recruitment during inflammatory conditions.

Objective: As FcεRI-expressing mast cell progenitors are the dominating mast cell type during acute allergic lung inflammation *in vivo*, we hypothesized that they are activated by IgE cross-linking.

Methods: Mouse peritoneal and human peripheral blood cells were sensitized and stimulated with antigen, or stimulated with anti-IgE, and the mast cell progenitor population analyzed for signs of activation by flow cytometry. Isolated peritoneal mast cell progenitors were studied before and after anti-IgE stimulation at single-cell level by time-lapse fluorescence microscopy. Lung mast cell progenitors were analyzed for their ability to produce IL-13 by intracellular flow cytometry in a mouse model of ovalbumin-induced allergic airway inflammation.

Results: Sensitized mouse peritoneal mast cell progenitors demonstrate increased levels of phosphorylation of tyrosines on intracellular proteins (pTyr), and spleen tyrosine kinase phosphorylation (pSyk) after antigen exposure. Anti-IgE induced cell surface-associated LAMP-1 in naïve mast cell progenitors, and prompted loss of fluorescence signal and altered morphology of isolated cells loaded with lysotracker. In human mast cell progenitors, anti-IgE increased pTyr, cell-surface associated LAMP-1 and CD63. Lung mast cell progenitors from mice with ovalbumin-induced allergic airway inflammation were induced to produce IL-13.

Conclusion: Mast cell progenitors become activated by IgE cross-linking may contribute to the pathology associated with acute allergic airway inflammation.

Key messages:

- IgE cross-linking induces activation of mouse and human mast cell progenitors
- Lung mast cell progenitors from mice with experimental asthma produce IL-13

Capsule Summary:

Human and mouse mast cell progenitors show hallmarks of IgE-mediated activation, and their lung counterparts in mice with experimental asthma produce IL-13, indicating that they contribute to acute allergic inflammation before acquiring the mature phenotype.

Key words: Activation; Allergic airway-inflammation; IgE cross-linking; Mast cells; Mast cell progenitors; Phosphorylation

Abbreviations: BAT: basophil activation test; FSC: forward scatter; IL: interleukin; MCp: mast cell progenitors; OVA: ovalbumin; PBMCs: peripheral blood mononuclear cells; pTyr: total tyrosine phosphorylation; Syk: spleen tyrosine kinase; SSC: side scatter; TNP: tri-nitrophenyl

Introduction

Distinct immune cell types differ in life span and in peripheral differentiation stages, from the short-lived neutrophils which mature in the bone marrow to the extremely long-lived mast cells that mature in peripheral tissues. Hematopoietic progenitors were previously considered an inert reservoir of immune cells. Lately, they were demonstrated to express functional toll-like receptors (1, 2), respond to cytokine signals such as type I interferons (3), and produce cytokines upon innate immune stimulation (4).

Mast cells reside in all vascularized tissues and are involved in allergic reactions. IgE-mediated cross-linking of FcεRI-receptors induces mast cell activation and release of granule-associated mediators, biogenesis and release of lipid mediators, as well as synthesis and release of cytokines. Mediator release is triggered by a signaling cascade in which tyrosine residues on intracellular proteins such as spleen tyrosine kinase (Syk) become phosphorylated (pSyk) (5). Mast cells develop from mast cell progenitors (MCp), which during embryogenesis appear in the yolk sac and seed the connective tissues. Later, bone marrow-derived MCp develop mainly into mucosal mast cells in the peripheral tissues (6, 7). Mouse MCp have a typical progenitor phenotype with a lymphocyte-like size and few granules (8). A similar human MCp population exists in the peripheral blood and bone marrow (9, 10). Both human and mouse MCp are extremely rare cells that make up ~0.005 % of enriched mononuclear cells in the blood.

Acute allergic stimulation triggers the activation of mast cells and the recruitment of FcεRI⁺ MCp to peripheral tissues (11, 12). Here, we investigated whether MCp from mouse peritoneum or human peripheral blood can be activated by IgE cross-linking *ex vivo*. Indeed, several hallmarks of activation could be detected early after incubation with IgE/antigen or anti-IgE, and lung MCp produced IL-13 *in vivo* during acute ovalbumin (OVA)-induced allergic airway inflammation in mice.

106

107 **Methods**

108 For detailed methods, please see the Methods section in this article's Online Repository at
 109 www.jacionline.org.

110

111 **Results and discussion**

112 The concept of the immune system consisting of differentiated immune cells solely responsible
 113 for immune functions has been challenged by observations of stem- and progenitor cells capable
 114 of effector cell behavior. However, the contribution of immune progenitor cells to allergic
 115 inflammation is largely unknown. An exception is CD34⁺ IL-5R α ⁺ eosinophil progenitors,
 116 described to be present along with mature eosinophils in the allergic inflamed lung *in vivo* (13).
 117 MCp equipped with the high affinity receptor for IgE (Fc ϵ RI), are recruited to the lung and
 118 dominate among the lung mast cell populations during the acute phase in experimental *in vivo*
 119 models of antigen-induced allergic lung inflammation (11, 12). Thus, here we hypothesized that
 120 MCp can participate actively in IgE-mediated reactions.

121

122 To test our hypothesis, mouse peritoneal cells from OVA-immunized mice were left untreated or
 123 stimulated with OVA (Fig E1, A). MCp were distinguished as lineage (Lin)^{-lo} c-kit^{hi}
 124 SSC^{lo} FSC^{lo} CD16/32^{int} integrin β ^{7hi} cells and constituted approximately 0.024 % of the singlets,
 125 whereas mature mast cells as Lin^{-lo} c-kit^{hi} SSC^{hi} FSC^{hi/int} CD16/32^{hi} integrin β ^{7lo/int} cells (Fig E1,
 126 B) (14). OVA stimulation induced an increased level of tyrosine phosphorylation (pTyr) in both
 127 mast cell populations (Fig 1, A, B), indicating that peritoneal MCp act like mature mast cells
 128 upon IgE-mediated activation. To corroborate these data, peritoneal cells from naïve mice were
 129 sensitized with mouse anti-TNP IgE *ex vivo* before stimulation with OVA-TNP (Fig E1, C). An
 130 increase in pSyk was demonstrated in mast cells and their progenitors 30 seconds after
 131 stimulation with OVA-TNP (Fig 1, C), a sign consistent with IgE-mediated mast cell activation
 132 (15). Naïve mouse peritoneal MCp and mast cells express Fc ϵ RI and have IgE bound to the

receptor (Fig 1, D). Thus, peritoneal cells from naïve mice were stimulated by anti-IgE or vehicle before analyzing the cell-surface presence of lysosomal-membrane protein LAMP-1. The fusion of LAMP-1 with the plasma membrane is a reliable marker of mast cell activation (16). Anti-IgE resulted in marked increase of surface-associated LAMP-1⁺ mast cells and MCp (Fig 1, E, F).

To ensure that the signaling events occurring in MCp after IgE cross-linking conditions was not an effect of secondary mediators from other peritoneal cells, a single-cell approach was chosen to investigate whether IgE cross-linking induces a response in isolated MCp. Peritoneal MCp isolated from naïve mice by fluorescence activated cell sorting (FACS) are smaller and much less granulated than the mature mast cells (Fig 2, A). Mast cell granules, and even lysosomal structures in T cells have been successfully visualized using lysotracker (17, 18). The peritoneal MCp were incubated with lysotracker to visualize granular intracellular compartments and the fluorescence imaged before and after addition of an anti-mouse IgE antibody, or vehicle. Most MCp became activated almost instantly after addition of anti-IgE as shown by a decrease in fluorescence and change in cellular morphology compared to vehicle control cells or non-responders (Fig 2, B-E; Video E1-6). Collectively, these data suggest that mouse MCp become activated by IgE cross-linking *ex vivo*.

To investigate whether human MCp also are activated by IgE cross-linking, freshly prepared peripheral blood mononuclear cells (PBMCs) were stimulated with an anti-human IgE antibody, or left unstimulated. Human MCp were identified as CD4⁻ CD8⁻ CD19⁻ CD14⁻ CD34^{hi} CD117⁺ FcεRI⁺ cells, and a population of CD4⁻ CD8⁻ CD19⁻ CD14⁻ CD34⁻ CD117⁻ FcεRI⁺ cells, likely basophils and dendritic cells, were used as positive controls (Fig E2). Morphologically, MCp found in human blood have only few granules (9, 10). The morphological difference between human blood MCp and mouse peritoneal MCp is not unexpected since peritoneal mast cell

turnover is extremely slow, (19) which may allow more acidic compartments to develop, and thus the peritoneal MCp may be more “mature” in their phenotype. Despite that, human blood MCp and the CD117⁻ FcεRI⁺ positive control population both demonstrated an increased total pTyr 30 seconds after anti-IgE stimulation of PBMCs (Fig 3, A; not shown). The rapid response appears to exclude MCp activation mediated by bystander cells responding to IgE cross-linking. Thus, anti-IgE has a direct activating effect on human MCp. However, quantification of pSyk using a similar experimental approach revealed increased pSyk in MCp and CD117⁻ FcεRI⁺ positive control population only in the same two out of five individuals (Fig 3, B; not shown). These data suggest that MCp from some individuals phosphorylate Syk, and that MCp from other individuals may be “non-responders” similar to basophils, which in a proportion of donors are unable to respond to stimulation with anti-IgE (20). Alternatively, the kinetics of pSyk may differ between individuals. Next, we tested whether anti-IgE could induce cell-surface associated CD63 and LAMP-1 (CD107a). Indeed, MCp from all four individuals tested responded with a higher level of cell surface-associated CD63 and LAMP-1 after anti-IgE treatment (Fig 3, C, D). Altogether, our data suggests that anti-IgE stimulation induced activation of the human MCp.

Since IgE cross-linking induced MCp activation *ex vivo*, we next asked whether MCp activation could be observed *in vivo*. As IL-13 is secreted by activated mast cells after IgE cross-linking (21), we investigated whether MCp can produce IL-13 in an allergic setting *in vivo*. Mice were subjected to acute OVA-induced allergic airway inflammation and the presence of IL-13⁺ MCp was analyzed by intracellular flow cytometry (Fig 4, A; Fig E3). Lung MCp identified as CD45⁺ Lin^{-/lo} c-kit^{hi} T1/ST2⁺ FcεRI⁺ CD16/32^{int} integrin β7^{hi} cells from OVA-sensitized and challenged mice were six times more frequent than in the control mice, which were only i.p. sensitized to OVA (Fig 4, B). On average 19±3 % of the lung MCp from mice with acute OVA-induced allergic airway inflammation were positive for IL-13, corresponding to around 3±0.1% of the IL-13 producing CD45⁺ cells, whereas few of the MCp from control mice showed a signal for IL-13

(Fig, 4C). The IL-13 production in lung MCp could be due to OVA-induced cross-linking of IgE to FcεRI, or to *in vivo* activation by other signals set off by the allergic inflammation. Recently, data from a single cell RNA sequencing project were re-clustered based on cells expressing beta-tryptase. This re-analysis revealed four mast cell clusters, one of which contained genes expressed in integrin $\beta 7^{\text{hi}}$ mouse lung mast cells, and the IL-13 gene (22). IL-13 is crucial for the development of airway hyperresponsiveness and mucus production in experimental models, and Dupilumab, which targets the IL-4 receptor alpha-chain and thereby both IL-4 and IL-13 pathways, improves FEV1 and decrease exacerbations in asthma patients (23). Furthermore, human MCp are more frequent in the blood circulation of asthma patients with reduced lung function (24). Thus, it appears likely that IL-13 production by MCp may be biologically relevant *in vivo*. Although we here demonstrate cytokine production by MCp in an allergic setting, hypogranular mast cells in the mesenteric lymph nodes with features resembling MCp have been described to produce IL-4 and IL-6 after *Trichinella Spiralis* infection (25). This hints towards a general contribution of MCp to cytokine production across settings.

In summary, we show that IgE cross-linking induces activation of mouse and human MCp, and suggest that these cells play an active role in allergic inflammation by producing cytokines such as IL-13.

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Author contributions

EM-E, MS, and JH designed the study and planned the experiments. EM-E and MS performed the experiments and analyzed the data. JE and MES were involved in the planning, execution, and analysis of one set of experiments. AM and CJ were responsible for the RHINESSA cohort and human sample collection. EM-E, MS and JH wrote the paper. All authors critically assessed and approved the manuscript.

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Figure Legends

Fig 1. IgE cross-linking induces activation of mouse peritoneal MCp. (A-B) Representative histograms (A) and the gMFI for pTyr (B) in mast cells (MC) and MCp in peritoneal cells from OVA-sensitized mice stimulated 2 minutes *ex vivo* with OVA. Each dot represents a sample of pooled peritoneal cells from 10-17 mice per experiment from 3 independent color-coded experiments. In (A) and (E), the dashed line and gray shadow represent the isotype control, and basal level (A) or level in vehicle-treated cells (E), respectively. (C) The gMFI for pSyk in MC and MCp from anti-TNP IgE sensitized peritoneal cells stimulated 30 seconds with OVA-TNP. Each dot represents a sample of pooled peritoneal cells from 8-10 mice per experiment in 4 color-coded experiments. (D) Representative histograms of FcεRI and IgE surface staining in peritoneal MC and MCp. (E-F) Representative histograms and the % MC and MCp with surface-associated LAMP-1 gated from peritoneal cells stimulated by anti-IgE or vehicle for 30 minutes. The data derives from 3 experiments in which peritoneal cells from 6-8 mice per experiment were pooled, divided into 4 samples, and incubated with vehicle or anti-IgE. Statistical significance was tested by paired Student's t-test (* $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$).

Fig 2. Isolated peritoneal MCp respond to anti-IgE stimulation. (A) Representative May-Grünwald Giemsa (left) or chloroacetate esterase (right) stained FACS-isolated mouse peritoneal MCp and MC. Bars represent 10 μ m (B-E) Isolated MCp were incubated with lysotracker and recorded by time-lapse microscopy 10 minutes before and after either vehicle or anti-IgE was added. (B) Representative pictures of MCp before incubation with anti-IgE or vehicle. (C) The fluorescence signal over time recorded for each cell. (D) Quantification of the normalized mean fluorescent intensity after the addition of vehicle or anti-IgE. The deviation from 100% in the vehicle is due to signal bleaching. The lowest data point in the vehicle group in H defined the cut-off. (E) Donut diagram illustrating the proportion of individual MCp responding to anti-IgE.

The data are derived from 2 experiments using peritoneal cells from 5-6 mice per experiment.

Statistical significance was tested by Mann-Whitney U-test (**p<0.01).

Fig 3. IgE cross-linking induces activation of human peripheral blood MCp. (A, B) The PBMCs were incubated with anti-IgE antibody for 30 seconds (A, B) or 10 minutes (C, D) before the reaction was stopped. In A, B the gMFI for the total pTyr (A) or pSyk (B) in MCp were determined by intracellular flow cytometry. Two representative histograms show the phosphorylation in comparison to an unstimulated sample (grey) from the same donor using the same gating strategy. Each color represents one experiment. Most points represent cells from one donor, except for 3 points where PBMCs from 2 donors were pooled. In C, D the level of cell surface-associated CD63 (C) or LAMP-1 (D) was analyzed by flow cytometry. The data in A, B derive from 14 experiments (9 for pTyr, 5 pSyk) using cells from 17 individuals. The data in C, D derive from 4 experiments using cells from the same 4 color-coded individuals. Each color reflects cells from a certain individual. Statistical significance was tested by a paired Student's t-test (ns - non-significant; *p<0.05; **p<0.01).

Fig 4. Lung MCp produce IL-13 upon allergen challenge. (A-C) Mice were sensitized on days 0 and 7 with OVA/alum i.p. and left untreated (controls) or challenged by OVA aerosol for 30 min per day on day 17-19, before all were euthanized directly after the final challenge. Lung cells left to rest in IL-3 and brefeldin before they were analyzed for IL-13 by intracellular flow cytometry. (A) Representative contour plots of IL-13⁺ lung MCp (CD45⁺ Lin^{-/lo} c-kit^{hi} T1/ST2⁺ FcεRI⁺ CD16/32^{int} integrin β7^{hi} cells) from control mice (top) and OVA challenged mice (down). (B) The quantification of lung MCp in mice with OVA-induced allergic airway inflammation and controls. (C) The percentage of IL-13⁺ lung MCp from mice with OVA-induced allergic airway inflammation and controls from three independent experiments, each labeled by color. The

343 points represent a single sample of pooled lung cells from 5-7 control mice, or 3 OVA
344 challenged mice. Statistical significance was tested by an unpaired Student's t-test (**p < 0.01).
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Figure 1

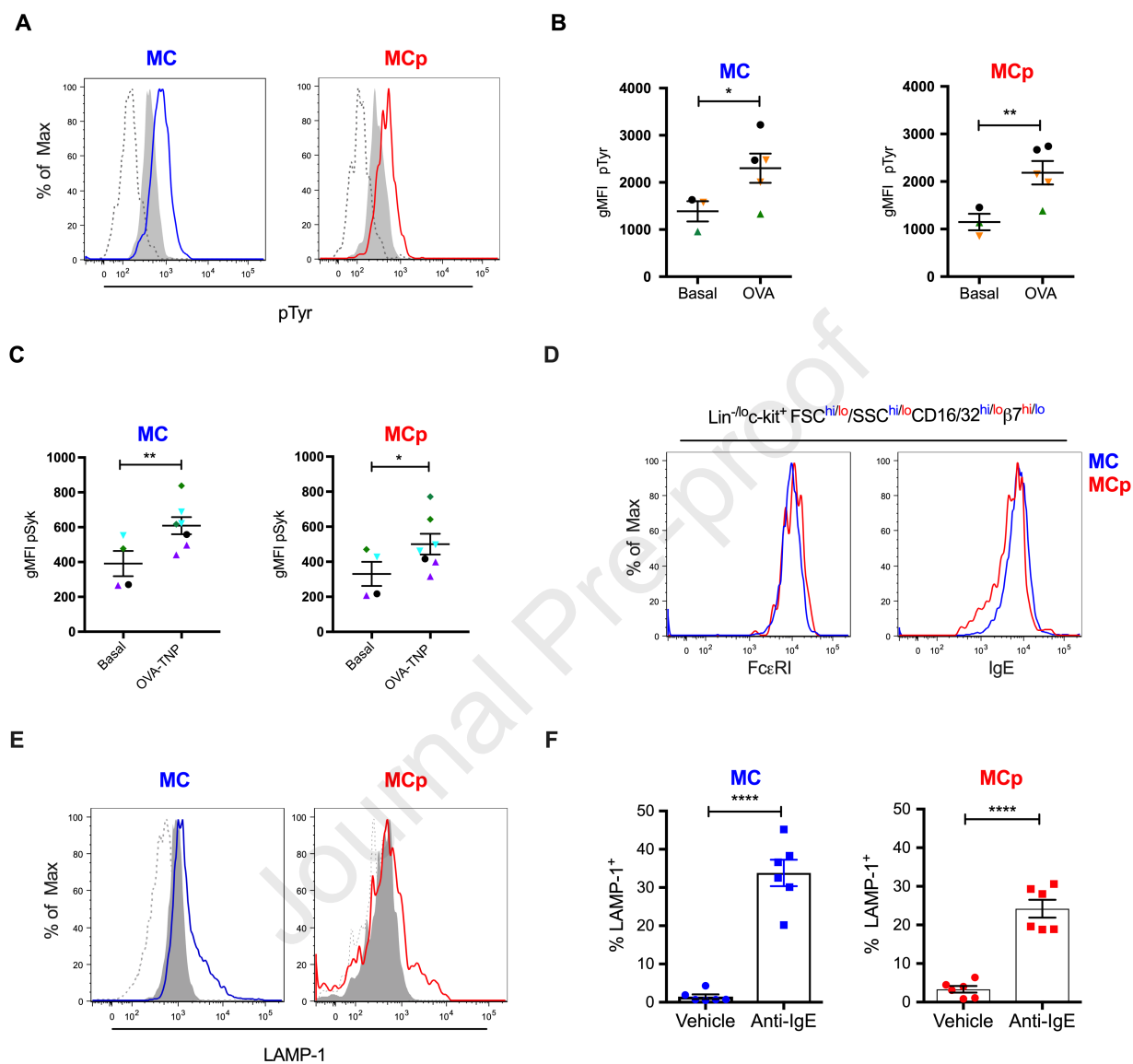


Figure 2

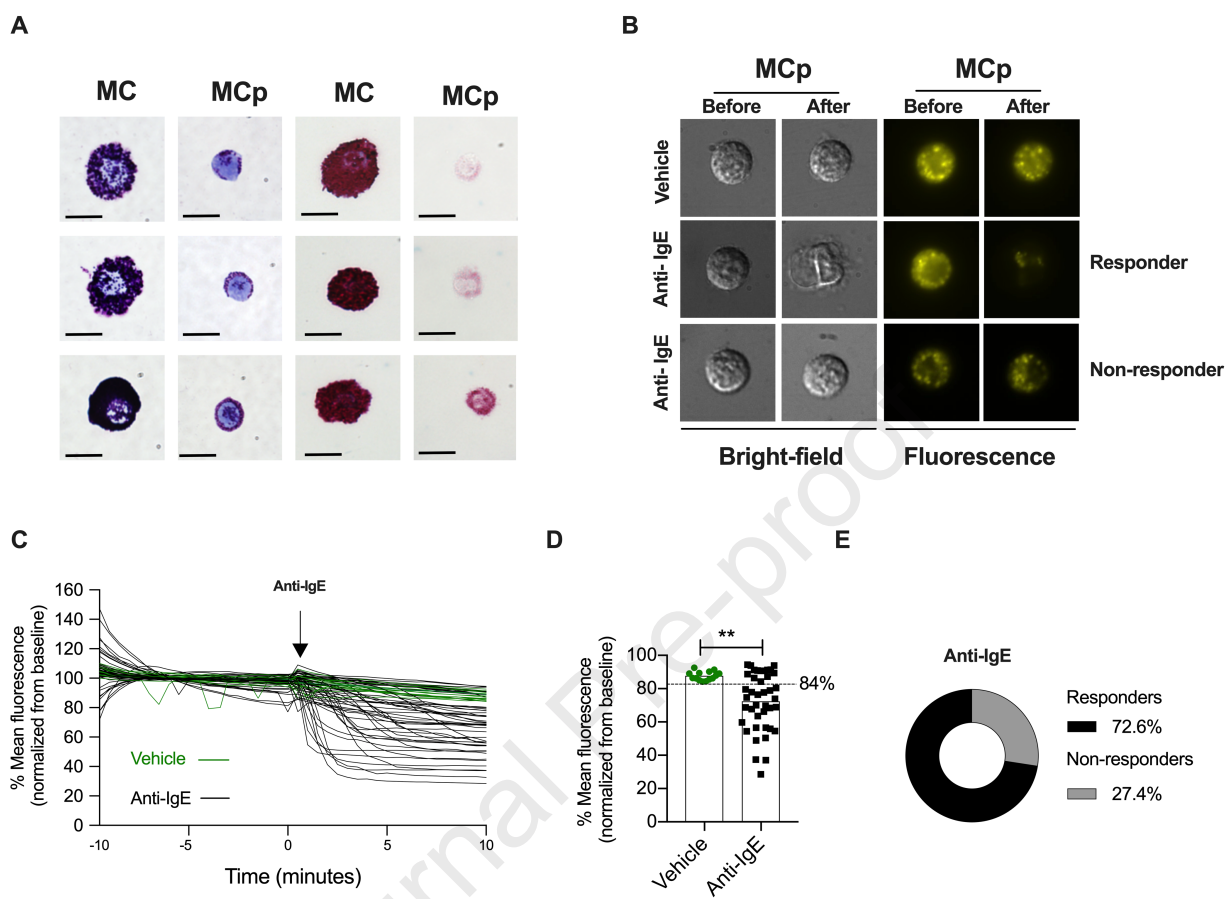


Figure 3

