Supplemental Materials

Figure S1. Cultured BMDCs express CD11c

BMDCs were generated *in vitro* from bone marrow cells cultured in 10 % RPMI supplemented with 15 ng/ml GM-CSF. Media was changed and fresh cytokine added on days 2 and 4, then the cells were washed on day 7. On day 12, cells were removed and stained with anti-CD11c to verify cell surface expression by flow cytometry. The cells demonstrated high levels of surface CD11c when compared to isotype control, indicating that BMDCs were generated.

Figure S2. NO oxidation and processing of GlyAg is fundamental for T cell activation.

Co-cultures of purified wt and $iNOS^{-/-}$ APCs (depleted with CD90.2 microbeads) and $CD4^+$ T cells were stimulated with 100 μg/ml GlyAg or PBS and subsequent IFN-γ production was measured six days later (eBioscience, San Diego, CA). wt $CD4^+$ T cells produce significantly more IFN-γ in response to GlyAg stimulation than do $iNOS^{-/-}$ T cells, demonstrating that NO production and subsequent oxidation of GlyAg is necessary for T cell activation. n = 3; mean ± SEM

Figure S3. LysoSensor Green fluorescence is pH dependent.

LysoSensor Green (1 μM) was added to 200 μl of Tris or citrate buffers at various pH values within a 96-well plate and the fluorescence was measured immediately (n = 3; mean ± SEM) using a $Victor^3V$ multilabel counter (Perkin-Elmer). Error bars represent SEM. Fluorescence intensity increased by a factor of nearly 4 when comparing neutral and acidic (pH < 6) pH values.

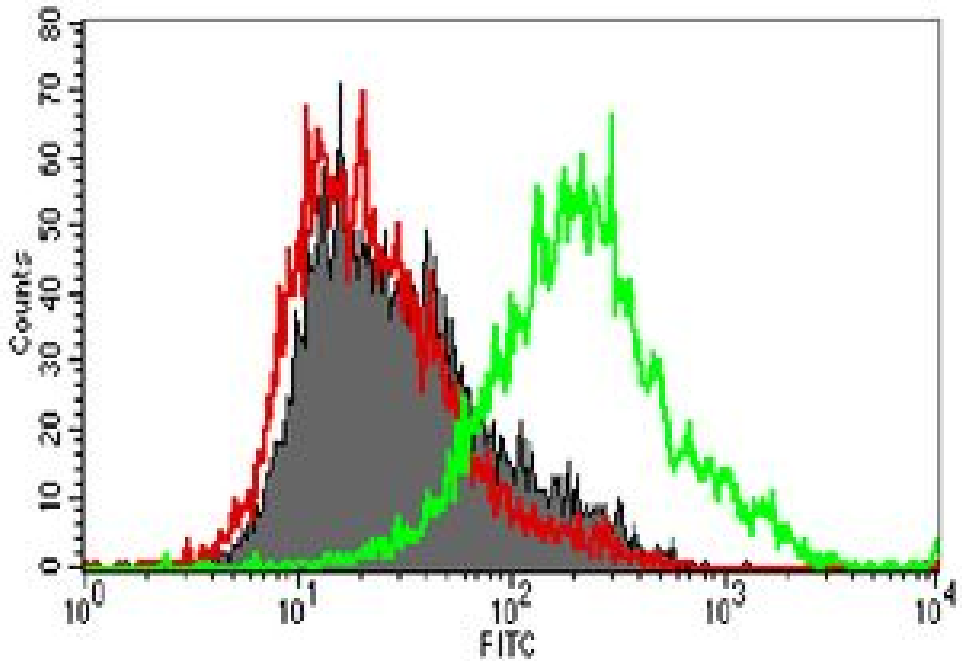
Figure S4. **The amount of GlyAg present in cells positively correlates to the amount of vesicular acidification**

(A) Wild type BMDCs were stimulated with 50 μg/ml labeled GlyAg overnight, washed, then treated with 2 μM LysoSensor Green for 25 minutes. Ten confocal images were captured and the mean intensity of the fluorescent signals (green = LysoSensor; red = GlyAg) of 100 independent subcellular ROIs were quantified using Leica Application Suite software. The mean fluorescent intensities were graphed in order of brightest to weakest GlyAg signal and Pearson Correlation coefficient was determined. There is a positive correlation (r = 0.4396) between the amount GlyAg present in the cells and the degree of acidification achieved (*i.e.* LysoSensor fluorescence) even in these cells, which retain V-type ATPase proton pump activity. Although this relationship is more apparent when constitutive acidification is blocked with BafA (Figure 4), these data demonstrate strong GlyAg oxidation-driven acidification above the normal proton pump-mediated acidification. (B) Confocal images were obtained for RAW macrophages treated overnight with or without 50 μg/ml unlabeled GlyAg, then stimulated with 50 μg/ml FITC-OVA for 90 minutes. Images shown are maximum projections of each z-stack; bars = 20 μm. Untreated macrophages readily endocytose the protein antigen as evidence by punctate staining within cells (top row). However, the uptake of FITC-OVA is greatly enhanced by the pre-treatment of the cells with GlyAg, resulting in significantly increased mean fluorescent intensity of 50 independent ROIs. These data show that under normal conditions (*i.e.*, without BafA), NO-mediated processing of GlyAg enhances pH-dependent receptor-mediated endocytosis and receptor-ligand dissociation within vesicular compartments.

Figure S5. BafA reduces protein antigen retention within endocytic vesicles

Confocal images were obtained for RAW macrophages treated overnight (~16 hours) with 40 nM BafA and 50 μg/ml GlyAg or with BafA only, then stimulated with 50 μg/ml FITC-OVA for 30 minutes. Images shown are maximum projections of each z-stack; bars = 20 μm. The mean

fluorescent intensity of 50 independent subcellular ROIs was quantified for each treatment group. Cells treated overnight with BafA only show primarily surface accumulation and little to no internalization of FITC-OVA (top row). However, the addition of GlyAg to BafA treated cells increased internalization and retention of FITC-OVA 4-fold as evidenced by the punctuate staining within the cell bodies (bottom row). These data support previously published findings that BafA alters antigen endocytosis by diminishing pH-dependent intravesicular receptor-ligand dissociation, leading to loaded receptors being recycled and accumulation of FITC-OVA on the cell surface. However, pretreatment with GlyAg overcomes this inhibition and promotes retention of FITC-OVA within the cells, likely through creating an acidic endosomal environment.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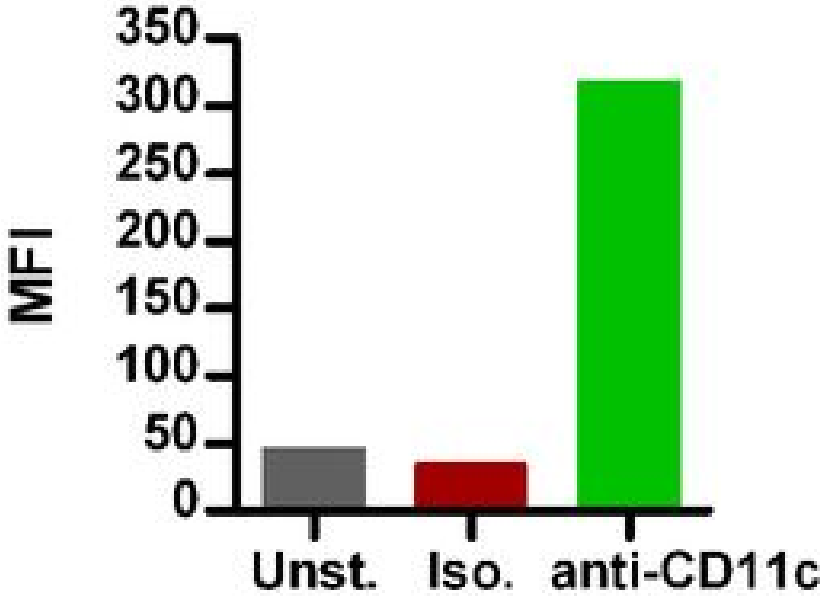
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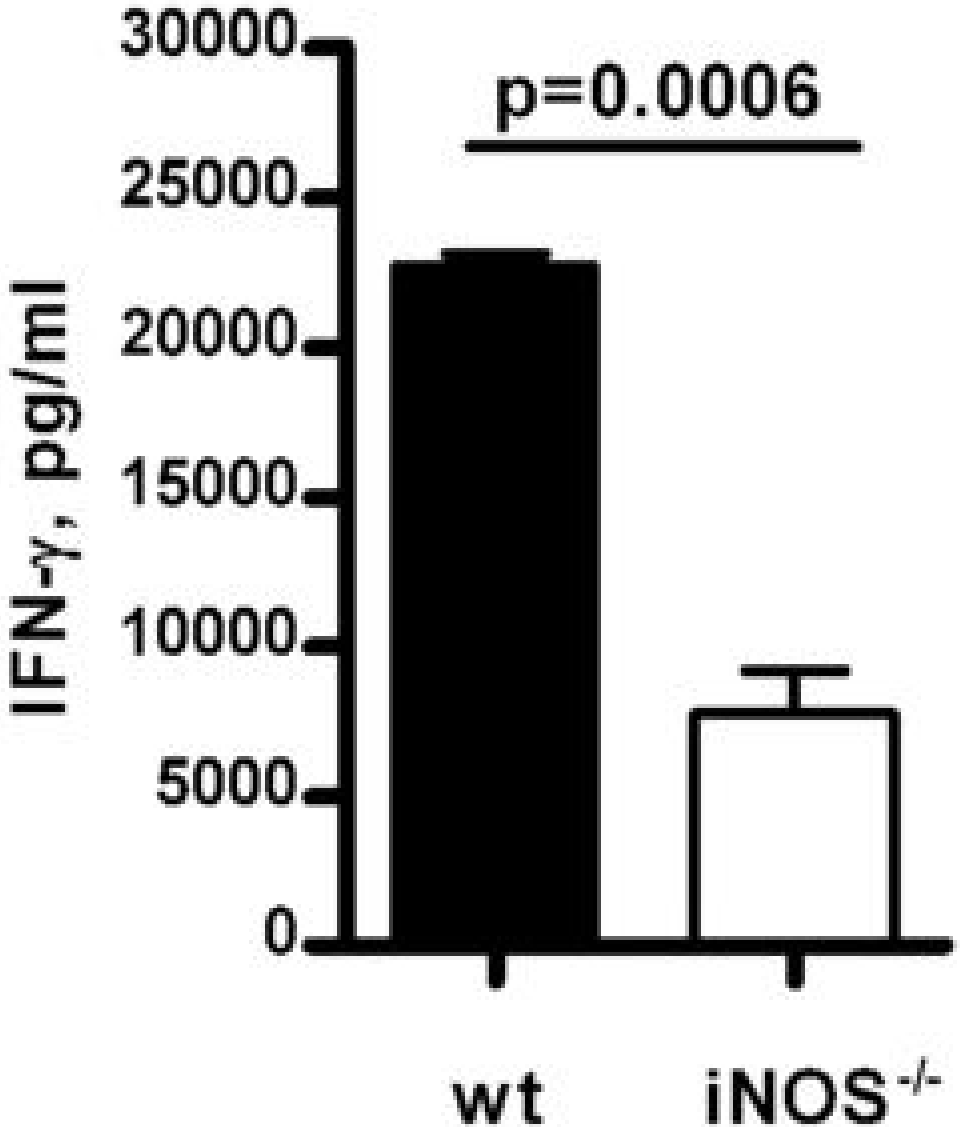
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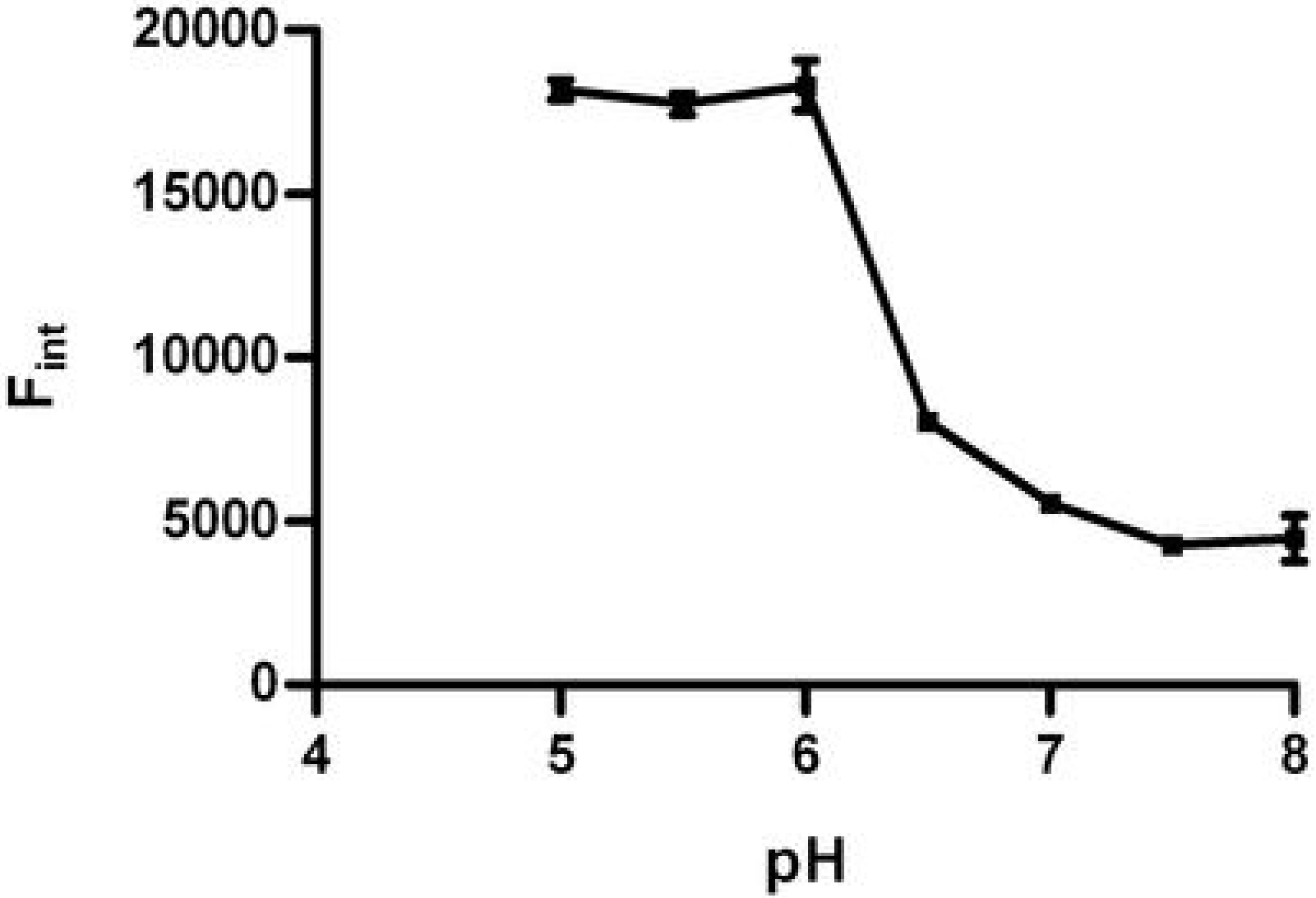
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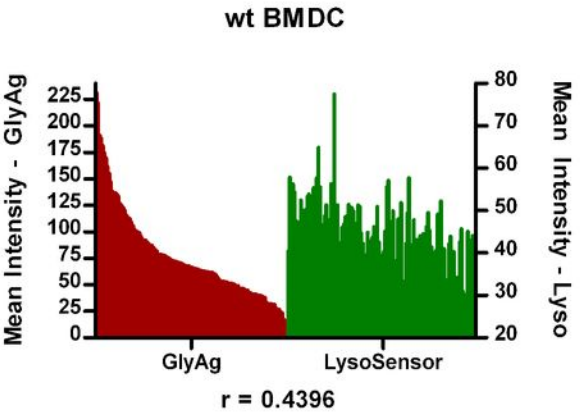
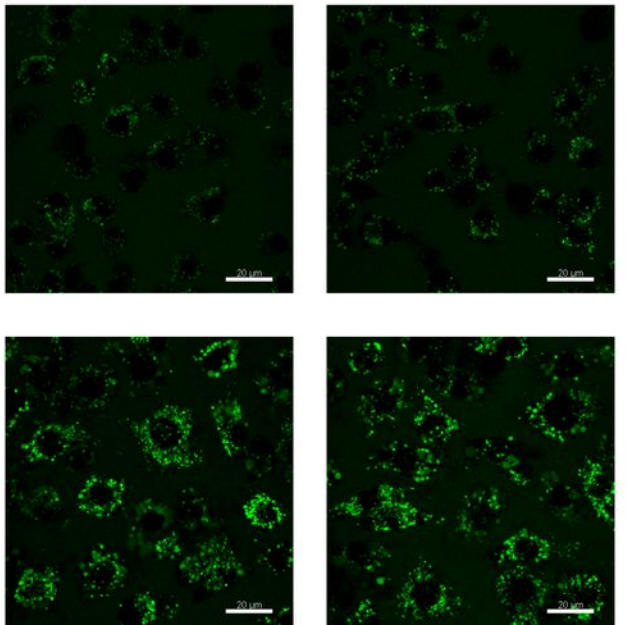
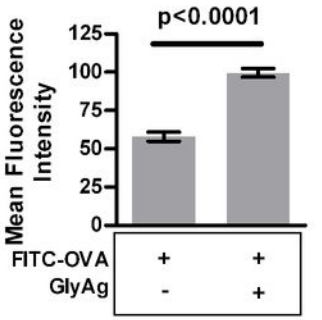


B

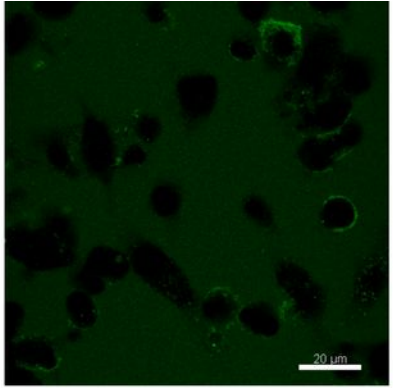
FITC-OVA

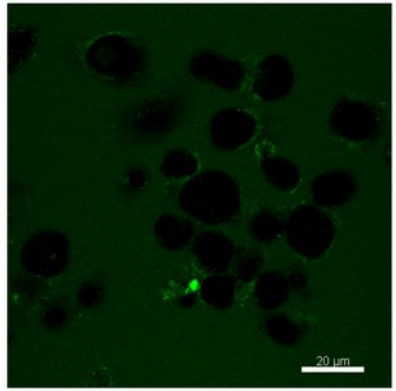
FITC-OVA & GlyAg





FITC-OVA with BafA





FITC-OVA with BafA & GlyAg