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IgE cross-linking impairs monocyte antiviral responses and inhibits influenza-driven Th1 differentiation.

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Capsule Summary: (35 words describing article's contribution to the literature) IgE-mediated allergic activation impairs the virus-induced Th1 lymphocyte priming capacity of human monocytes in response to influenza exposure. These findings represent one potential mechanism contributing to the link between viral infections and allergic disease.

Key words: IgE, IgE cross-linking, monocytes, influenza, Th1, T cell differentiation, co-stimulation

Abbreviations:

DCs – Dendritic cells

FcεRI – High-affinity IgE receptor

HLA – Human leukocyte antigen

Flu – influenza A virus

IFN – Interferon

IgG – Rabbit whole IgG antibody

IL – Interleukin

MHC – Major histocompatibility complex

TCR – T cell receptor

Th – helper T cell

TLR – Toll-like receptor

αIgE – Rabbit anti-human IgE antibody

To the editor:

Mechanisms underlying exacerbations of allergic asthma, although poorly defined, are clearly linked with viral infections¹. Rhinoviruses are the most common viruses associated with asthma exacerbations^{E1-E3}. Influenza virus infections have a similar association^{E4-E6}, resulting in increased disease severity in this population^{E7,E8} (Table E1). Defects in innate cell antiviral responses represent one potential mechanism underlying the link between viral infections and atopic disease exacerbations. For example, high-affinity IgE receptor (FcεRI) stimulation on plasmacytoid dendritic cells inhibits influenza-induced interferon-α secretion² and suppresses TLR7² and TLR9 expression³. Decreasing serum IgE with omalizumab in allergic asthmatics restores *ex vivo* antiviral interferon-α responses, and correlates with improved clinical outcomes⁴. Monocytes are also recruited to respiratory sites during viral infections^{E17} and allergen exposure^{5,E18}, and implicated in food allergy development⁶. Additionally, allergic stimulation via IgE cross-linking impacts monocyte functions including apoptosis^{E19}, cytokine secretion and phagocytosis⁷. Defining IgE-mediated effects on monocyte antiviral responses could reveal additional pathways involved in atopic disease pathogenesis.

Antigen presentation, which drives T cell activation and differentiation, represents a critical monocyte antiviral function. Influenza exposure increased monocyte-driven Th1 priming of naïve CD4⁺ lymphocytes in primary human monocyte-T cell co-cultures, but did not significantly affect Th2 differentiation (Figure 1A and E1). Influenza-driven Th1 differentiation inversely correlated with monocyte surface FcεRI expression (Figure 1B), a surrogate for serum IgE^{E20}, suggesting that IgE-mediated stimulation may impact monocyte-directed Th1 priming during viral infection. Confirming this concept, IgE cross-linking of influenza-exposed monocytes significantly disrupted Th1 differentiation (Figure 1C and E1), a finding consistent across all donors (Figure 1D). Similarly, IgE cross-linking decreased IFNγ secretion (Figure 1E) and T cell proliferation (Figure 1F and E1), but had no effect on Th2 development (Figure E2). The decrease in Th1 cell stimulation was not due to IgE-mediated inhibition of influenza virus infection of monocytes as IgE cross-linking did not affect percentages of monocytes expressing the influenza proteins hemagglutinin and nucleoprotein (Figure 1G and H). These data suggest that allergen-mediated IgE cross-linking on monocytes during concomitant viral infection could result in impaired development of virus-specific Th1 cells in sensitized individuals *in vivo*.

We evaluated potential roles for multiple cytokines in IgE-mediated inhibition of monocyte CD4⁺ T lymphocyte development, including IL-12, a critical factor in Th1

differentiation (Figures E3 and E4). Our results indicate that IL-12, TNF- α , IL-6, and IL-10 do not regulate IgE-mediated suppression of monocyte-driven antiviral Th1 priming.

Up-regulation of MHC and co-stimulatory molecules is required for engagement and activation of the T cell receptor complex. These signals direct CD4+ T cell maturation; stronger antigen signal strength favors Th1 differentiation while weaker signals promote Th2 or regulatory phenotypes^{E21}. We evaluated the effect of IgE cross-linking on influenza-induced monocyte maturation. Influenza exposure significantly up-regulated surface CD80, CD86, MHC Class I (HLA-ABC) and II (HLA-DR) expression with levels significantly diminished by IgE-mediated stimulation (Figure 2A-C). We hypothesized that IgE-mediated down regulation of HLA-DR and CD86 may disrupt monocyte-T cell interactions. Visualization and quantitation of monocyte-T cell interactions revealed that monocyte-T cells clusters were significantly increased by influenza exposure, but impaired by IgE cross-linking (Figure 2D & E). Since cell proliferation directly reflects T cell activation, the IgE-mediated decrease in cell division (Figure 1F) provides additional evidence that IgE cross-linking prevents monocytes from forming high affinity interactions needed to activate multiple T cells simultaneously. These data support the model that allergic stimulation significantly impairs up-regulation of proteins essential for antigen presentation, resulting in weaker T lymphocyte stimulation and diminished antiviral Th1 responses (Figure E5).

IgE-mediated inhibition of virus-induced Th1 priming was remarkably conserved across all donors, and may reflect an evolutionarily maintained mechanism. Parasite-produced proteins have effects on APCs similar to IgE cross-linking on monocytes - down regulation of antigen presenting molecules, altered CD4 T cell activation, and decreased Th1 responses^{E22,E23}. Parasite infection can even alter viral immune responses in co-infection models^{E24-E27}. Parasite-specific IgE is protective during parasitic infections^{E28,E29}; diminished Th1 responses induced by IgE cross-linking could thus be immunomodulatory in the setting of either parasitic or atopic diseases. Along these lines, many epidemiological studies reveal that children with parasitic infections have decreased atopy^{E30-E32}. Parasitic infections are characterized by Th2-mediated immunity. Activation of IgE-mediated pathways by parasites, or allergen-specific IgE, could thus negatively regulate Th1 development, creating a Th2-predominant, more optimal anti-parasitic response. Decreased Th1 differentiation could then dampen pro-inflammatory responses during parasitic (or even viral) infections, resulting in decreased inflammation and tissue damage^{E33-E36}, and providing potential benefit in allergic airway disease.

Alternatively, IgE-mediated disruption of antiviral Th1 responses could promote more severe viral disease via dysregulation of normal Th1/Th2 balance and contribute to exacerbations of allergic disease. The samples in our study were predominantly from unknown donors obtained via a local blood bank; no information regarding atopic status or serum IgE levels was available. We can thus only extrapolate our findings to those with defined atopic disease. However, the range of surface FcεRI expression suggests a spectrum of IgE levels in our study participants, and the inverse correlation with influenza-induced Th1 differentiation supports the concept that elevated IgE may contribute to defective monocyte-directed antiviral Th1 responses *in vivo*. Consistent with our findings, others have reported blunted Th1 responses to viral infections associated with atopy. Following rhinovirus infection of asthmatics, blood and airway CD4 T cells secreted decreased IFNγ⁸. Similarly, atopic dermatitis patients had decreased virus-specific Th1 cells after transcutaneous live virus vaccination, which inversely correlated with serum IgE levels⁹. In future studies, it will be important to evaluate effects of IgE cross-linking on T cell priming by monocytes exposed to other viruses, including rhinoviruses, and compare responses in atopic (high IgE) versus non-atopic individuals.

To our knowledge, this is the first study demonstrating IgE-mediated impairment of human monocyte antiviral responses, via suppression of virus-induced monocyte maturation and Th1 differentiation. This extends our understanding of how allergen-mediated pathways alter host antiviral responses, highlighting a potential role for monocytes in virus-associated exacerbations of allergic disease. Further studies investigating the mechanisms underlying IgE-mediated suppression of virus-induced monocyte responses will enhance our comprehension of allergic disease pathogenesis and potentially uncover novel targets for therapeutic development.

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Figure Legends:

Figure 1. IgE cross-linking inhibits virus-driven monocyte priming of Th1 cells

(A) Naïve CD4 T cells polarized with monocytes exposed to no virus (mock) or influenza A virus (Flu). (B) Linear regression analysis was used to determine correlation between baseline monocyte FcεRI expression (x-axis) and Th1 priming capacity (% IFNγ+ cells, y-axis) of influenza-exposed monocytes with p values and r^2 values noted. N=15. (C and D) % IFNγ+ T cells in monocyte-T cell co-cultures under IgE cross-linking conditions: (C) mean and (D) individual donor pairs. N=15. (E) Mean IFNγ concentration in T cell supernatants (N=5). (F) IgE cross-linking diminishes T cell proliferation. Naïve CD4 T cells labeled with VPD450 cellular dye prior to co-culture. % divided defined as cells with lower VPD450 fluorescence compared to unstimulated T cell controls, N=3. Error bars represent the standard error of the mean (SEM). Pertinent p values are shown and were obtained with two-way ANOVA analysis. (G-H) Influenza A virus protein expression in monocytes at 6 and 24 hours post infection by (G) flow cytometry of Flu positive cells (NP/HA double positive) and (H) flow cytometry imaging at 6 hours post infection (NP green, HA red). Shown is representative data from N=3 experiments. Error bars represent SEM, and p values represent results of one-way ANOVA. All Material and Methods can be found in the Online Repository.

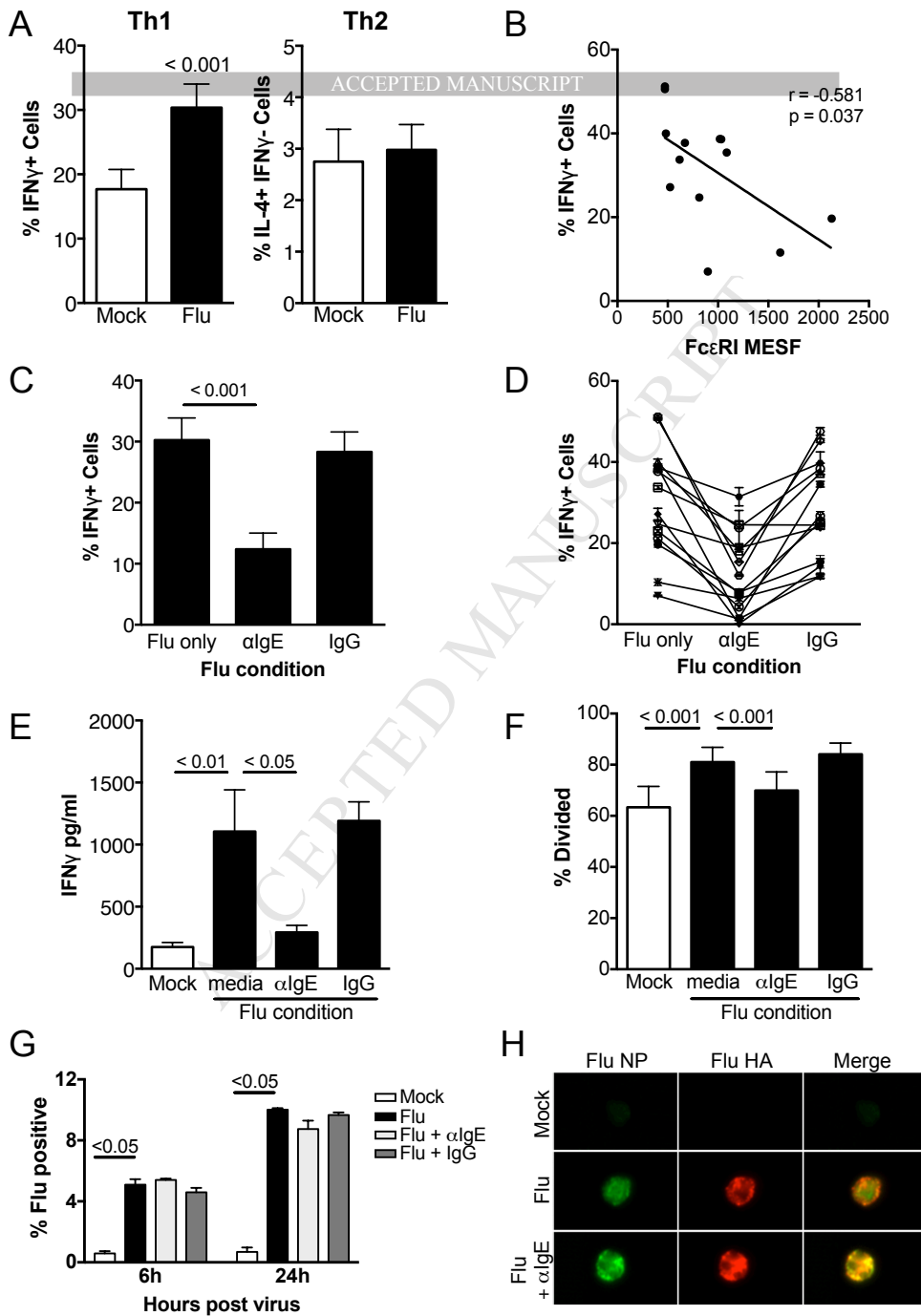
Figure 2. IgE cross-linking inhibits virus-induced monocyte maturation and monocyte-T cell interactions

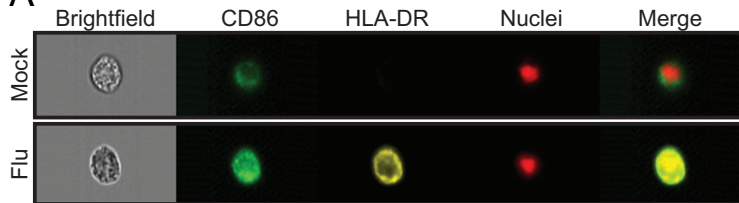
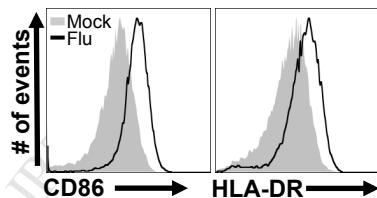
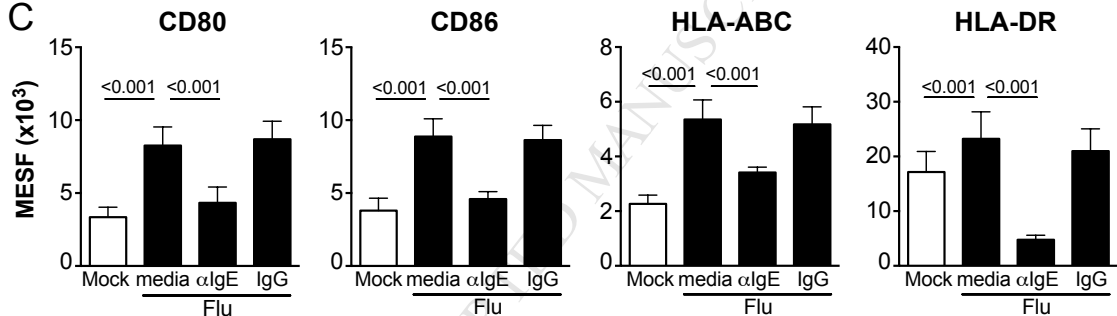
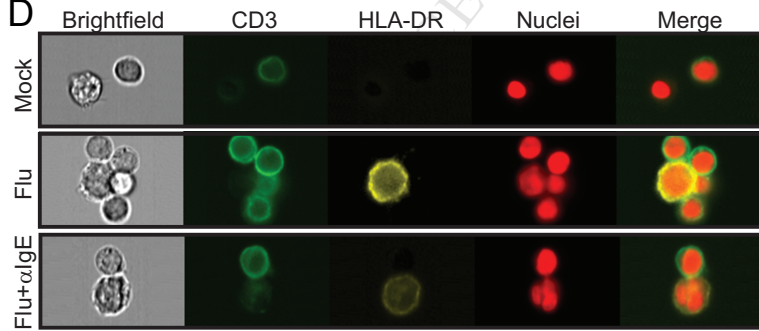
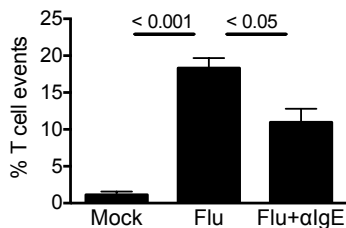
(A-C) Monocytes treated with media alone (mock) or influenza A virus (Flu) +/- IgE cross-linking or IgG control antibody and stained for various cell surface markers. Representative flow cytometry (A) imaging analysis and (B) histograms for CD86 and HLA-DR staining are shown for mock and Flu only (C) Mean cell surface marker expression by flow cytometry analysis of the mean equivalent soluble fluorochrome (MESF, expressed as $\times 10^3$ units) value were determined under the various conditions, N=3 (D) Flow cytometry imaging analysis of monocyte-T cell interactions. Cell clusters from monocyte-T cell co-cultures stained with anti-CD3 (green), anti-HLA-DR (yellow), and nuclei (red) were visualized, 60x magnification on the Amnis ImageStream flow cytometer. (E) Quantitation of total monocyte-T cell clusters was performed using the Ideas Software (Amnis), (N=3). Error bars represent SEM and p values represent results of one-way ANOVA. All Material and Methods can be found in the Online Repository.

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A**B****C****D****E**

Online Repository Materials:

Table E1: Influenza virus associations with allergic disease

- Influenza viruses are associated with severe disease in individuals with allergic asthma, and have been linked to exacerbations^{E4-E6}.
- During the 2009 influenza pandemic, increased disease severity was observed in asthma patients, with many requiring intensive level care^{E7,E8}.
- However, other comorbidities besides asthma have higher rates of severe outcomes, including death, among hospitalized individuals with influenza^{E9-E11}.
- The Centers for Disease Control reports asthma is the most common underlying comorbidity in children and in the top 5 for adults hospitalized with influenza^{E12}.
- More severe infections have been noted in atopic versus non-atopic children in general^{E13}.
- Influenza is also isolated more frequently from adults with allergic rhinitis compared to healthy controls^{E14}.
- Murine models of allergic sensitization demonstrate enhanced allergic phenotypes after influenza infection^{E15, E16}.

Investigating IgE-mediated effects on antiviral immune responses to influenza virus may shed light on key mechanisms modulating allergen-virus interactions.

Figure Legends:

Figure E1. IgE cross-linking inhibits virus-driven monocyte T cell priming and proliferation.

Representative flow cytometry plots for Figure 1. Naïve CD4 T cells polarized with monocytes exposed to no virus (mock) or influenza A virus (Flu) +/- IgE cross-linking antibody (α IgE) or isotype IgG control. T cells were labeled with the cell proliferation dye, VPD450, prior to co-culture. (A) Zebra plots for IFN γ expression versus VPD450 show decreased IFN γ positive cells in the presence of IgE cross-linking. (B) Histograms show cell proliferation (measured by loss of VPD450 intensity, x-axis) of T cells stimulated with monocytes (black line) compared to unstimulated T cells in gray.

Figure E2. IgE cross-linking does not alter Th2 polarization

Naïve CD4 T cells polarized with monocytes exposed to no virus (mock) or influenza A virus (Flu) +/- IgE cross-linking antibody (α IgE) or isotype IgG control. Graphs shown are (A) Mean percentages of IL-4+ IFN γ - cells for the indicated conditions. N=15 donor pairs. (B) Mean IL-4 concentration in monocyte-T cell co-culture supernatants. N=5 donor pairs; error bars represent SEM, and p values represent results of two-way ANOVA.

Figure E3. IL-12 is not involved in Th1 polarization by influenza-exposed monocytes

IL-12 is a critical factor in Th1 development^{E21}. Neither blocking antibodies nor exogenous addition of IL-12 restored the IgE-mediated defect in Th1 differentiation (A) IL-12p70 production was measured in supernatants from monocytes treated +/- IFN γ for 18h followed by 24h incubation with media alone (mock), influenza A virus (Flu), or lipopolysaccharide (LPS). IL-12p70 was not detectable after influenza exposure, despite robust production under positive control conditions. Dashed line denotes the limit of detection (LOD). (B) Monocyte-induced Th1 priming; mean % IFN γ + T cells in depicted conditions treated +/- isotype control (black bars) or neutralizing IL-12 antibody (white bars). N=3. (C) % IFN γ + T cells +/- recombinant human IL-12. Representative data (of N=3 experiments) is shown; error bars represent SEM and p values represent results of one-way ANOVA.

Figure E4. IgE cross-linking suppresses Th1 priming independently of TNF- α , IL-6, and IL-10

Cytokine secretion from monocyte supernatants at 18 hours post influenza virus +/- IgE cross-linking was measured for (A) TNF- α , (B) IL-6 or (C) IL-10. These were secreted by monocytes upon IgE cross-linking⁷, and in the presence of both IgE cross-linking and influenza. (D-F) Neutralization of these cytokines did not impact influenza-induced Th1 priming or restore inhibition by IgE cross-linking. Mean percentages of IFN γ + T cells in monocyte-T cell co-cultures treated with isotype controls (black bars) or neutralizing antibodies (white bars) to (D) TNF- α , (E) IL-6 or (F) IL-10. Representative data from of N=3 experiments are shown; error bars represent SEM and p values represent results of one-way ANOVA.

Figure E5: Model of IgE-mediated inhibition of monocyte-driven Th1 priming

Upon monocyte influenza exposure, the normal antiviral response results in phenotypic maturation and up-regulation of monocyte cell surface molecules, downstream antigen presentation, and strong TCR signal culminating in Th1 differentiation. In the setting of allergic stimulation, via IgE receptor (FcεRI) engagement, virus-induced monocyte maturation is inhibited, resulting in weak TCR signal and poor Th1 differentiation. Impaired Th1 production would have significant downstream effects, including decreased antiviral cytokine secretion (e.g. IFNγ), cytotoxic CD8+ T cell development, and antigen-specific antibody production.

Materials and Methods:

Human Subjects

Leukocyte-enriched blood samples were acquired predominantly from unknown donors from a local blood bank, or peripheral blood samples were obtained from known volunteers. Unknown donors were deemed healthy enough for donation by the local blood bank; neither atopic status nor serum IgE levels were obtained. Studies were approved by the UT Southwestern institutional review board (Study # STU 122010-139). Written informed consent and assent were obtained.

Reagents and Media

Phosphate Buffered Saline (PBS) with fetal bovine serum (FBS) and EDTA (complete PBS, cPBS) and complete Roswell Park Memorial Institute Medium 1640 (cRPMI) were prepared as previously described⁷. Rabbit anti-human IgE (α IgE) was purchased from Bethyl Laboratories (Montgomery, TX). Rabbit whole IgG control antibody (IgG) was purchased from Jackson ImmunoResearch (Westgrove, PA). Unless specified, recombinant human (rh) cytokines, anti-human cytokine and receptor antibodies were purchased from R&D Systems (Minneapolis, MN).

Purification of Immune Cells from Blood

Monocytes were isolated from blood as previously described⁷. Naïve CD4⁺ T cells were purified using the EasySepTM Negative Selection Human Naïve CD4⁺ T Cell Enrichment Kit (STEMCELL Technologies Inc., Vancouver, Canada) according to manufacturer recommendations. Purity of isolated cells was assessed by flow cytometry; monocytes were identified as CD14⁺ and enriched naïve CD4⁺ T cells as CD3⁺ CD4⁺ CD45RA⁺. Samples with <90% purity were excluded.

Monocyte Culture Conditions

Monocytes were cultured in cRPMI in the presence of recombinant human (rh) M-CSF (1 ng/ml) at a concentration of $1-2 \times 10^6$ cells/ml. α IgE (10 μ g/ml), control rabbit IgG (10 μ g/ml), or IFN γ (20 ng/ml) was added to monocyte cultures as indicated. Influenza (Influenza A/PR/8/34 H1N1; 0.1 multiplicity of infection (MOI), Charles River Laboratories International, Inc.) or LPS (lipopolysaccharide; 200 ng/ml, Sigma-Aldrich, St. Louis, MO) was added to cultures. Cultures

were incubated at 37°C for indicated times and cells and supernatants were harvested for analysis.

Monocyte-T Cell Co-Culture

Monocytes were cultured as described above in the presence or absence of αIgE, control IgG, or influenza at 37°C for 18-24 hours. Pretreated monocytes were mixed at a 1:1 ratio with allogeneic naïve CD4⁺ T cells pre-loaded with VPD450 Proliferation Dye (BD Biosciences, San Jose, CA) prior to co-culture. Cells were cultured at 2x10⁶ cells/ml in media containing 50 Units/mL recombinant human IL-2. For select experiments, mouse anti-human antibodies against IL-10, IL-6, IL-12, TNF-α, IL-10Rα, IL-6R, or TNFR1 and IgG1 or IgG2b isotype controls were added to monocyte cultures in various combinations at 5-10μg/mL each for a total mouse IgG concentration of 10 μg/ml prior to co-culture with T cells. As indicated, rh IL-12 (1 ng/ml) was added at the initiation of the co-culture. After 3 days of co-culture, T lymphocytes were diluted 1:10 and cultured for an additional 4 days. T cells were then rested overnight at 37°C in media without IL-2, and subsequently stimulated with either: 1) 6 hours of phorbol 12-myristate 13-acetate (PMA, 50 ng/ml, Sigma, St. Louis, MO), ionomycin (1μM, ThermoFisher, Grand Island, NY), and monensin (1x, BioLegend, San Diego, CA) followed by cell harvesting and staining for intracellular cytokines, or 2) 24h on anti-CD3-coated plate (OKT3 clone, provided by David Farrar, UT Southwestern) followed by harvesting of supernatants for analysis of secreted cytokines by ELISA^{E37, E38}.

Flow Cytometry Analysis of Surface Antigens

The following fluorochrome-conjugated anti-human antibodies or molecules were used: CD3-AlexaFluor®700, CD3-Fluorescein isothiocyanate (FITC), CD4-allophycocyanin (APC), CD14-V450, CD45RA-phycoerythrin (PE), CD80-FITC, CD86-PE-CyTM5, FcεRI-PE, HLA-A,B,C-APC, HLA-DR-APC-CyTM7, and HLA-DR-PE, CD86-biotin, and Streptavidin-FITC (BD Pharmingen, San Diego, CA). Staining and flow cytometry were performed as previously described⁷.

Flow Cytometry Analysis of Intracellular Cytokines and T cell Proliferation

The following antibodies were used: IFNγ-PE-Cy7 and IL-4-PE (BD Biosciences, San Jose, CA). Cells were fixed in 2% paraformaldehyde and permeabilized with 0.1% saponin in PBS to detect intracellular antigens. VPD450 Proliferation dye was used per manufacturer's instructions to measure T cell proliferation. Proliferated cells were defined as cells with lower VPD450

intensity than non-stimulated control T cells. Samples were acquired on a BD LSR II flow cytometer (BD Biosciences) and analyzed with the data analysis software, FlowJo (FLOWJO, LLC, Ashland, OR). Gating of cytokine-expressing cells was guided by staining negative control (unstimulated cells) and positive control T cells differentiated under Th1- or Th2-inducing conditions^{E37, E38}. Percentages of total populations that were cytokine-positive were then determined.

Influenza infection of monocytes and Flow cytometry analysis of influenza proteins

Monocytes were isolated as above, and exposed to influenza A virus at MOI of 0.1 with or without IgE cross-linking or IgG control. Cells were infected for 2 hours at 37°C in complete RPMI media. Cells were washed twice with complete PBS to remove non-absorbed virus and resuspended in complete RPMI with or without IgE cross-linking or IgG control antibody as above. At various times post infection, cells were washed, fixed in 2% paraformaldehyde, and stained for total cell (permeabilized with 0.1% saponin to detect intracellular fraction) influenza nucleoprotein (mouse anti-influenza A virus nucleoprotein FITC, clone 431, Abcam (Cambridge, MA)) and hemagglutinin (mouse anti-influenza hemagglutinin H1N1, Alexa Fluor 647, clone 102, Novus Biologicals (Littleton, CO). Cells were analyzed on by either flow cytometry on the BD LSR II (BD Biosciences), or flow cytometry imaging with the ImageStream flow cytometer (Amnis, Millipore/Sigma).

Cytokine Secretion Analysis

Supernatants were stored at -80°C until use. The following ELISA kits were used according to manufacturer recommendations: READY-SET-GO! Human ELISA kits (eBioscience, Inc., San Diego, CA) for IL-12p70, IFN γ , and IL-4; ELISA Max human ELISAs for IL-10, TNF- α , and IL-6 were obtained from Biolegend (San Diego, CA). Absorbance was measured on a Biorad iMark microplate reader (Biorad, Hercules, CA). TNF- α was also measured with the V-plex human cytokine 30-plex assay per manufacturer's instructions for increased sensitivity (Meso Scale Diagnostics, Rockville, MD).

Flow cytometry imaging analysis

Flow cytometry imaging was performed using the Amnis ImageStream flow cytometer (Amnis, Seattle, WA). Briefly, monocytes and naïve CD4 T cells were co-cultured as above described. At 24h post-co-culture, cells were gently removed from the plate to preserve cell-cell interactions, pelleted at 200 x g, and fixed in 4% paraformaldehyde for 10 minutes. Cells were stained with anti-CD3-FITC and anti-HLA-DR-PE antibodies. Nuclei were counterstained with DRAQ5 (1:2000 dilution, Invitrogen, ThermoFisher, Grand Island, NY) per manufacturer's recommendations. Images were analyzed using the IDEAS analysis software (Amnis, Seattle, WA). CD3-HLA-DR double positive T cell-monocyte cell clusters were identified using a multi-step gating strategy. Events were analyzed based on nuclear intensity to obtain number of nuclei per cluster. Monocyte-only staining was performed as above for surface expression with anti-HLA-DR-PE and anti-CD86-Biotin antibodies, followed by incubation with a streptavidin-FITC conjugate.

Data Analysis and Statistics

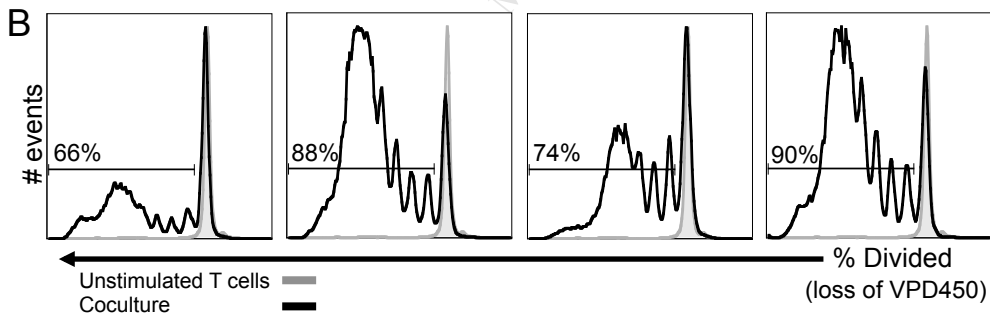
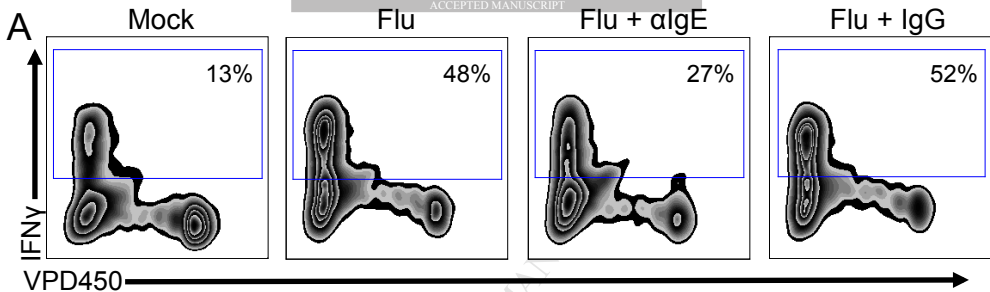
Data are presented as means $\pm$ standard error of the mean (SEM). In experiments containing 3 or more conditions, one-way or two-way (for experiments with multiple donors) repeated measures ANOVA and pairwise Tukey's post hoc comparisons were performed. Two monocyte-T cell experiments (of 17 total) were eliminated from analysis due to a lack of influenza-induced Th1 response. For experiments comparing 2 conditions, paired t tests were performed with Holm-Sidak correction where appropriate. $p < 0.05$ was considered significant, pertinent p values are noted in figures; significant as < 0.05 , < 0.01 , and < 0.001 , or > 0.05 for non-significant values. All statistical analyses were performed using GraphPad Prism version 6.

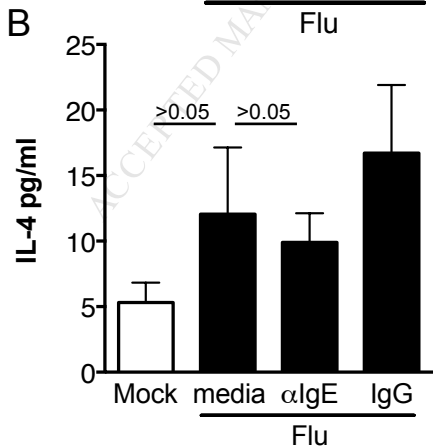
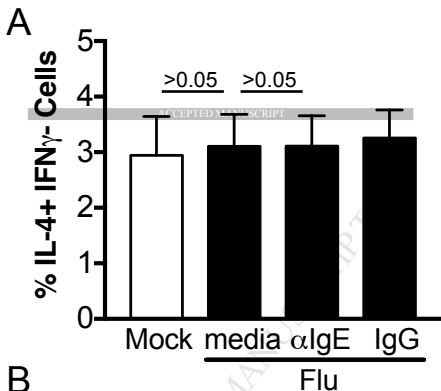
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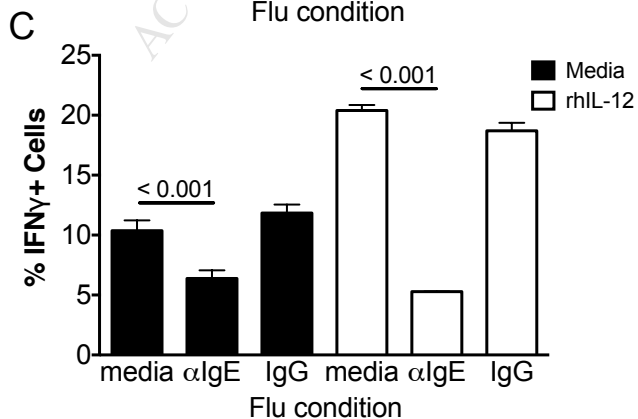
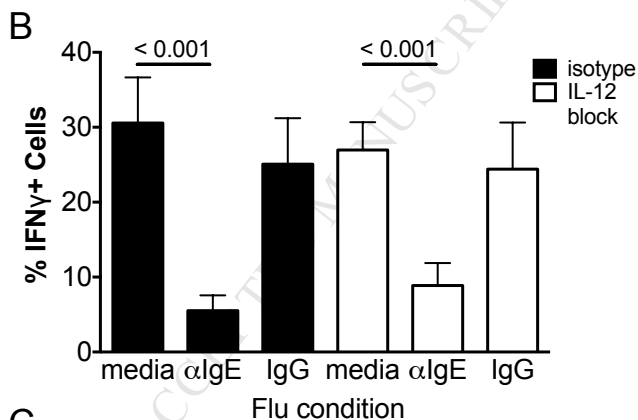
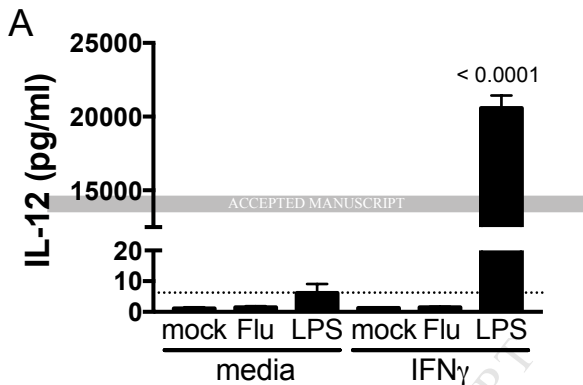
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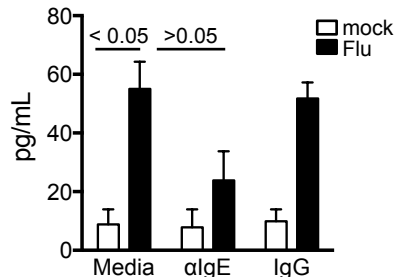
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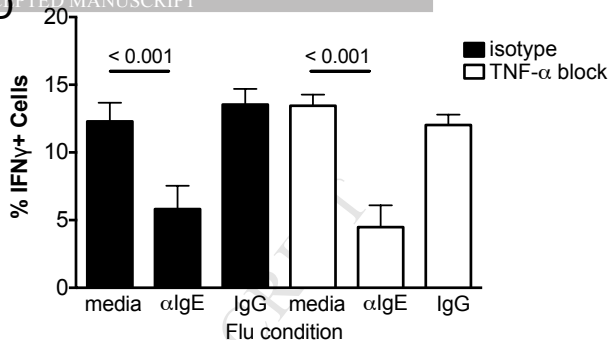




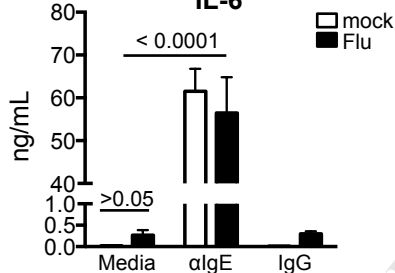
A

TNF- α 

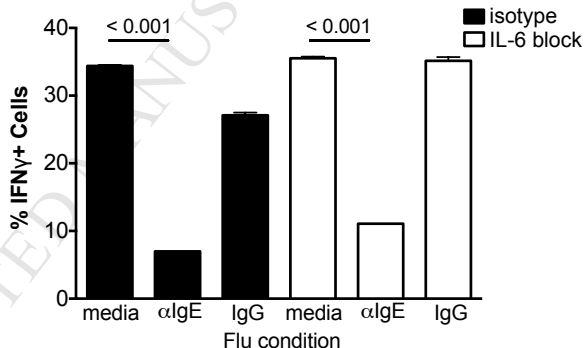
D



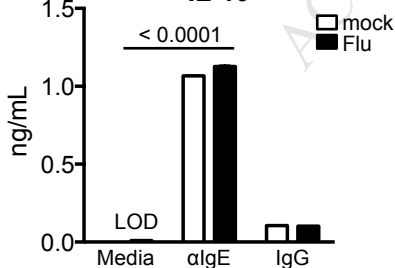
B

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E



C

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