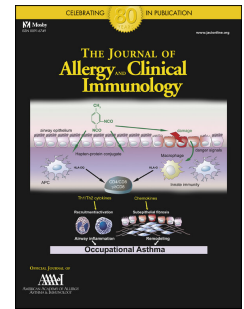


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A humanized mouse model of anaphylactic peanut allergy

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ABSTRACT

Background: Food allergy is a growing health problem with very limited treatment options. Investigation of the immunological pathways underlying allergic sensitization to foods in humans has been greatly constrained by the limited availability of intestinal tissue and gut-resident immune cells. While mouse models have offered insights into pathways of food sensitization, differences between rodent and human immune physiology limit the extension of these findings to our understanding of human disease.

Objective: To develop a strategy for the generation of mice with humanized adaptive immune systems, complete with tissue engraftment by human mast cells that are competent to mount specific IgE-mediated responses and drive systemic anaphylaxis upon ingestion challenge.

Methods: Non-obese diabetic (NOD) severe combined immunodeficient (SCID) mice lacking the cytokine receptor common gamma chain ($\gamma_c^{-/-}$) and carrying a human stem cell factor (SCF) transgene were engrafted with human hematopoietic stem cells (HSC). The impact of peanut (PN) feeding and IgE neutralization on the development of immune responses, mast cell homeostasis and anaphylactic food allergy was assessed in these animals.

Results: NSG SCF (huNSG) mice exhibited robust engraftment with functional human T and B lymphocytes and human mast cells were found in significant numbers in their tissues, including the intestinal mucosa. Following gavage feeding with PN they mounted specific antibody responses, including PN-specific IgE. When enterally challenged with PN, they exhibited mast cell mediated systemic anaphylaxis, as indicated by hypothermia and increases in plasma tryptase levels. Anti-IgE (omalizumab) treatment ablated this anaphylactic response.

Conclusions: huNSG mice provide a novel tool for studying food allergy and IgE-mediated anaphylaxis.

Key Words: IgE, mast cells, anaphylaxis, humanized mice, tryptase, peanut allergy

Abbreviations: NOD, non-obese diabetic; SCID, severe combined immunodeficiency; NSG, NOD SCID common gamma chain-deficient; SCF, stem cell factor; IL-3, interleukin-3; HSC, hematopoietic stem cell; PN, peanut; CAE, chloroacetate esterase; LAMP-1, lysosomal-associated membrane protein-1, Th, T helper; Treg, regulatory T cell; Teff, effector T cell; huNSG, humanized NSG SCF mouse; MMCP-1, murine mast cell protease-1; OIT, oral immunotherapy.

INTRODUCTION

Food allergy is a rising health challenge with a lack of effective treatments. As such, new tools are required to probe the earliest cellular and molecular signals driving allergic food sensitization and those responsible for maintaining the response. Furthermore, innovative approaches are needed for the preclinical evaluation of treatment strategies emerging from basic research. While substantial advances in delineating pathways of allergic sensitization to foods and effector mechanisms of hypersensitivity have recently been made in human subjects, progress has been constrained by the limited availability of the key tissues required to analyze local intestinal responses (e.g. intestinal mucosa, mast cells and gut-associated lymphoid tissue).

Mouse models have been applied with significant success but, for the most part, have required non- or less physiologic methods of sensitization (*i.p.* priming or using adjuvant) or genetic manipulations to enhance sensitivity. In addition, inherent differences in the immune physiology of rodents and humans limit the interpretation of findings from such models. Notably, the high affinity IgE receptor, FcεRI, displays a wider distribution on human leukocytes, including antigen-presenting cells; and soluble CD23 (FcεRII) complexed to IgE can stimulate IgE synthesis in human but not mouse B cells via CD21^{1, 2}. The differences in antibody Fc biology extend to the interaction between IgG and FcγRIIb, which exerts a critical brake on degranulation and anaphylaxis and is generally stronger in mice than humans,^{3, 4} with human skin mast cells expressing primarily activating FcγRIIa⁵.

We reasoned that a model in which rodents harbor a fully humanized adaptive immune system capable of generating food-specific IgE following allergen ingestion, as well as fostering the development of the human innate effector cells of anaphylaxis, would provide such a tool. Here we describe the conditions for the generation of such mice, their physiological response to peanut (PN) allergen and their utility as a source of human mast cells. Furthermore, we demonstrate the impact of omalizumab-mediated IgE neutralization on PN-induced anaphylaxis.

METHODS

Mice.

NSG SCF (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl} Tg(PGK1-KITLG*220)441Daw/SzJ) mice were purchased from The Jackson Laboratory, and bred in a biosafety level 2 facility at Boston Children's Hospital. NSG mice were maintained in autoclaved cages with Sulfatrim oral suspension (Sulfamethoxazole/Trimethoprim, HiTech Pharmacal) in the sterilized drinking water. All procedures were carried out under protocols approved by the local institutional animal care and use committee.

Stem cells.

Cord blood-derived flow-sorted human CD34⁺ hematopoietic stem cells were purchased from AllCells. Such cells are obtained under Institutional Review Board (IRB)- or Human Subject Committee-approved protocols. Cells (5×10^4 - 10^5) were injected intravenously via the retro-orbital sinus into 3-6 week old mice. Engraftment was monitored in samples of peripheral blood using flow cytometry monthly for the four month engraftment period preceding allergen sensitization.

Flow Cytometry.

Cells were stained with the following antibodies: Brilliant Violet 421-conjugated anti-human CD45 (clone HI30), PE-Cy7 anti-mouse CD45 (30-F11), Alexa Fluor 700 anti-CD3 (HIT3a), FITC anti-CD4 (OKT4), PE-Dazzle594 anti-IL-4 (MP4-25D2), Alexa Fluor 647 anti-Foxp3 (259D), PerCP-Cy5.5 anti-CD127 (A019D5), PE-Cy7 anti-CD25 (BC96), PerCP-Cy5.5 anti-IFN γ (4S.B3), PE anti-IL-10 (JES3-19F1), APC anti-CD19 (HIB19), PE-Cy7 anti-HLA-DR (L243), Brilliant Violet anti-c-Kit (104D2), and PE anti-Fc ϵ RI (AER-37 (CRA-1)) were purchased from Biolegend. APC anti-IgE (Ige21) was obtained from Affymetrix eBioscience. Anti-mouse

CD16/32 (clone 93) and TruStain FcX Fc receptor blocking solution (both Biolegend) were used to prevent non-specific binding. Dead cells were excluded using fixable viability dye eFluor 780 (Affymetrix eBioscience). Intracellular cytokine staining was performed after a 4hr stimulation at 37°C with 500ng/ml ionomycin, 500ng/ml phorbol 12,13-dibutyrate and 1µg/ml brefeldin A (all Sigma-Aldrich). Coordinate analysis of transcription factors and cytokine production was performed using BD Biosciences Cytofix and Cytoperm reagents as previously described⁶. Intestinal leukocyte isolation was performed according to established protocols⁷.

Food allergen sensitization and anaphylaxis.

After four months of stem cell engraftment, mice were sensitized by intragastric feeding with 22.5mg (5mg protein) Skippy creamy peanut butter (Hormel Foods) in 250µl 0.2M sodium bicarbonate pH 8.0 weekly for eight weeks. Control mice were sham-sensitized with sodium bicarbonate alone. Allergen challenge was performed by gavage feeding with 350mg peanut butter suspended in 0.2M sodium bicarbonate. Temperature measurements were performed using microchip transponders implanted subcutaneously 48hrs prior to challenge, as we have previously described⁸. Omalizumab (αIgE) was administered weekly by i.p. injection 48hrs prior to PN feedings at 120mg/kg. Dosing was calculated to correspond to the 10mg/kg used in humans, based upon surface area conversion recommendations from the Food and Drug Administration and other sources^{9,10} and was designed to optimize the potential for IgE neutralization rather than an attempt to mimic therapeutic use in human patients.

ELISAs.

PN-specific IgE was quantified by ELISA, capturing IgE onto plates coated with 3µg/ml anti-human IgE (clone G7-18, BD Biosciences), and detecting with biotinylated PN extract (200ng/ml, see reference¹¹). A standard curve was constructed using PN-allergic patient sera

that had been previously quantified by Immunocap. Tryptase measurements were performed by ELISA as previously described¹².

Statistics.

Data were plotted and analyzed in Prism 5.0f (GraphPad Software, Inc.). Temperature curves were analyzed using repeated measures two-way ANOVA, with matching for each individual mouse across the time course. Tryptase and IgE values were log transformed prior to analysis by ANOVA with Bonferroni post-tests. Data are represented as mean±SEM, with an individual point for each mouse where applicable.

RESULTS

PN-fed huNSG mice exhibit PN-specific IgE responses, PN-induced systemic anaphylaxis and intestinal mast cell expansion.

A number of murine models of food allergy have been developed by our group and others to probe mechanisms underlying both allergic sensitization to foods and the induction of tolerance^{8, 13-18}. One such model, in which an atopic tendency including susceptibility to food allergy is conferred by targeted insertion of a gene expressing an activated form of the IL-4 receptor α -chain, revealed the importance of IgE antibodies and mast cells in mediating food anaphylaxis⁸. Although there is a great deal of overlap in the pathways leading to immunological sensitization and tolerance in humans and rodents there are inherent differences that limit the application of findings from animal models to human disease. However, the direct study of human intestinal immune responses to food allergens has been hindered by the lack of available tissue. Food allergic subjects are generally otherwise healthy and have no clinical indications for endoscopy and biopsy, which would be necessary to obtain primary tissue for investigational purposes. We reasoned that a compromise would be to reconstitute immunodeficient mice with human hematopoietic progenitor that would develop into the human

adaptive immune cells capable of generating Th2 and IgE responses as well as the effector innate immune cells (mast cells) of immediate hypersensitivity. Such humanized mice would permit us to test food allergy mechanisms and specifically the role of IgE antibodies and mast cells in a humanized setting.

A number of approaches have been described for the generation of humanized mice, generally using immunodeficient mice reconstituted with human PBMC or stem cells¹⁹⁻²³. We elected to use a stem cell repopulating model, in which CD34⁺ cord blood hematopoietic stem cells (5x10⁴) are injected into NOD SCID common gamma chain (γ_c)^{-/-} (NSG) mice and develop into a human immune system. We predicted that, in addition to reconstituting full T and B cell adaptive immune function, it would be necessary in an allergy model for the recipient mice to be populated with functional human mast cells. Mast cells are dependent on stem cell factor (SCF), which binds to c-Kit, for their expansion and survival in tissues so we selected NSG mice with a human membrane-bound stem cell factor (SCF) transgene (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl} Tg(PGK1-KITLG*220)441Daw/SzJ) as recipients. SCF expression in this strain had previously been shown by Takagi *et al.* to support functional mast cell development and enhanced immune engraftment without requiring irradiation^{21, 24}.

This approach provided robust engraftment and, after 12-20 weeks, human CD45⁺ leukocytes were predominant in the peripheral blood, spleen, mesenteric lymph nodes, bone marrow and small intestinal lamina propria (**Fig 1** and data not shown). Adaptive immune lymphocytes were exclusively of human origin. Human CD45⁺ cells included CD4⁺ effector T cells exhibiting Th1 (IFN γ ⁺) and Th2 (IL-4⁺) T-helper phenotypes following stimulation as well as Foxp3⁺CD127^{low}CD25⁺ regulatory T cells. An IL-10⁺Foxp3⁺ population of T cells was observed as well. The B cell compartment was also well reconstituted with abundant CD19⁺HLA-DR⁺ B cells in the spleen and jejunum.

When repeatedly fed small doses of commercial peanut butter (22.5mg, 5mg protein content) by gavage weekly for eight weeks (**Fig 2A**), the mice with human immune systems

developed anaphylactic sensitivity to the PN. Upon enteral challenge with a high dose of PN (350mg, 80 mg protein), most animals displayed a rapid and sustained loss of core body temperature ($3.4^{\circ}\text{C} \pm 2.1^{\circ}\text{C}$ SD at nadir), indicative of the vasodilation and peripheral blood flow diversion characteristic of anaphylaxis (**Fig 2B**). In human subjects undergoing anaphylaxis tryptase levels are often elevated, reflecting mast cell degranulation²⁵. PN-sensitized and challenged but neither mock-sensitized nor unchallenged mice exhibited marked increases in human tryptase in serum 1hr after PN challenge (**Fig 2C**). PN-allergic humanized mice made PN-specific human IgE in their serum, with a maximal response around 2.5 kU/L (**Fig 2D**). Neither human tryptase nor human IgE were detected in the sera of mice that did not receive HSC.

The tissues of the huNSG mice were efficiently reconstituted with human mast cells and these cells were brightly positive for human IgE (**Fig 3A**). A significant mast cell expansion was evident in the jejunum of PN-fed mice, concordant with murine models of food allergy (**Fig 3B**). Mast cell counts were also elevated in the spleen following PN sensitization. Histological examination revealed that hematopoietic stem cell transfer gave rise to robust human mast cell engraftment in the small intestine (**Fig E1**). Comparison with literature-reported values for human mast cell counts in biopsy samples suggested that mast cell densities in these huNSG mice might be somewhat elevated relative to healthy human tissues (**Table E2**) although it is possible that some of these differences might be due to variations in the tissue fixation and staining techniques used in the cited studies.

Anaphylaxis in PN-fed huNSG mice is mediated exclusively by engrafted human mast cells and human IgE, with no contribution from host murine mast cells.

While these findings were strongly consistent with PN anaphylaxis mediated by human IgE interacting with human mast cells, we performed additional experiments to completely rule out the possibility that murine mast cells might be mediating the human IgE effect. To this end we looked for human and murine mast cells in the huNSG mice both by histology and by flow

cytometric analysis of human IgE and FcεRI staining. Human IgE does not bind to murine FcεRI, and the absence of γ_c signaling in cells of murine origin in these chimeric mice would be expected to greatly reduce the viability and function of murine FcεRI-bearing effector cells²⁶⁻²⁹. Histological analysis by chloroacetate esterase (CAE) staining, which detects mast cell granules by chymotryptic protease activity, revealed a complete absence of mast cells in the small intestine of mice that did not receive human stem cells (**Fig E1**). In contrast, some murine mast cells were observed in skin. Following HSC engraftment, unsensitized huNSG mice exhibited elevated numbers of mast cells in the stomach, small intestine and skin but not the lungs (**Table E2**). We further quantified human and murine mast cells by flow cytometry in the spleen and jejunum, finding that human mast cells outnumbered murine FcεRI⁺ cells by at least 100-fold in the spleen and 60-fold in the small intestine (**Fig E3**). No human IgE was detected on the surface of murine FcεRI⁺ cells. Consistent with the absence of murine B and T cells in NSG mice, murine IgE was undetectable in serum. We monitored the possibility of murine mast cell activation by quantification of murine mast cell protease (MMCP)-1, which, similarly to tryptase in human anaphylaxis, is readily detected in serum following mast cell degranulation in murine food allergy and anaphylaxis models^{8, 13, 14}. MMCP-1 was undetectable in NSG mice, irrespective of engraftment or PN-induced anaphylaxis (**Fig E3**).

We further tested whether human IgE could elicit anaphylaxis or mast cell activation in mice by passive sensitization with human IgE and systemic challenge with antigen. For these experiments we used IgE-deficient (*Igh7*^{-/-}) mice, which are immunocompetent and have an intact mast cell compartment. Because of their lack of endogenously produced IgE, these mice are highly sensitive to passive systemic anaphylaxis mediated by injected IgE and antigen. While mice passively sensitized with murine IgE exhibited a robust loss of core body temperature and elevated MMCP-1 levels upon antigen challenge as expected, no response was observed in mice that received human IgE (**Fig E3**). In contrast, antigen challenge of a stem cell-engrafted huNSG mouse sensitized with human IgE did result in loss of core body

temperature (**Fig E3**). This confirms that human IgE cannot elicit responses by murine mast cells and, taken together, these findings strongly implicate engrafted human mast cells triggered by human IgE antibodies produced by engrafted B cells as the sole inducers of anaphylactic responses to PN exhibited by the humanized mice.

IgE neutralization blocks anaphylaxis in PN-fed huNSG mice

Leung and colleagues have previously reported that PN anaphylaxis can be blocked by treatment with anti-IgE³⁰ and we have found that murine PN anaphylaxis is fully dependent on the presence of IgE and on signaling via FcεRI^{6, 8, 11}. We directly assessed the requirement for human IgE in PN-induced anaphylaxis in huNSG mice by neutralization of IgE with omalizumab (αIgE). While omalizumab does not interact with receptor-bound IgE, it can reduce the amount of soluble IgE that is available to bind to cellular receptors^{31, 32}. Mice receiving standard PN sensitization developed specific IgE responses and exhibited anaphylaxis upon gavage challenge (**Fig 4A**). In contrast, animals receiving omalizumab 48 hours prior to each of the sensitizing PN were completely protected from the loss of core body temperature observed in response to PN challenge (**Fig 4A**). Omalizumab treatment resulted in a significant increase in serum levels of total IgE (**Fig E4A**), an effect that is commonly observed in human subjects treated with the antibody that is attributable to the persistence of stable circulating omalizumab:IgE complexes³³. However, we observed variable effects on the production of PN-specific IgE with omalizumab treatment. We suspect that reductions in detection might be related to interference with the anti-IgE capture antibody in our PN-specific IgE ELISA (**Fig 4B**). Although there was a trend towards lower tryptase levels in the omalizumab-treated animals, following PN challenge this did not achieve statistical significance. Overall the tryptase responses were low in this experiment, likely due to variations in efficiency of reconstitution.

Antigen-specific immune responses in huNSG mice are induced by the huMHC-restricted interaction of donor-derived human antigen presenting cells (APC) and T cells.

One issue requiring consideration with humanized mouse models is the possibility that developing human T cells might receive their training and selection upon murine major histocompatibility (MHC) molecules in the murine host's thymus in addition to—or instead of—on human MHC expressed on donor APC. In characterizing models such as the huNSG one under study here, it is important to understand whether T cells emerging following antigen exposure exhibit human MHC-restricted recognition, which is a requirement for effective T cell help in the cognate T:B cell interactions leading both to specific antibody production and to IgE isotype switching. We therefore investigated the presence of murine and human dendritic cells in huNSG mice and their contributions to specific immune responses.

The vast majority of murine leukocytes present in huNSG mice expressed the dendritic cell marker CD11c, and most also expressed the NOD-specific I-A^{g7} MHCII molecule (**Fig E5**). CD11c⁺ leukocytes in the human CD45⁺ compartment almost universally expressed the human MHCII molecule HLA-DR in both the spleen and small intestine. Modest upregulation of MHCII was observed in human dendritic cells from PN-sensitized mice in the spleen and small intestine, as well in murine intestinal dendritic cells, an effect consistent with the stimulatory effect of IgE and mast cell-derived cytokines on dendritic cell activation^{34, 35}.

In order to assess the competence of the murine and human dendritic cells coexisting in huNSG mice to present antigen to human T cells, we examined recall proliferative responses to PN and tetanus toxoid in CD4⁺ T cells. When cultured with isolated, antigen-pulsed human CD11c⁺ dendritic cells from huNSG mice, CellTrace Violet-labeled CD4⁺ T cells from immunized huNSG mice exhibited dye dilution (indicative of proliferation) (**Fig E6**). In contrast only minimal cell division was evident in the same CD4⁺ T cells cultured with murine CD11c⁺ cells, or when the T cells were isolated from naïve huNSG mice. No cross reactivity between the human and murine anti-CD11c antibodies used for isolation was observed. While not definitive, these results strongly suggest that in the long-term (6 month) engraftment used in these experiments, CD4⁺ T cells in huNSG mice respond to presentation of antigens by human MHCII. The

consistent presence of low levels of all human immunoglobulins except IgA, as well as the production of PN-specific IgG antibodies in PN-fed mice (**Fig E4**), is consistent with previous reports that antibody responses are enhanced by increased engraftment times^{36, 37}.

DISCUSSION

Humanized mouse models have proven useful in a number of settings including the study of human-specific pathogens such as HIV and research on mechanisms of cancer and autoimmunity^{19, 38}. They have provided invaluable tools both for the dissection of disease mechanisms and for preclinical assessments of novel therapies. In this report we describe a strategy for the investigation of PN allergy, using humanized NSG mice engrafted with human CD34⁺ stem cells. The mice exhibited robust reconstitution of the T and B cell compartments of the adaptive immune response and developed PN-specific hIgE responses and anaphylactic sensitivity to ingested PN following sensitization. Like humans undergoing anaphylaxis, their plasma tryptase levels were markedly elevated, consistent with a human mast cell-mediated reaction. Anti-IgE treatment with Omalizumab blunted their anaphylactic reactions as expected. In each of its features, this model nicely recapitulates the typical features of human food allergy.

The use of animals transgenic for the obligate mast cell growth factor, SCF, in this model ensured the population of their tissues by human mast cells. Un-reconstituted NSG mice had no intestinal tissue mast cells whereas engrafted animals had jejunal mast cell densities of about 1-2 times those reported in normal human biopsies. Murine mast cell protease was undetectable in huNSG mice undergoing PN-induced anaphylaxis, and administration of human IgE to mice without human immune systems failed to elicit any discernable reaction upon antigen challenge. Thus, our data are consistent with a human IgE-mediated anaphylactic reaction produced by human mast cells, without any involvement of murine effector cells.

In this study we found evidence that PN allergens (as well as tetanus toxoid) can be presented to human CD4⁺ T cells by engrafted human APCs in huNSG mice. In contrast,

resident host APCs, expressing murine MHC antigens were not efficient inducers of recall responses in T cells from the humanized mice, consistent with a dominant role for human MHC restriction. Current understanding concerning the education of developing T cells holds that selection occurs in the thymus on MHC molecules expressed by stromal cells of nonhematopoietic origin^{39, 40}. While human hematopoietic cells bearing human MHC molecules might enter the mouse thymus in our huNSG mice, some published studies have predicted that these cells would not efficiently select developing human T cells^{19, 39, 40}. Consistent with this dogma, initial reports describing the development of human leukocytes from hematopoietic stem cells in NSG mice concluded that the human T cells were restricted to murine MHC and dysfunctional, failing to respond to anti-CD3 stimulation⁴¹. These past conclusions contrast strongly with our findings that human MHC-restricted T cell development and HLA-restricted antigen presentation are functional in these mice. Consistent with our findings, several studies have demonstrated HLA-restriction of cytotoxicity of CD8 T cells⁴² and delayed type hypersensitivity reactions⁴³. Most tellingly, direct testing for human HLA restriction of human T cells in humanized mice by mixed leukocyte reaction demonstrated strong responses to allogeneic human DCs, but weak reactions to mismatched murine MHC⁴⁴, consistent with human MHC restriction.

The discordance in findings regarding human vs. murine MHC restriction in the immune responses of huNSG mice suggests the existence of as yet unresolved complexities. It also points to potential strategies for the further improvement of such humanized mouse models. The surgical implantation of human organoids (bone, liver and thymus) or transgenic expression of human HLA alleles has been shown to enhance human T cell development and function^{19, 38, 45, 46}, and it is likely that the PN allergy model described here could be improved by either of these approaches.

Normally, allergen ingestion by humans or mice results in tolerance. Thus the default tendency of PN-fed huNSG mice to exhibit allergy rather than tolerance came as a bit of a

surprise. We speculate that several factors might contribute towards the allergic predisposition of these animals. These include the mismatch between thymic stromal murine MHC and human APC MHC described above, as well as the relative lymphopenia of these animals; a condition associated with loss of tolerance perhaps either because of low Treg numbers or repertoire mismatch between Teff and Treg⁴⁷⁻⁴⁹. The generally naïve T and B cell repertoire related to short-term immune reconstitution could additionally be contributing to this dysregulation^{50, 51}. We speculate that altered colonization might also be affecting the immune response of these mice. The prolonged antibiotic treatment necessary to maintain immunodeficient NSG mice in our facility could skew their intestinal microbiota in a pro-allergic direction. We and others have observed that the food allergic phenotype of mice is linked to the complexity and community structures of their intestinal microbiota and it has been observed that germ-free animals have a tendency towards allergy⁵²⁻⁵⁵.

Finally we have considered the possibility that both the high density of tissue mast cells (higher than reported in normal human tissues) and a potentially activated phenotype related to the constant provision of SCF (both a driver of mast cell differentiation and a stimulus for activation) might together give rise to an expanded and very sensitive intestinal mast cell compartment. Such densely packed and sensitive mast cells might mediate hypersensitivity reactions even in the context of modest IgE levels or antigenic stimuli. In this way the mice might behave similarly to humans with mast cell activation disorders who can sometimes present with frequent and intense allergic reactions⁵⁶.

The abundant and relatively activated mast cells likely also create an environment conducive to allergic sensitization. Using a mouse model of PN allergy, we have shown that the presence of mast cells and their activation by antigen-specific IgE antibodies are both conducive to the induction of Th2 responses and suppress the emergence of Treg-mediated tolerance. Thus a combination of inherent immune dysregulation, altered microbiota and amplified mast cell responses might render the huNSG mice particularly allergy prone. The high frequencies of

skin-resident mast cells in these huNSG mice might also render them susceptible to allergic sensitization through cutaneous or epicutaneous exposure, routes that are effective in standard mouse models and have been considered to be physiologic routes of sensitization for human patients^{14, 20, 35, 57, 58}.

The efficient adaptive immune reconstitution of the huNSG mice described in this report along with their inherent predisposition to allergic sensitization and mast cell-mediated anaphylactic responses render them ideally suited for the study of cellular and molecular interactions in the human food allergy response. The experiments presented here establish that both the processes of sensitization (antigen presentation and T cell activation) and of effector responses (mast cell activation) are mediated exclusively by cells of human origin and without significant contributions by murine APCs or mast cells. The results of blocking studies with omalizumab clearly demonstrate that human IgE mediates the observed responses. We anticipate that this novel system will provide a powerful platform for future efforts to identify key checkpoints in allergic sensitization and tolerance to food allergens as well for testing innovative strategies for manipulating these responses.

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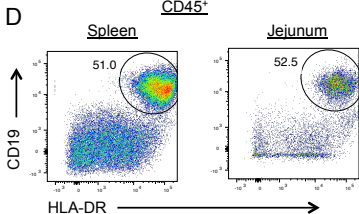
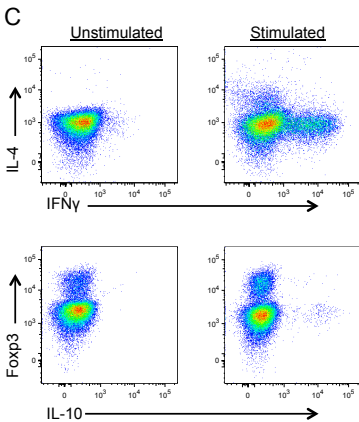
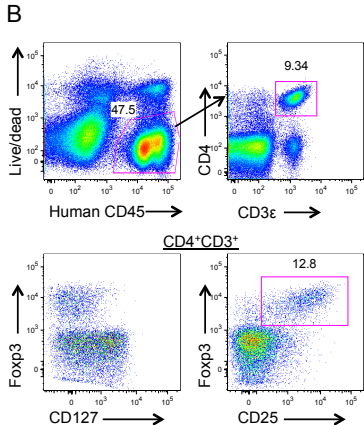
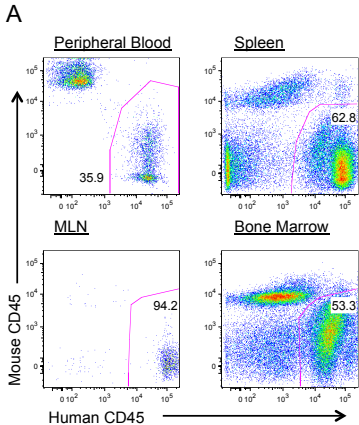
FIGURES AND FIGURE LEGENDS

FIG 1. A. Flow cytometry identifying human leukocytes in peripheral blood, spleen, mesenteric lymph nodes (MLN) and bone marrow. **B.** Intracellular staining of splenic CD4⁺CD3⁺ T cells identifying regulatory T cells (Foxp3⁺CD127^{low}CD25⁺) and **C.** T helper subsets (IFN γ ⁺ or IL-4⁺). **D.** Identification of CD19⁺HLA-DR⁺ B lymphocytes in the spleen and small intestinal lamina propria. Data are representative of three independent experiments.

FIG 2. Peanut (PN) allergy in humanized mice. **A.** Experimental design. SCF-transgenic NSG mice are engrafted with human CD34⁺ hematopoietic stem cells (5×10^4) by i.v. injection. After 4 months, mice are sensitized by weekly gavage feedings with PN (22.5mg) for 2 months. **B.** Core body temperature after enteral PN challenge in mice sensitized as in A and then challenged with 350mg PN (n=6-10), $P=0.0105$ by repeated measures 2-way ANOVA. **C.** Serum levels of human tryptase. $*P<0.05$, $***P<0.001$ by Bonferroni post-test on ANOVA of log-transformed values. **D.** Serum PN-specific IgE levels.

FIG 3. A. Flow cytometry for total human IgE on Fc ϵ RI⁺c-Kit⁺ splenic mast cells. **B.** Human Fc ϵ RI⁺c-Kit⁺ mast cell numbers in the spleen and jejunal lamina propria as assessed by flow cytometry. Data are representative of three independent experiments.

FIG 4. Impact of omalizumab on PN-induced anaphylaxis. Mice were sensitized as in Fig 2. Omalizumab (α IgE) was injected i.p. weekly 48h prior to sensitization dosing at 120mg/kg. **A.** Core body temperature loss after enteral PN challenge in sensitized animals (n=3-5), $P=0.0004$ by repeated measures 2-way ANOVA for an effect of α IgE. **B.** Serum PN-specific IgE levels. **C.** Serum levels of human tryptase. $*P<0.05$, $**P<0.01$, $***P<0.0001$ by Bonferroni post-test on ANOVA of log-transformed values. Data are representative of two independent experiments.



A

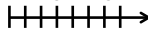
5×10^4 CD34⁺ stem cells i.v.



16 weeks

Check
engraftment

Peanut butter
gavage



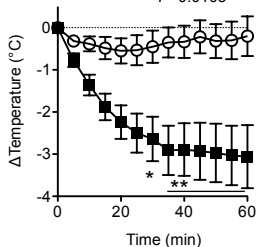
8 weeks

- Challenge
- Assess immune responses

B

Temperature

$P=0.0105$

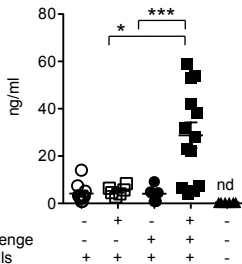


huNSG PN

huNSG saline

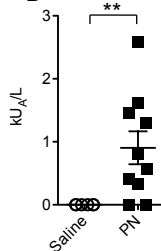
C

Tryptase



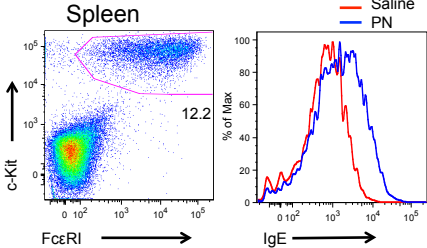
D

IgE

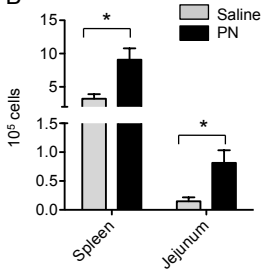


A

Mast cells

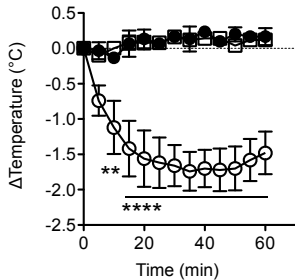
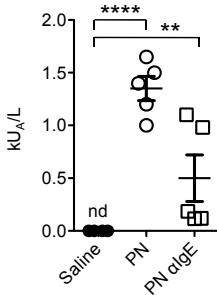
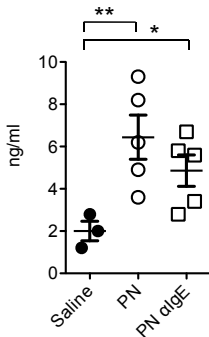


B



A**Temperature**

● huNSG □ huNSG PN α IgE
○ huNSG PN

**B****IgE****C****Tryptase**

Online Repository Material for: *A humanized mouse model of anaphylactic peanut allergy*

METHODS

Mice

WT BALB/c and IgE^{-/-} (*Igh*-7^{-/-}) mice were bred under specific pathogen-free conditions in a biosafety level 1 facility. Age-matched controls were used for experiments at 8-12 weeks of age.

Passive anaphylaxis

IgE-mediated anaphylaxis was assessed following passive sensitization of IgE^{-/-} mice with mouse or human IgE. Mice were injected intravenously via the retro-orbital sinus with 10µg IgE from mouse (IgE anti-DNP clone SPE-7, Sigma-Aldrich) or human (chimeric IgE anti-NP clone JW8/1, AbD Serotec), and challenged 72hrs later by intraperitoneal injection with 500µg of the respective antigen (TNP-albumin from Sigma-Aldrich or NP-OVA from Biosearch Technologies).

Mast Cell Culture and Degranulation.

Mast cells were cultured from humanized mouse splenocytes or bone marrow under stimulation with recombinant human IL-3 and SCF (100ng/ml each, Shenandoah Biotechnology) with weekly passage. Purity was assessed by flow cytometry, staining against human FcεRI and c-Kit. Sera from peanut-allergic donors were collected under IRB protocols approved by Boston Children's Hospital, with written informed consent from all donors¹. Mast cell degranulation assays using LAMP-1 as a read-out were performed according to procedures we have established for mouse and human basophil activation¹. Mast cells were sensitized overnight with peanut-allergic serum containing 12 kU/L anti-PN IgE in 100µl final volume. Cells were stimulated for 10min at 37°C with 4 µg/ml aqueous peanut extract in the presence of anti-LAMP-1 antibody (0.5µg/ml). Cells were promptly washed with cold buffer containing azide and analyzed by flow cytometry, gating on c-Kit⁺ viable cells.

Immunization and recall

huNSG mice were immunized to tetanus by i.p. injection with 1µg tetanus toxoid (Calbiochem) adsorbed to 1mg alum (aluminum hydroxide gel, Sigma-Aldrich) in saline on days 0 and 14, followed by weekly i.p. injections with 1µg tetanus toxoid. After 8 weeks, mice were sacrificed and splenocytes were fractionated using magnetic selection into mouse CD11c⁺, human CD11c⁺ and human CD4⁺ cell types. For this purpose, mouse and human Fc gamma receptors were blocked using anti-mouse CD16/32 (clone 93) and human TruStain FcX, followed by staining with PE-conjugated antibodies against mouse CD11c (N418), human CD11c (Bu15) or human CD4 (OKT4) (all Biolegend). Anti-PE microbeads were then used prior to isolation over LS columns (Miltenyi Biotec). Dendritic cells (10⁴) were pulsed overnight with 1µg/ml tetanus toxoid or 100µg/ml aqueous peanut extract (made as previously described²). Isolated T cells were labeled with CellTrace Violet (Invitrogen/ThermoFisher) according to the manufacturer's instructions, and 2x10⁵ cells from immune or non-immune mice were plated with pulsed dendritic cells. Soluble anti-CD3 (2µg/ml, OKT3, Biolegend) was added as a positive control. After 7 days, cells were stained for CD3, CD4 and viability, and proliferation was assessed in the CD4⁺CD3⁺ T cells.

Flow Cytometry

The following additional antibodies were used in flow cytometry experiments for the data in the online repository: APC anti-CD19 (HIB19, Biolegend), PE anti-IL-10 (JES3-19F1, Biolegend), Alexa Fluor 647 anti-LAMP-1 (H4A3, Biolegend), FITC anti-mouse I-A^k/I-A^{g7} (10-3.6, Biolegend), PE-Cy7 anti-HLA-DR (L243, Biolegend), PE anti-human CD11c (Bu15, Biolegend), PE-Dazzle594 anti-mouse CD11c (N418, Biolegend), FITC anti-mouse c-Kit (2B8, BD Pharmingen), PerCP-Cy5.5 anti-mouse CD49b (DX5, Biolegend), PE-Cy7 anti-mouse FcεRI (MAR-1, Biolegend), and PE-Cy7 anti-human CD4 (OKT4, Biolegend).

ELISAs

Total serum immunoglobulin levels were assessed by direct ELISA. Immunosorp ELISA plates (Nunc) were coated overnight with sera diluted in PBS, washed, blocked with 2% BSA and specific isotypes were detected with the following biotinylated isotype-specific antibodies: IgG1 (G17-1, BD Pharmingen), IgG2 (G18-21, BD Pharmingen), IgG3 (HP6047, Invitrogen/Thermo Fisher), IgG4 (G17-4, BD Pharmingen), IgA1/2 (G20-359, BD Pharmingen), IgD (goat polyclonal, Southern Biotechnology), IgM (goat polyclonal, Southern Biotechnology), and IgE (G7-26, BD Pharmingen). Purified IgM (Sigma-Aldrich), IgD (Athens Research), IgG1 (Sigma-Aldrich), IgG3 (Sigma-Aldrich), IgE (Abcam) and IgG4 (Affymetrix eBioscience) were purchased for use as standards. Chimeric IgG2 (clone JW183) and IgA (JW393A) for use as standards were obtained from AbD Serotec. PN-specific IgG isotypes were assessed by capture onto plates coated with peanut extract and detected using the isotype-specific antibodies described above. PN-specific IgG levels were quantified relative to a standard curve developed from peanut-allergic patient serum for which the isotype-specific levels of anti-peanut immunoglobulins had previously been roughly determined¹. Total murine IgE was measured using goat polyclonal anti-mouse IgE (Southern Biotechnology) to capture and biotinylated anti-mouse IgE (R35-92, BD Pharmingen) to detect with IgE anti-TNP as a standard (clone C38-2, BD Pharmingen). Mouse mast cell protease-1 (MMCP-1) was quantified by ELISA according to the manufacturer's instructions (Affymetrix eBioscience).

Histology.

Tissue samples were fixed overnight in 10% formalin (Sigma-Aldrich) prior to processing and paraffin embedding at the Beth Israel Deaconess Medical Center Histology Core. Chloroacetate esterase staining with hematoxylin counter stain was performed as previously described³. Photomicrographs were taken through a Nikon E800 Eclipse microscope. Mast cells were

counted by an observer unaware of the samples' identities, and normalized to tissue area as measured using Image J software.

FIGURE LEGENDS

Figure E1. Analysis of mast cells in NSG SCF mice. CAE-stained sections of paraffin-embedded tissue are shown with insets highlighting example mast cells. Jejunal micrographs were taken at 20x magnification (scale bar indicates 100µm).

Table E2. Comparison of mast cell burden in huNSG mice to literature-reported values on adult humans.

	<u>Human literature values</u>		<u>Experimental mouse values</u>	
	Healthy	Atopic/Disease	NSG SCF	huNSG
Lung	44±8 ⁴ 44.7 ⁵ 155±21 ⁶	84±7 ⁴ 91-102 ⁵ 270±51 ⁶ 250±60 ⁷	4±1	67±55
Intestine	187±23 ⁸ 146±29 ⁹ 38±13 ¹⁰ 268 ^{#11} 47 ^{§11}	413±139 ⁸ 243±41 ⁹	0 (n.d.)	256±140*
Stomach	60±25 ¹²	135±103 ¹²	13±8	111±24*
Skin	38.4±4 ¹³ 48±3 ¹⁴ 50±2 ¹⁴	71.8±13 ^{13A} 596.5±278 ^{13B} 720.6±176 ^{13C} 142±69 ¹⁵	57±10	98±6*

Values indicate mean±SEM counts of mast cells per mm² by histological analysis. n.d., not detected.

*Statistically significant increase compared to unengrafted NSG SCF mouse.

⁴Farmer's lung.

⁵Extrinsic allergic alveolitis (hypersensitivity pneumonitis), 2 patients only.

⁶Nonfatal asthma.

⁷Severe asthma.

⁸Gastritis.

^{11#}Carnoy's fixative. [§]Formalin fixation.

¹²Atopic/oral allergy syndrome.

¹³A) Unexplained anaphylaxis, B) urticaria pigmentosa, C) systemic mastocytosis.

¹⁵Atopic dermatitis.

Figure E3. Analysis of possible contributions of murine mast cells and IgE to anaphylaxis in NSG SCF mice. **A.** Representative panels showing flow cytometric identification of residual murine mast cells in humanized NSG SCF mice. **B.** Quantification of human and murine mast cells by flow cytometry. **C.** Serum levels of murine IgE and MMCP-1 in NSG SCF and immunocompetent mice. Values from huNSG mice are from sera post-challenge with PN. **D.** Assessment of the anaphylactogenic capacity of human and mouse IgE in the murine system. IgE-deficient *Igh7^{-/-}* BALB/c mice ($n=5$) were passively sensitized with human α NP IgE or mouse IgE α NP IgE and challenged. Anaphylaxis was assessed by measurement of core body temperature and mast cell degranulation followed by determining serum MMCP-1 levels. $P<0.0001$ by 2-way ANOVA. **E.** Anaphylactogenic capacity of human and murine IgE in NSG SCF mice ($n=4$) without human immune engraftment. **F.** Anaphylaxis elicited by human α NP IgE and antigen challenge in a CD34⁺ stem cell (HSC)-engrafted huNSG mouse. **G.** Serum MMCP-1 levels elicited by antigen challenge of human or murine IgE-sensitized mice. Data are representative of two independent experiments. *** $P<0.001$ by unpaired t test (B) or Bonferroni post-test on ANOVA (E). n.d., not detected.

Figure E4. Serum antibody levels in humanized mice. **A.** ELISA determination of total serum levels of human IgE, IgA, IgD and IgM. **B.** Serum levels of IgG isotypes. Note that omalizumab (α IgE) is an IgG1 monoclonal. **C.** Serum levels of PN-specific IgG isotypes. The data shown summarize three independent experiments.

Figure E5. Analysis of murine and human dendritic cells in humanized NSG SCF mice. Flow cytometric identification of murine CD11c⁺MHCII⁺ dendritic cells, and quantification of MHCII expression in **A.** the spleen and **B.** the jejunum. Representative flow cytometry plots showing human CD11c⁺HLA-DR⁺ dendritic cells in the spleen and quantification of HLA-DR expression in **C.** the spleen and **D.** the jejunum. Data are representative of two experiments.

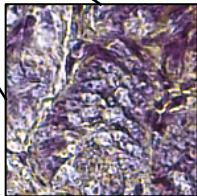
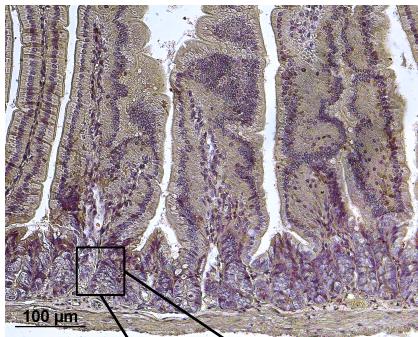
Figure E6. Presentation of antigens to human CD4⁺ T cells in humanized NSG SCF mice. Human CD4⁺ T cells from immunized or naïve humanized mice were assessed for recall proliferative responses following presentation of antigen by isolated CD11c⁺ dendritic cells of murine or human hematopoietic origin. Representative flow plots show the frequency of dividing (Violet CellTrace^{low}) cells among CD4⁺CD3⁺ T cells after 7 days of culture. Similar data were obtained in a second experiment.

Figure E7. Culture techniques for *in vitro* experiments with human mast cell. **A.** Flow cytometry showing c-Kit and Fc ϵ RI expression in cultured human splenic mast cells. **B.** CAE-stained cultured human splenic mast cells **C.** *In vitro* degranulation (LAMP-1) following sensitization with peanut-allergic patient serum and peanut stimulation using culture splenic and bone marrow-derived mast cells. Data are representative of three independent experiments.

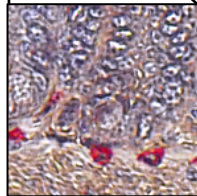
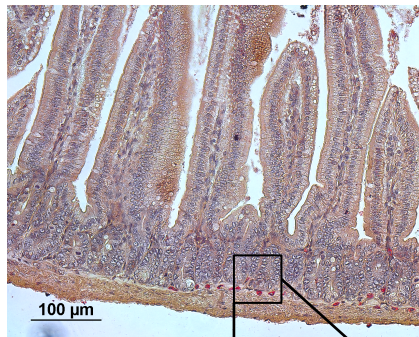
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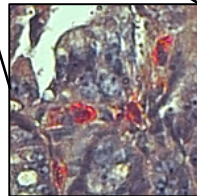
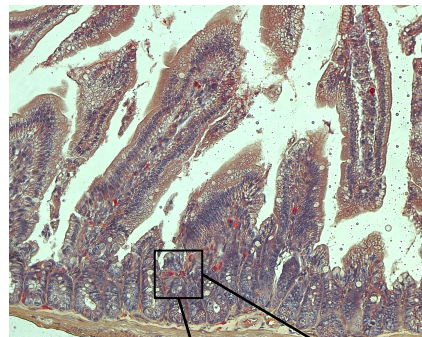
No HSC



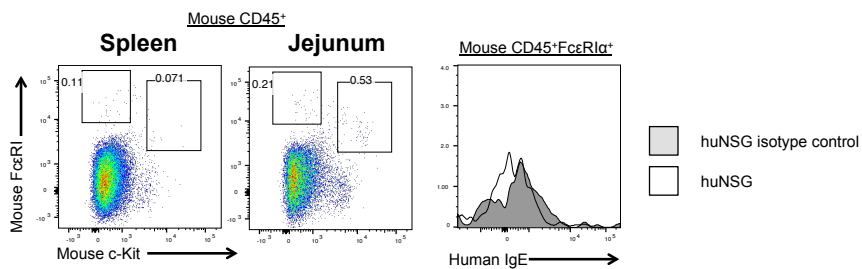
HSC, saline



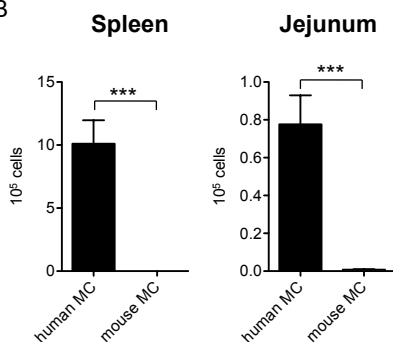
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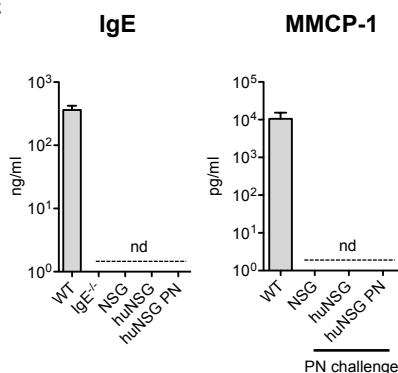
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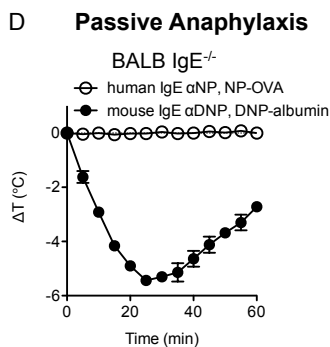
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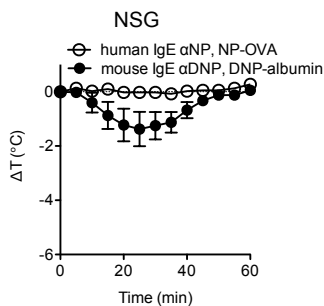
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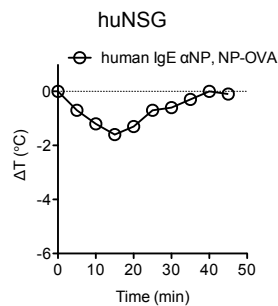
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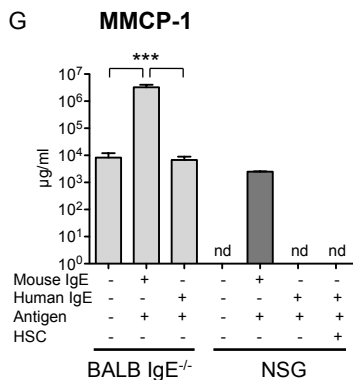
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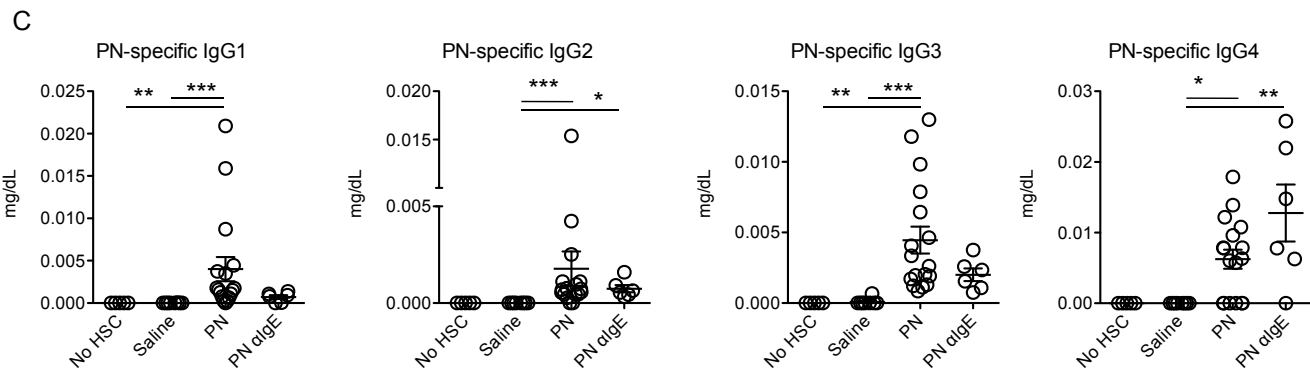
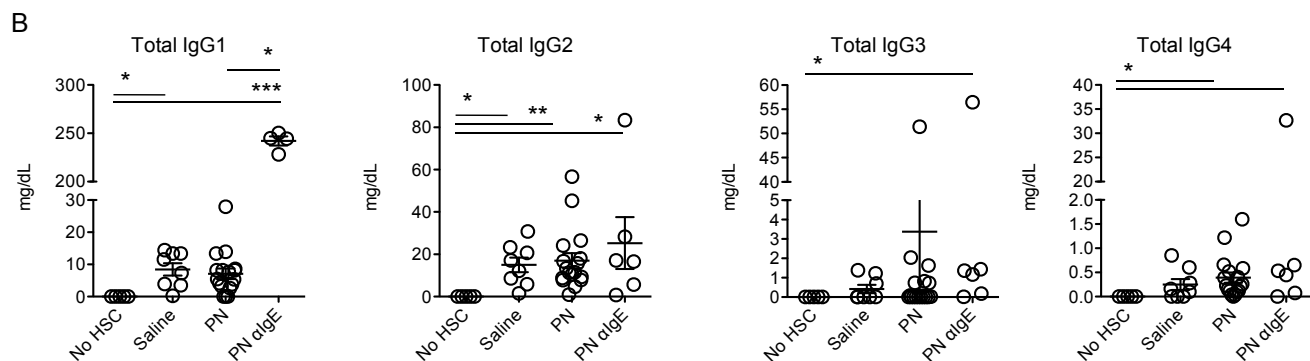
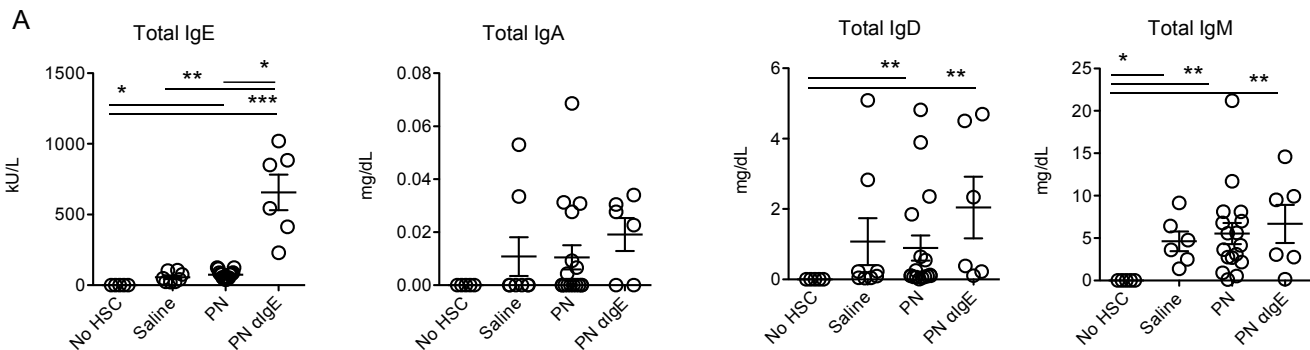


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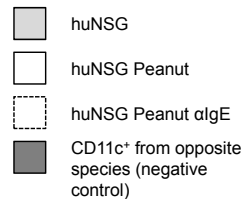
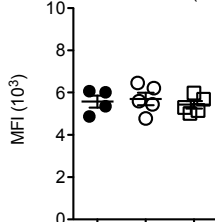
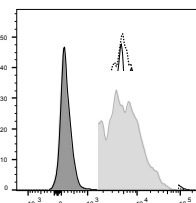
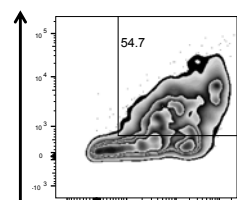
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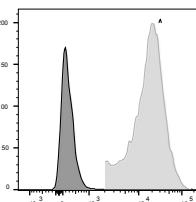
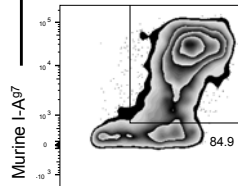
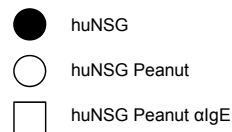
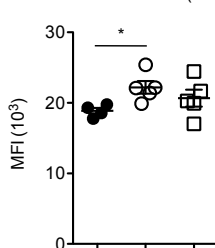


A

Murine

SpleenMurine CD45⁺Murine CD45⁺CD11c⁺MHCIIMurine MHCII (I-A^{g7})

B

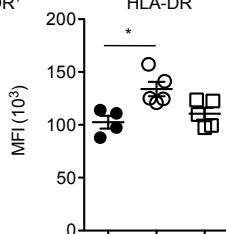
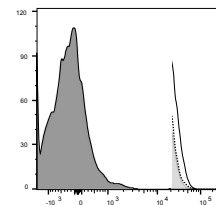
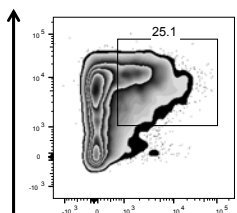
JejunumMurine MHCII (I-A^{g7})

C

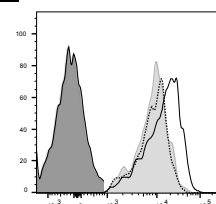
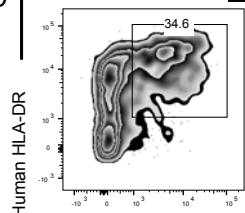
Human

SpleenHuman CD45⁺Human CD45⁺CD11c⁺HLA-DR⁺

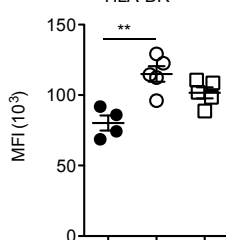
HLA-DR



D

Jejunum

HLA-DR



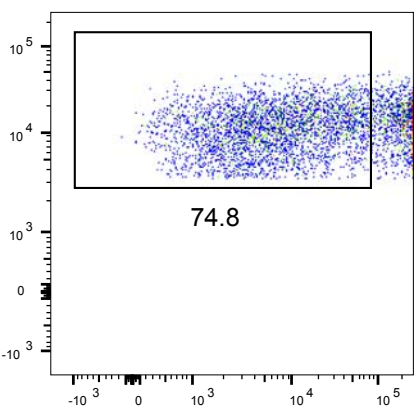
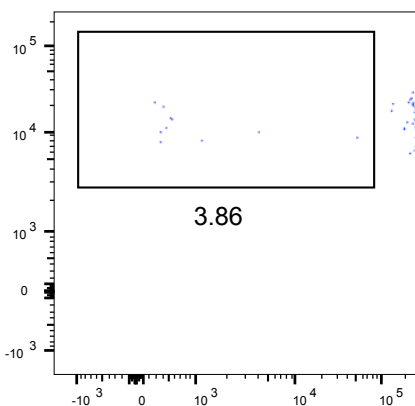
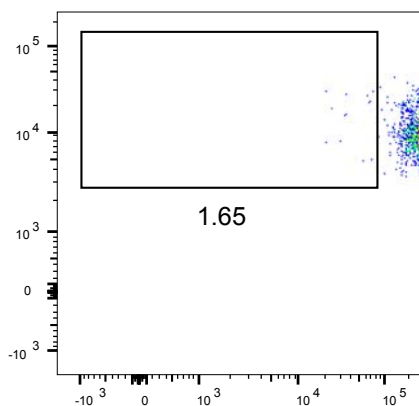
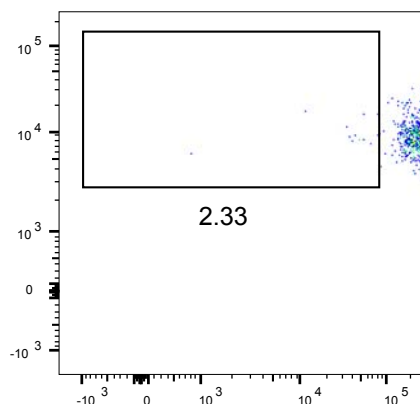
Human HLA-DR

Human CD11c →

Human HLA-DR →

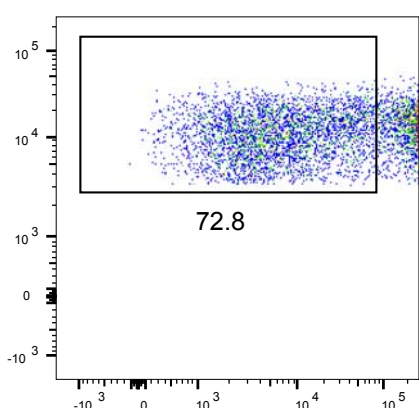
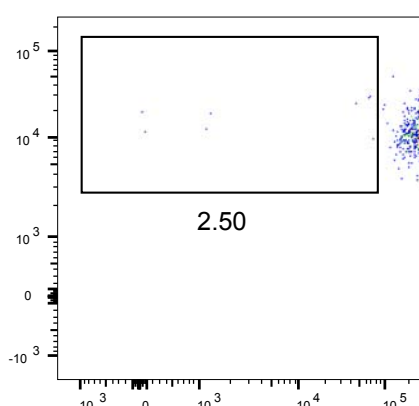
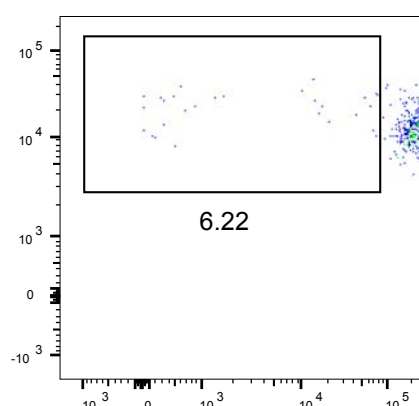
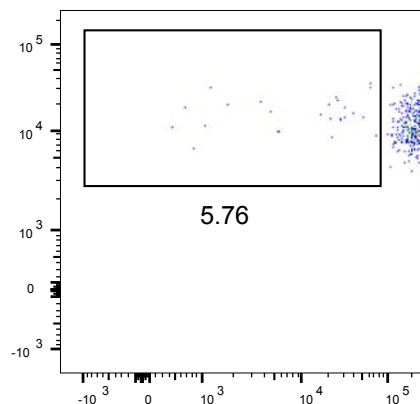
Unimmunized

Human CD11c⁺

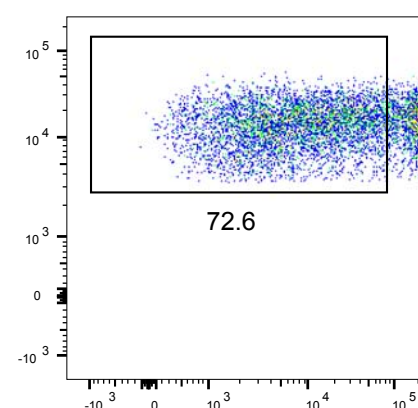
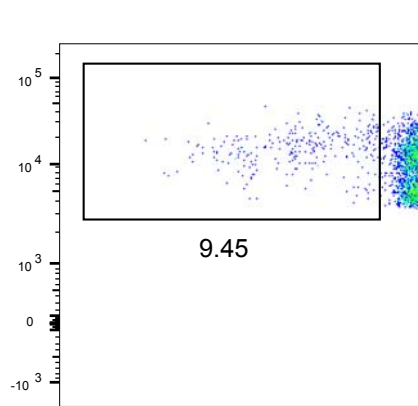
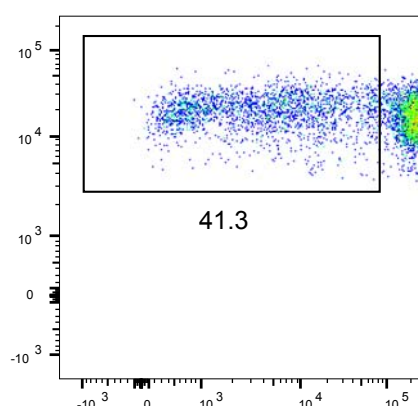
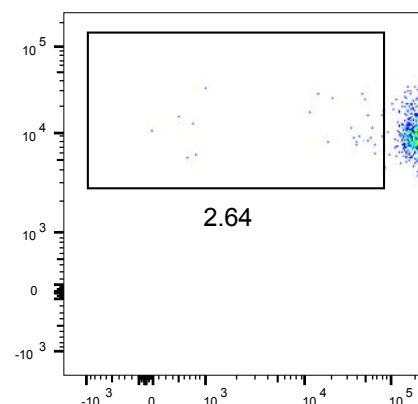


PN- & Tetanus-immunized

Murine CD11c⁺



Human CD11c⁺



unstimulated

Tetanus

PN

α CD3

CellTrace Violet (Proliferation)

