MSc Thesis

Bovine Milk IgG–House Dust Mite Immune Complexes Engage Human Monocyte FcγRIIa: Toward Understanding Trained Immunity Mechanisms

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Abstract

House dust mite (HDM) allergen sensitization is a major risk factor for allergic asthma, yet epidemiological studies consistently associate the early-life consumption of unpasteurized cow milk with a protective effect against the development of allergic asthma. Bovine milk immunoglobulins G (bIgG), which bind HDM allergens, are hypothesized to form immune complexes (ICs) that engage human monocyte Fcγ receptors, potentially reprogramming them toward trained immunity or tolerance. This study investigated the formation of bIgG–HDM ICs, their binding mechanisms, and their capacity to reprogram human monocytes. Using flow cytometry, it was demonstrated that bIgG forms large ICs with HDM allergens that bind to $CD14^+$ monocytes. However, IC formation was restricted with single allergens (Der p1 or p2), likely due to the polyclonal nature of the bIgG stock, which may have contained a limited fraction of single-allergen-specific bIgG. In contrast, the multi-allergen HDM extract enabled robust ICs binding, leading to a >20-fold increase in FcγRIIa engagement on $CD14^+$ monocytes. The blocking of FcγRIIa abolished IC binding. To assess whether bIgG–HDM ICs reprogram monocytes, an established *in vitro* trained immunity model was employed. The initial exploratory analysis found that, while restimulation elicited sporadic donor-specific increases in TNF-α/IL-6 production, along with decreases in IL-10 levels, definitive trends were limited by a high donor variability, small sample sizes, elevated baseline cytokine levels, and inconsistent responses to the training controls. Further optimization of the training model will address these limitations and potentially strengthen the mechanistic link between raw milk consumption and asthma protection. This work confirms the formation of bIgG–HDM ICs and suggests the monocyte FcγRIIa to be a central mediator of IC binding.

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1. Introduction

Asthma is a chronic inflammatory disease of the lungs, characterized by airway hyperresponsiveness to a variety of physical and environmental stimuli such as stress, exercise, weather, allergens, irritants, and viral infections (Au-Doung et al., 2024; Scadding et al., 2024). The response to these stimuli is marked by bronchial narrowing and swelling (remodeling), along with edema and production of mucus, leading to episodic airflow obstruction (Au-Doung et al., 2024; Doeing & Solway, 2013). Symptoms include recurrent episodes of wheezing, coughing, shortness of breath, chest tightness and reduced expiratory outflow (Au-Doung et al., 2024). The global prevalence of asthma is estimated to be 7–20% among both adults and children, and it is the most common chronic disease in childhood (Au-Doung et al., 2024; Licari et al., 2023; Pleșca et al., 2024).

1.1 Development of Asthma – Genetics & Environment

Asthma is increasingly recognized as a multifaceted syndrome, comprising an array of distinct diseases (endotypes) with varying phenotypes (Kuruvilla et al., 2019). The most common form of asthma is associated with allergic sensitization via T helper 2 (Th2) cells, referred to as allergic or type 2 (T2) asthma (Gonzalez-Uribe et al., 2023). Allergic asthma is caused by both genetic and environmental factors (Mukherjee & Zhang, 2011). Twins studies suggest that genetic factors account for 60–80% of the variation in asthma susceptibility, however this is reduced to 25% in relation to the overall severity of symptoms (McGeachie et al., 2013; Thomsen, 2014, 2015; Thomsen et al., 2010). Notably, genetic factors accounted for only 6% of the variation in house dust mite (HDM) sensitivity (Thomsen, 2014). The role of environmental factors in asthma susceptibility is further emphasized by the hygiene hypothesis, which postulates that the depletion of microorganisms (e.g., gut microbiota, viruses, helminths; 'old friends') from the urbanized environment interferes with the normal education of the immune system, which relies on early-life microbial exposure (Murdaca et al., 2021). This disruption may lead to dysfunctional immune responses to harmful or harmless stimuli and the dramatic increase observed in the incidence of autoimmune and allergic diseases (Bloomfield et al., 2016). Akdis (2021) expanded on this by introducing the epithelial barrier hypothesis, proposing that the rising prevalence of autoimmune and allergic diseases is, in part, explained by a disrupted epithelial barrier function due to the increased presence of epithelial barrier-damaging agents (e.g., 'urban allergens' such as mold spores, pet dander, and house dust mite, as well as surfactants, enzymes and emulsifiers in processed food, cigarette smoke, particulate matter, diesel exhaust, ozone, nanoparticles, and microplastics). For instance, HDM allergen Der p1 exhibits proteolytic activity and is known to disrupt tight junctions, impacting epithelial barrier integrity (Hellings & Steelant, 2020). Bronchial epithelial barrier dysfunction may enable HDM allergens to interact more readily with components of the immune system (Gandhi et al., 2013). Indeed, HDM allergen sensitization is most commonly linked to allergic asthma (Gandhi et al., 2013; Illi et al., 2006; Lodge et al., 2011; Ouyang et al., 2024; Platts-Mills et al., 2009; Posa et al., 2017; Vrtala, 2022).

1.2 House Dust Mite Allergens

HDM major allergens Der p1, Der p2, and Der p23—along with moderately relevant allergens Der p5, Der p7, and Der p21—derived from *Dermatophagoides pteronyssinus*, are among the most clinically significant triggers of allergic asthma and rhinitis (Gregory & Lloyd, 2011; Vrtala, 2022). This pathogenic effect is partially attributable to the small size of mite fecal pellets, which fragment into inhalable particulates capable of depositing in the bronchial mucosa, rather than being confined to the nasopharyngeal mucosa (Tovey et al., 1981; Weghofer et al., 2013). This deep penetration heightens localized exposure to allergenic components (e.g., Der p1) and promotes proximal interaction with immune cells (Gregory & Lloyd, 2011). Der p1, a cysteine protease, compromises epithelial barrier integrity by cleaving tight junction proteins (e.g., occludin; Wan et al., 1999), facilitating allergen penetration and promoting Th2 immune responses through protease-activated receptors (PARs) on immune cells (Ouyang et al., 2024). Der p2 mimics the MD-2 co-receptor in toll-like receptor 4 (TLR4) signaling, amplifying TLR4-mediated proinflammatory responses (Trompette et al., 2009). Der p23 is a peritrophin-like protein found in the peritrophic membrane of mite fecal pellets and exhibits high sensitization frequences, similar to Der p1 and Der p2 (Celi et al., 2019; Muddaluru et al., 2021; Weghofer et al., 2013). Next to these single allergens, HDM whole body extract (WBE) is a complex mixture of HDM allergens, including Der p1 and other proteases/chitinases, and is often used in experimental models to mimic natural HDM exposure (Buday & Plevkova, 2014).

1.3 Pathophysiology of Allergic Asthma

Allergic asthma induction requires an initial exposure to an inhaled allergen, known as the sensitization phase, where mast cells and basophils are loaded with allergen-specific immunoglobin E (IgE). Upon re-exposure to the allergen, termed the effector/challenge phase, these innate immune cells will evoke an inflammatory response, giving rise to typical asthma symptoms (León & Ballesteros-Tato, 2021). In detail, upon aeroallergen exposure, lung epithelial cells release alarmin cytokines interleukin (IL)-25, IL-33, and thymic stromal lymphopoietin (TSLP; Figure 1). These alarmins stimulate type-2 innate lymphoid cells (ILC2s) to produce type-2 cytokines IL-5, and IL-13 (Chen et al., 2020), however alarmins may also directly promote the polarization of the Th2 type immune response (Van der Ploeg et al., 2019; Yang et al., 2017). Simultaneously, the alarmins stimulate dendritic cells (DCs) in the epithelia to capture the allergen and transport it to the lymph node for presentation to naïve allergen-specific $CD4^+$ T cells. Atopic individuals are predisposed to prime a type 2 immune response after allergen exposure via T helper 2 (Th2) cells (León, 2023). This DC–$CD4^+$ T cell interaction will initiate differentiation of $CD4^+$ T cell into Th2 cells through MHC-II mediated allergen-derived antigen presentation (signal 1), co-stimulation provided by DC CD80/CD86 activating T cell CD28 (signal 2), in combination with Th2-polarizing type 2 cytokines produced by ILC2s along with IL-4 produced by follicular T helper (T_{FH}) cells in the lymph node (signal 3; León, 2023). Principally, IL-4 activates the Signal Transducer and Activator of Transcription 6 (STAT6), which in turn induces upregulation of transcription factor GATA Binding Protein 3 (GATA3), the key driver of Th2 cell differentiation (Ho et al., 2009; Zhu et al., 2004).

The Th2 cells will migrate to the lungs while producing signature type-2 cytokines IL-4, IL-5, IL-9, and IL-13 (Lambrecht et al., 2019). Next to Th2 cells and ILC2s, $CD8^+$ T cells, as well as mast cells,

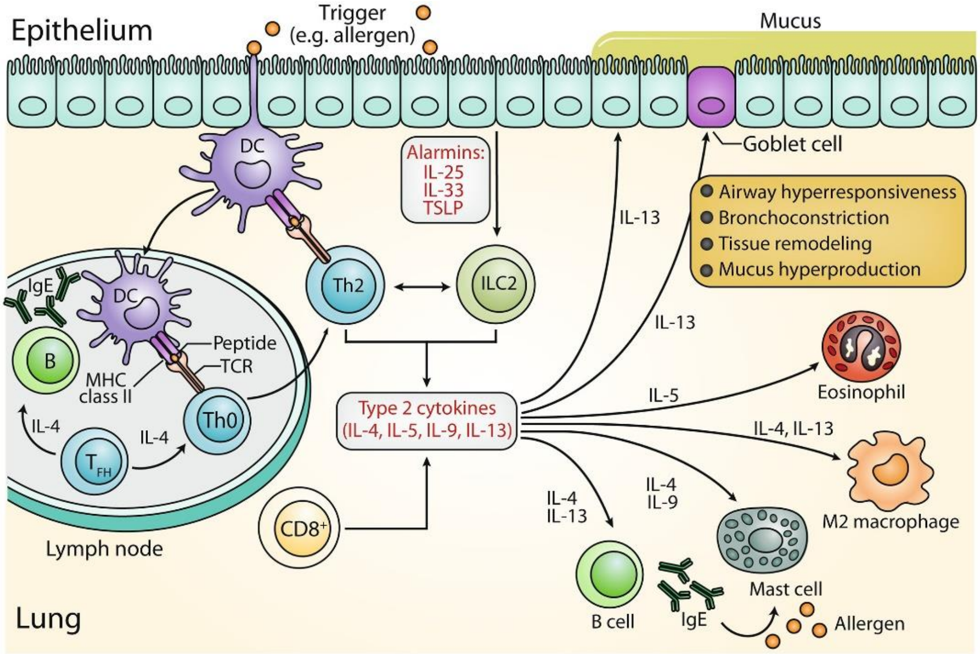

Figure 1. **Allergic sensitization and pathophysiology of allergic asthma**. In atopic individuals, exposure to aeroallergens can trigger the release of alarmins TSLP, IL-33, and IL-25 by epithelial cells which in turn leads to the secretion of type-2 cytokines by type-2 innate lymphoid cells (ILC2s). Simultaneously, nearby dendritic cells (DCs) are activated and in turn capture the allergen and present it to naïve allergen-specific CD4$^+$ T cells in the lymph node. Allergen-derived antigen presentation, in combination with type 2 cytokines by ILC2s and follicular T helper (T_{FH}) cells, will induce differentiation of CD4$^+$ T cells into T helper 2 (Th2) cells. The Th2 cells will migrate to the lungs while producing signature type 2 cytokines, which stimulate effector functions of B cells, basophils, mast cells, M2 macrophages, eosinophils, and epithelial cells. Notably, IL-4 induces B cell heavy chain isotype switching to initiate production of allergen-specific IgE. These allergen-specific IgE sensitize mainly mast cells and basophils, which will activate and degranulate upon re-exposure to the allergen, giving rise to acute inflammation and the vascular smooth muscle response. Illustration by Stikker et al. (2023).

basophils and eosinophils, produce type-2 cytokines (Stikker et al., 2023). IL-5 promotes eosinophil development and their migration to the lungs (Cusack et al., 2021). IL-9 supports mast cell survival and proliferation, induces epithelial cell differentiation into mucin-producing goblet cells (metaplasia), and contributes to airway remodeling (Koch et al., 2017). IL-13 compromises epithelial barrier function by impairing tight junctions, in addition to inducing airway hyperresponsiveness, mucus hyperproduction, and airway remodeling (Schmidt et al., 2019). Crucially, IL-4 is a key driver of naïve T cell differentiation into Th2 cells, composing a strong positive feedback loop for Th2 cell differentiation. Furthermore, IL-4 induces B cell heavy chain isotype switching to initiate the production of allergen-specific IgE antibodies in B cells found in the lymph node as well as in inducible bronchus-associated lymphoid tissue(Chvatchko et al., 1996). These allergen-specific IgE antibodies will enter circulation and bind to high affinity Fc epsilon receptors (FcεRIs) expressed on mast cells and basophils, but also to low affinity Fc receptors (FcεRIIs) found on eosinophils, monocytes, and platelets (Méndez-Enríquez & Hallgren, 2019). The pulmonary innate immune cells bound to IgE are now sensitized to the allergen and will readily activate and degranulate upon allergen re-exposure, specifically via cross-linking of the IgE–FcεRI complex induced by allergen binding (Nagata & Suzuki, 2022). The activation and degranulation of mast cells and basophils result in the release of mediators of inflammation, referring to cytokines, chemokines and growth factors, reviewed by Méndez-Enríquez and Hallgren (2019) and Mukai et al. (2018). These mediators include histamine, serotonin, bradykinin, and lipid mediators like leukotrienes and prostaglandins, all of which are responsible for the immediate inflammatory/vascular smooth muscle response underlying typical asthma symptoms. The concomitant production of cytokines such as tumor necrosis factor alpha (TNF-α) is responsible for late-phase inflammation and is thought to further drive the development of allergic airway inflammation (Nakae et al., 2007; Reuter et al., 2008).

1.4 Allergic Sensitization & Early Life Exposures

Primary sensitization to aeroallergens mainly occurs in toddlers (1–3 years of age), however allergen exposure can begin as early as the prenatal period (Järvinen et al., 2023; Wärnberg Gerdin et al., 2022). Allergic sensitization is associated with the development of allergic diseases and asthma in later life, though often emerging before the age of five (Illi et al., 2006; Lodge et al., 2011). Children are at a higher risk of developing allergic asthma as they are predisposed to prime a Th2-skewed cell immune response, conducive to allergic sensitization, as opposed to a Th1-like immune response more commonly observed in adulthood (Debock & Flamand, 2014; Zaghouani et al., 2009). While the underlying mechanisms and the multitude of factors influencing allergic asthma remain poorly understood (Bisoffi et al., 2024), epidemiological studies have consistently associated rural areas and farms with a substantially lower prevalence of asthma compared to urban areas (Pechlivanis & von Mutius, 2020). The traditional farming environment provides natural exposure to livestock, microbes, manure, straw, lipopolysaccharide (LPS)-containing aeroallergens in farm dust, and bacterial CpG DNA (Gerretsen & Schuijs, 2022; Pechlivanis & von Mutius, 2020). Indeed, the supposed protective effect of such early-life exposures against asthma aligns with the hygiene hypothesis. Furthermore, consuming unpasteurized cow milk has also been associated with a reduced risk of developing asthma (Lluis et al., 2014; Pfefferle et al., 2010; Riedler et al., 2001; Song et al., 2023; Waser et al., 2007). Notably, microbial components in bovine milk have not been found to play a significant immunological role in asthma prevention, and milk total fat content has also not been associated with asthma development (Gehring et al., 2008; Loss et al., 2011; Pfefferle et al., 2010).

1.5 The Protective Effect of Bovine Milk on Asthma Susceptibility

Meanwhile, bovine milk contains a variety of bioactive compounds, including vitamins, minerals, and immunoglobulins (Igs; Esch et al., 2020; Foroutan et al., 2019; Neerven et al., 2012). Igs provide immunological protection against bacteria, viruses, allergens, and toxins in the gastrointestinal (GI) and respiratory tract of the offspring (Greenspan & Cavacini, 2019; O'Riordan et al., 2014). However, there is currently no evidence indicating that intact bovine Igs can be absorbed into the human systemic circulation (Jasion & Burnett, 2015; Shaw et al., 2016). Interestingly, bovine Ig class G (bIgG) has been shown to bind a range of human GI and respiratory pathogens (Hartog et al., 2014; Korhonen et al., 2000; Porbahaie et al., 2022; Weiner et al., 1999) and common aeroallergens, including asthma-associated HDM allergens (Collins et al., 1991; Knol et al., 2024). Ulfman et al. (2018) extensively reviewed potential mechanisms through which bIgG can exert various protective effects via the GI tract in humans. Remarkably, various studies have demonstrated that bIgG can bind to human Fc gamma receptors, primarily FcγRII (CD32), either as aggregate or when complexed with antigens (an immune complex; IC), inducing effector functions and even trained immunity (Hartog et al., 2014; Hellinga et al., 2020; Jungi et al., 1989; Korhonen et al., 2000; Nederend et al., 2020; Porbahaie et al., 2022; Van Splunter et al., 2018).

1.6 Immune Complex Formation: Molar Equivalence & Lattice Assembly

ICs are formed when antibodies bind antigens, resulting in antibody–antigen complexes that may trigger immune responses, including phagocytosis, antigen presentation, and cellular maturation and activation (Lu et al., 2018). IC formation is fundamentally governed by the molar equivalence of antibody and antigen, a principle described in the classic Heidelberger-Kendall framework (Aibara & Ohyama, 2020; Heidelberger & Kendall, 1929, 1935). At optimal antibody–antigen ratios (*equivalence zone*), bivalent IgG and multivalent antigens cross-link to form large lattice structures (Figure 2). This lattice formation is formed because the two fragment antigen-binding (Fab) regions of each antibody bind distinct antigenic epitopes, while antigens with multiple epitopes reciprocally bridge multiple antibodies, creating a three-dimensional network. In contrast, neither extreme of antibody–antigen ratio permits stable IC formation. In antibody excess (*prozone*), antigen epitopes are fully occupied by antibodies, leaving no free epitopes to bridge monovalently-bound antibodies. Conversely, in antigen excess (*postzone*), antibody binding sites are fully saturated, leaving no monovalently-bound antibodies available for crosslinking. Both scenarios yield only small complexes that are incapable of crosslinking the Fcγ receptors to initiate downstream signaling (Hui, 2019; Lu et al., 2018). Experimental design to produce ICs must therefore determine the optimal antibody–antigen ratio, ensuring maximal lattice assembly for functional studies. The lattice structure orients the Fc regions of the IgG molecules outward, enabling them to engage and crosslink FcγRs on leukocytes, triggering receptor signaling and effector functions (Patel et al., 2019).

1.7 A Brief Overview of Fcγ Receptors: Classes, Expression & Functions

The FcγR family comprises transmembrane glycoprotein receptors which are expressed on both human lymphoid and myeloid cells, and in various non-immune cells such as neurons and liver sinusoidal endothelial cells (Hamdan et al., 2020). There are three main FcγR classes, which exhibit different affinities for monomeric IgG. First, FcγRI (CD64) is a high-affinity FcγR (10^{-9} M Kd) that is expressed on monocytes, macrophages, neutrophils, and eosinophils, and mainly involved in antibody-dependent cellular phagocytosis (ADCP), antigen presentation, and cytokine production (Bournazos et al., 2020; Holtrop et al., 2022). Secondly, FcγRIII (CD16) and FcγRII (CD32) are both low-affininty FcγR (10^{-6} M Kd) that can only interact with multimeric IgG–antigen ICs (Dekkers et al., 2017). FcγRIII mediates antibody-dependent cellular cytotoxicity (ADCC) in both monocytes and NK cells (Mandelboim et al., 1999; Yeap et al., 2016), but is also expressed on eosinophils, neutrophils, and mast cells (Bournazos et al., 2020). FcγRII exists in three subtypes: FcγRIIa, FcγRIIb, and FcγRIIc. FcγRIIa contains an immunoreceptor tyrosine-based activation motif (ITAM), enabling activating functions, while FcγRIIb possesses an immunoreceptor tyrosine-based inhibitory motif (ITIM), mediating inhibitory signals (Bournazos et al., 2020). FcγRIIc, though structurally similar to FcγRIIa with an ITAM, is less ubiquitously expressed (Nagelkerke et al., 2019). FcγRIIa is expressed on monocytes, macrophages, dendritic cells, neutrophils, and platelets, whereas B cells solely express FcγRIIb-1 (Bournazos et al., 2020; Nagelkerke et al., 2019). In monocytes, FcγRIIa facilitates immune processes such as ADCP, ADCC and antigen presentation (Anania et al., 2019; Kramski et al. 2012). Next to these IgG-interacting FcγRs, the neonatal FcR (FcRn) facilitates the recycling of internalized IgG, thereby

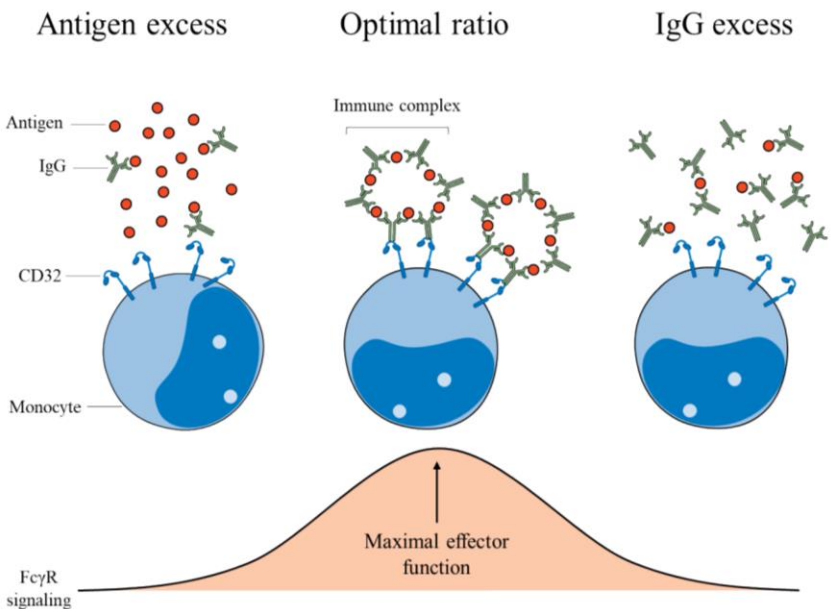

Figure 2. **Optimal antibody-antigen ratio for IC formation.** IC formation requires molar equivalence (Heidelberger-Kendall curve) to generate lattice structures via bivalent IgG and multivalent antigen crosslinking. When IgG is in excess (prozone) or the antigen is in excess (postzone), the resulting small complexes fail to effectively crosslink and activate low-affinity FcγRs. At the optimal antibody-to-antigen ratio, large ICs robustly engage and crosslink low-affinity FcγRs, triggering receptor signaling and downstream effector functions. Illustration by Porbahaie (2022).

prolonging IgG half-life, while also mediating transcytosis of IgG-opsonized antigens across cellular barriers and playing a crucial role in directing large ICs toward antigen processing and loading onto presentation pathways (Pyzik et al., 2019, 2023; Qiao et al., 2008; Ravetch & Bournazos, 2024).

Centering on monocytes, the binding and crosslinking of several FcγRIIa by ICs is required for ADCP and the initiation of the ITAM-mediated signaling pathway. Crosslinking can only occur when large, multivalent ICs interact with multiple FcγRIIa simultaneously, leading to the induction of effector functions and, as mentioned, trained immunity (Lux et al., 2013; Porbahaie et al., 2022; Shakib, 2016; Van Splunter et al., 2018).

1.8 Trained immunity

Trained immunity, as established by Netea et al. in 2011, refers to the long-term functional reprogramming of innate immune cells. This innate immune 'memory' is characterized by an initial inflammatory challenge, such as an infection, leading to an enhanced, non-specific innate immune response towards secondary challenges (Netea et al., 2020). The reprogramming is largely mediated via epigenetic modifications of transcriptional pathways and can last for several months up to one year (Netea et al., 2020). Perhaps the best known example of trained immunity conferring non-specific protection against various human pathogens is via the Bacillus Calmette-Guérin (BCG) vaccine (Benn et al., 2013; Kleinnijenhuis et al., 2012, 2014). Randomized controlled trials involving human participants have shown BCG vaccination to non-specifically protect against yellow fever (Arts et al., 2018) and malaria (Walk et al., 2019), even inducing anti-tumor effects in monocytes *in vitro* (Buffen et al., 2014). Furthermore, Namakula et al. (2020) demonstrated that BCG-trained newborn cord blood monocytes produce a similar cytokine profile to that of adult monocytes when exposed to a second challenge. Accordingly, Hellinga et al. (2020) argued that trained immunity may serve as a compensatory mechanism for the underdeveloped adaptive immune system in early life, thereby providing sufficient immunological protection. Trained human monocytes show an increased production of pro-inflammatory cytokines including IL-6 and TNF-α, following a secondary non-specific stimulation (Ifrim et al., 2014).

1.9 Trained tolerance

In contrast, the primary stimulation can also reprogram the monocyte towards an anti-inflammatory state, known as trained tolerance (Bindu et al., 2022; Divangahi et al., 2021). Tolerance is a protective response of the body meant to limit collateral tissue damage caused by an excessive immune response (Medzhitov et al., 2012). Trained tolerance is characterized by overall functional defects, including downregulated expression of LPS-sensing TLR4, downregulated production of pro-inflammatory cytokines such as TNF-α and IL-6, and upregulated expression of anti-inflammatory cytokines like IL-10, following restimulation (Fernando et al., 2000; Nomura et al., 2000; Pena et al., 2011; Ziegler-Heitbrock et al., 1992). Whether trained immunity or tolerance is induced depends on the type and intensity of the primary stimulus (Dagenais et al., 2023). Notably, some studies suggest that trained tolerance may be partially reversed when stimulating trained monocytes with β-Glucan (Novakovic et al., 2016) or low concentrations of TLR ligands (Ifrim et al., 2014).

1.10 Linking Trained Immunity to the Protective Effect of Bovine Milk

As discussed, the traditional farming environment, including the consumption of unpasteurized cow milk, protects children against developing allergic asthma (Pechlivanis & von Mutius, 2020; Song et al., 2023). The cord blood mononuclear cells (CBMCs) of these asthma-protected children exhibit upregulated expression of TLRs (Ege et al., 2006) and increased production of proinflammatory cytokines TNF-α and interferon (IFN)-γ compared to ‘nonfarm’ children (Pfefferle et al., 2010). Recent findings by DeVries et al. (2022) support this by showing that the production of IL-6 and TNF-α in CBMCs of children was inversely associated with asthma development later in life. In contrast, Lechner et al. (2022) documented immune-trained T2 macrophages with elevated, TNF-driven inflammatory effector functions in asthma patients and HDM-allergic mice. The timing-dependent enhancing or inhibitory role of TNF on the differentiation of T2 inflammatory macrophages was also highlighted (Lechner et al., 2022).

While trained immunity is associated with an enhanced immune response, tolerance represents an anti-inflammatory state. Various studies have associated innate immune tolerance with a protective effect against developing asthma (Lechner et al., 2022; Stein et al., 2016). Remarkably, mouse studies have found that the maternal milk from mother mice who were exposed to asthma aeroallergens during lactation, especially when pre-sensitized, contained allergen–IgG ICs that could induce immune tolerance (Ohsaki et al., 2017) and even prevent asthma development in progeny mice (Mosconi et al., 2010; Verhasselt et al., 2008). Taken together, these findings indicate that early-life immune modulation is shaped by competing immunological influences, which may protect against the development of allergic asthma or exacerbate existing asthma.

1.10.1 Bridging the Gap: How Bovine Milk IgG–HDM Immune Complexes May Reprogram Monocytes & Confer Asthma Protection

Considering that: (1) HDM allergen sensitization is strongly associated with allergic asthma susceptibility, (2) raw cow milk consumption is associated with a reduced risk of developing asthma, (3) bovine milk IgG can bind to HDM allergens, and (4) bIgG–antigen ICs can induce trained immunity by interacting with the human monocyte receptor FcγRIIa, we hypothesize that bIgG–HDM ICs may induce trained immunity or tolerance in human monocytes. This hypothesis will be investigated via the following research questions.

Main research question

1) Can bovine IgG form large ICs with HDM allergens and do these bIgG–HDM ICs reprogram human monocytes toward a trained immunity or tolerance phenotype?

Research subquestions

1.1) Can bIgG form large ICs with single HDM allergens (Der p1, Der p2) or HDM whole body extract, as assessed by flow cytometry detecting IC-bound $CD14^{+}$ monocytes?

1.2) Does FcγRIIa mediate bIgG–HDM IC binding to human monocytes, as demonstrated by blocking monocyte FcγRIIa and assessing binding via flow cytometry?

1.3) Do bIgG–HDM ICs induce trained immunity or tolerance in human monocytes, as assessed by the production of TNF-α, IL-6, and IL-10 upon restimulation?

2. Materials & Methods

2.1 Isolation of PBMCs

Isolation of peripheral blood mononuclear cells (PBMCs) was performed as described by Porbahaie et al. (2022). In brief, PBMCs were isolated from buffy coats (Sanquin, Nijmegen, The Netherlands) by gradient centrifugation on Ficoll Paque Plus (Cytiva, 17-1440-02, Marlborough, MA, USA). Ficoll (15 mL/tube) was transferred to Leucosep tubes (Greiner Bio-One, #227290, Kremsmünster, Austria) and briefly spun down (1000g, 30s, room temperature [RT]). The buffy coats were then added to the Leucosep tubes after being diluted 1:1 with warm (37 °C) RPMI 1640 medium containing GlutaMAX (Gibco, #61870010, Waltham, MA, USA). This was centrifuged (1000g, 10min, RT) in a swinging bucket centrifuge without breaking during deceleration (Eppendorf, Centrifuge 5810R, #5811000015, Hamburg, Germany). Subsequently, the enriched PBMC fraction was transferred by pouring the supernatants above the porous barrier into new 50 mL Falcon tubes (Corning, #352070, Corning, NY, USA). Warm phosphate-buffered saline (PBS; Gibco, #20012027, Waltham, MA, USA) was used to wash the cells (250g, 7min, RT, brakes on). After centrifugation, the supernatant was discarded and the cell pellet was resuspended and washed twice more with RPMI 1640 GlutaMAX. Following the third wash, the cells are counted and either used directly for experiments or frozen (in FBS + 10% dimethyl sulfoxide [DMSO]) and stored in liquid nitrogen.

2.2 Cryopreservation of PBMCs

The cryopreservation of PBMCs is based on Nazarpour et al. (2012). The PBMCs were diluted to a maximum concentration of 4×10^7 cells/mL using FBS medium. An equal amount of ice-cold medium (80% FBS + 20% DMSO) was added in a dropwise manner while the tube was gently mixed by shaking. The cells were transferred to cryovials in aliquots of 1−1.5 mL. The cryovials were placed in a pre-cooled isopropyl alcohol freezing container (4 °C) which was subsequently stored in a −80 °C freezer for 8−12hr. After this period, the cryovials were transferred to liquid nitrogen for long-term storage.

2.3 Thawing & Counting of PBMCs

The cryovials were removed from the liquid nitrogen storage and put in a warm water bath (37 °C) under continuous agitation. When the cryovials were nearly thawed, they were disinfected and placed in a laminar flow cabinet. Here, the PBMCs were transferred to a tube containing warm RPMI 1640 GlutaMAX medium with a volume of approximately 10 times the volume of the cryovials. The cells were subsequently washed twice (250g, 7min [first wash] and 5min [second wash], RT) and finally resuspended in 5 mL RPMI 1640 GlutaMAX. From this, a sample (20−50 µL) was taken and diluted (5−10 times). A fraction of this diluted sample was mixed with an equal volume of 0.4% Trypan Blue and subsequently counted (Invitrogen, Countess 3, #AMQAX2000, Waltham, MA, USA).

2.4 Reagents

Three different stocks of bovine Immunoglobulin G (bIgG) were used, bIgG-1 and bIgG-2 were obtained from FrieslandCampina (Amersfoort, The Netherlands) and bIgG-3 was a kind gift from Dr. E. Knol and C. den Hartog Jager (UMC Utrecht, Utrecht, The Netherlands). Purified HDM allergens Der p1, Der p2, along with the whole body extract (WBE) were obtained from ALK-Abelló (Hørsholm, Denmark). For bIgG detection, AlexaFluor 647−conjugated α-bIgG was used (Jackson ImmunoResearch, #101-605-165, Ely, Cambridgeshire, UK).

2.5 Formation of Immune Complexes

The optimal antibody–antigen ratio must be determined experimentally to obtain ICs. Antibody–antigen ratio optimization was performed for each of the allergens tested (Der p 1, Der p 2, HDM WBE) by titrating bIgG while keeping the allergen concentration constant. First, purified bIgG was spun down (17g, 5min, RT) to remove antibody aggregates and obtain the monomeric form. The supernatant of the spun-down bIgG was used for a serial dilution, which was incubated 1:1 with a constant allergen concentration on a sterile NUNC plate (Thermo Scientific, #267245, Waltham, MA, USA). The tested bIgG concentrations varied across experiments, ranging from 0−960 µg/mL, while the concentrations for the three allergens ranged from 0.01−10 µg/mL. After combining bIgG and the allergen 1:1 (total volume 40 µL), the NUNC plate was pre-incubated (30min, RT) to allow for the formation of ICs. During the pre-incubation period, the plate was wrapped in plastic foil to minimize the escape of vapors. The plate was cooled (5min, 4 °C) and thawed PBMCs were exposed to the ICs (40 µL/well of 3.75×10^6 cells/mL). The plate is then foil wrapped and incubated (30min, 4 °C) to allow for IC binding to the cells.

Following incubation, the cells were washed twice with cold FACS buffer to remove any unbound antibody/antigen residuals. To reduce any potential shear stress on IC-bound cells, the pipette tips were cut to increase the tip diameter during resuspension. The cells were then incubated (10min, RT) with a Fc receptor blocking solution (Biolegend, #422302, San Diego, CA, USA) to reduce non-specific staining effects. Afterwards, the cells were stained with AlexaFluor 647−conjugated α-bIgG for bIgG detection, as a proxy for detecting bound ICs (Jackson ImmunoResearch, #101-605-165, Ely, Cambridgeshire, UK), FITC-conjugated α-CD14 antibody for monocyte identification (Biolegend, #325604, San Diego, CA, USA), and Brilliant Violet 785−conjugated α-CD19 antibody for B cell identification (Biolegend, #302240, San Diego, CA, USA). The same bIgG concentrations without any allergen (bIgG only), and allergen without bIgG (e.g., 'Der p 1 only), along with unstained and stained cells, were included as experiment controls and for background values. After washing twice, the cells were measured using a CytoFLEX LX flow cytometer (Beckman Coulter, #C11186, Brea, CA, USA). The data were processed using FlowJo v10.10 according to the gating strategy in Figure S7 (FlowJo, Ashland, OR, USA), and MS Excel (Microsoft, Redmond, WA, USA) was used to plot the bIgG detection median fluorescent intensity (MFI). The experiments were performed using bIgG–allergen conditions in technical triplicates, with PBMCs from at least three different donors.

2.6 Blocking of Immune Complex Binding

For the blocking experiments, PBMCs were pre-incubated with the anti-human FcγRIIa monoclonal antibody (Bio X Cell, #BE0224, clone IV.3, Lebanon, NH, USA) for 20min at RT. After washing (400g, 4min), the PBMCs were exposed to the pre-formed IC mixture (30min, 4 °C). The concentration of α-FcγRIIa needed to block the receptor was <1 μg per 1×10^6 cells in 100 μL of whole blood. This was based on the recommendation from STEMCELL Technologies regarding their α-FcγRIIa from the same antibody clone (STEMCELL Technologies, #60012, clone IV.3, Vancouver, BC, Canada), as the manufacturer of the α-FcγRIIa used in the experiment (Bio X Cell) did not suggest a concentration. The remaining steps were performed as described in section 2.5.

2.7 Pan Monocyte Isolation

Human monocytes were negatively selected from PBMCs using the pan human monocyte isolation kit with a magnetic cell separator (Miltenyi Biotec, autoMACS NEO, kit: #130-096-537, Bergisch Gladbach, Germany) according to the procedure as specified by the manufacturer (protocol in Figure S9). In short, cells were resuspended in the separation buffer, after which FcR blocking reagent and biotin-antibody cocktail were added. After cooled incubation (5 min, 5 °C), more separation buffer was added, along with anti-biotin microbeads. Following cooled incubation (10 min, 5 °C), the mixture was applied to a rinsed column in the magnetic field of the MACS. The flow-through containing the unlabeled cells represented monocytes. Finally, the cells were counted, and purity was determined using flow cytometry. The monocytes are used directly, or cryopreserved for long-term storage.

2.8 Trained Immunity Experiment

The *in vitro* trained immunity experiment (Figure 3) is adapted from Domínguez-Andrés (2021) and Porbahaie (2022). First, fresh negatively-selected CD14$^+$ monocytes were seeded (100 μL/well of 1.5×10^6 cells/mL) on a flat-bottom culture plate (Corning, #3585, Corning, NY, USA) in warm RPMI 1640 GlutaMAX supplemented with 1% penicillin/streptomycin and 1 mM pyruvate (hereafter referred to as RPMI$^+$). The majority of the cells employed for the training were cryopreserved CD14$^+$ monocytes, which were rested (2h, 37 °C, 5% CO_2) before training to allow the cells to adhere to the culture plate. For training, the monocytes were exposed to ICs formed from bIgG (30, 60, or 120 μg/mL) and Der p1 (0.1 μg/mL) or HDM WBE (4 μg/mL) for 24h (37 °C, 5% CO_2). Additionally, the monocytes were exposed to controls consisting of bIgG only, Der p1 only, and HDM only, at their respective concentrations used in the formation of ICs. RPMI$^+$ was used as negative control, while 200 μg/mL aggregated bIgG (which contains the pellet fraction of centrifuged bIgG-1 [17g, 5min, RT]) and 5 μg/mL curdlan (Megazyme, #P-CURDL, Bray, Ireland) served as training-inducing controls, and 0.5 μg/mL LPS (Sigma-Aldrich, #L2880, St. Louis, MO, USA) acted as a tolerogenic control. Two technical replicates per stimuli were included to normalize the number of cells and cytokine levels at the end of the protocol. Following 24h stimuli incubation, on day 2, the monocytes were washed twice (150g, 5min, RT) with DPBS (Gibco, #14040117, Waltham, MA, USA), after which RPMI$^+$ supplemented with 10% pooled human serum was added. Human serum contains factors such as GM-CSF that drive differentiation into macrophages. Microscopy images confirmed morphological changes consistent with macrophage differentiation (Figure S8). On day 4, the medium was refreshed. On day 7, monocytes were stimulated using 0.01

µg/mL LPS as TLR-4 ligand, representing gram-negative bacteria, and 10 µg/mL R848 (InvivoGen, #tlrl-r848-1, San Diego, CA, USA) as TLR-7/8 ligand, representing single-stranded RNA viruses. After incubation (24h, 37 °C, 5% CO_2), the culture plate was spun down (300g, 5min, RT) and the supernatant was stored (-20 °C) for later cytokine measurement.

2.9 Cytokine Assay

Cytometric bead array (CBA) cytokine Flex Sets were used for measuring IL-6 (BD, #558276, Franklin Lakes, NJ, USA), IL-10 (BD, #558274, Franklin Lakes, NJ, USA) and TNF-α (BD, #558273, Franklin Lakes, NJ, USA) in the culture supernatant of the cells. The experiments were performed with negatively-selected CD14$^+$ monocytes isolated from 2–7 different donors.

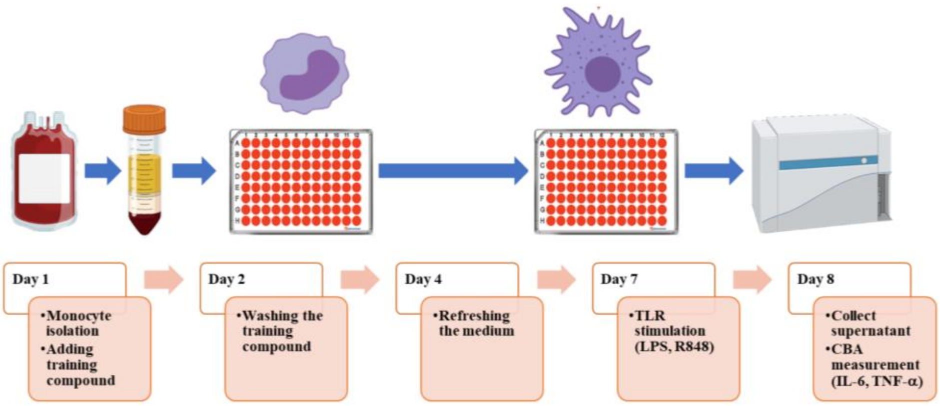

Figure 3. **Schematic representation of the *in vitro* innate immune training model.** PBMCs were isolated from buffy coats, of which the CD14$^+$ monocytes were negatively selected and subsequently exposed to the training compounds for 24h. Then, the cells were rested for six days to allow for differentiation into macrophages. On day 7, the cells were restimulated for 24h with RPMI, LPS, and R848. After 24h, the supernatant was collected and the poduction of IL-6, IL-10 and TNF-α was quantified. Illustration by Porbahaie et al. (2022).

2.10 Statistical Analysis

CBA data was analyzed by FCAP array v3 (BD, Franklin Lakes, NJ, USA) and then transferred to GraphPad Prism v10.3.0 (GraphPad Software, Boston, MA, USA) for statistical analysis and preparing the figures. The data were expressed as fold change relative to the untrained monocytes (control group RPMI$^+$). The Shapiro-Wilk test was used to assess normality. A one-way ANOVA was performed to evaluate overall differences among group means, followed by Dunnett's *post hoc* test for multiple comparisons to compare each experimental group with the control group. The differences are considered significant when the p-value is <0.05 (*), <0.01 (**), or <0.001 (***), as indicated in the graphs.

3. Results

3.1 The Formation of Large bIgG–Der p1 Immune Complexes

The formation of large ICs is dependent on the antibody–antigen ratio approaching molar equivalence. This optimum was determined experimentally by titrating bIgG with a constant concentration of Der p1 allergen, followed by incubation with human PBMCs. Bound immune complexes on CD14⁺ monocytes were quantified via flow cytometry using an AlexaFluor 647-conjugated detection antibody against bIgG. This approach leverages the principle that immune complexes—comprising multiple bIgG and Der p1 molecules arranged in a lattice structure—amplify the fluorescent signal compared to monomeric bIgG. The lattice configuration inherently increases the number of detectable bIgG molecules per IC, thereby correlating detection intensity with immune complex binding capacity.

The bIgG–Der p1 condition, representing the combination of these components in a single well, exhibited a higher MFI compared to the bIgG-only curve across several of the tested bIgG concentrations (Figure 4A). This increased fluorescent detection of bIgG suggests that ICs had formed and were bound to $CD14^+$ human monocytes of a representative donor. The MFI of the Der p1-only condition was comparable to the buffer control (0 µg/mL bIgG), indicating that the Der p1 allergen alone did not contribute to the increased MFI observed in the IC condition. Substracting the bIgG-only MFI from the bIgG–Der p1 MFI yielded a bell-shaped curve, reflecting the optimal bIgG–Der p1 ratio for large IC formation (Figure 4B). This optimal ratio was observed at 64 µg/mL bIgG to 0.1 µg/mL Der p1, with a marginally lower ΔMFI peak at 32 µg/mL bIgG. IC formation was absent at 250 µg/mL bIgG. These results were replicated in two additional donors (Figure S1), including a Der p1 titration for one donor (Figure S1A,B). Similar optimal ratios were observed for $CD19^+$ B cells (Figure S2A), which express an inhibitory isoform of FcγRIIb. Unlike FcγRIIa-expressing $CD14^+$ monocytes, the ΔMFI peak for bIgG–Der p1 on $CD19^+$ B cells was 8-fold greater compared (Figure S2B). Another major HDM allergen, Der p2, was also tested; however, consistent binding was observed only on $CD19^+$ B cells (Figure S2).

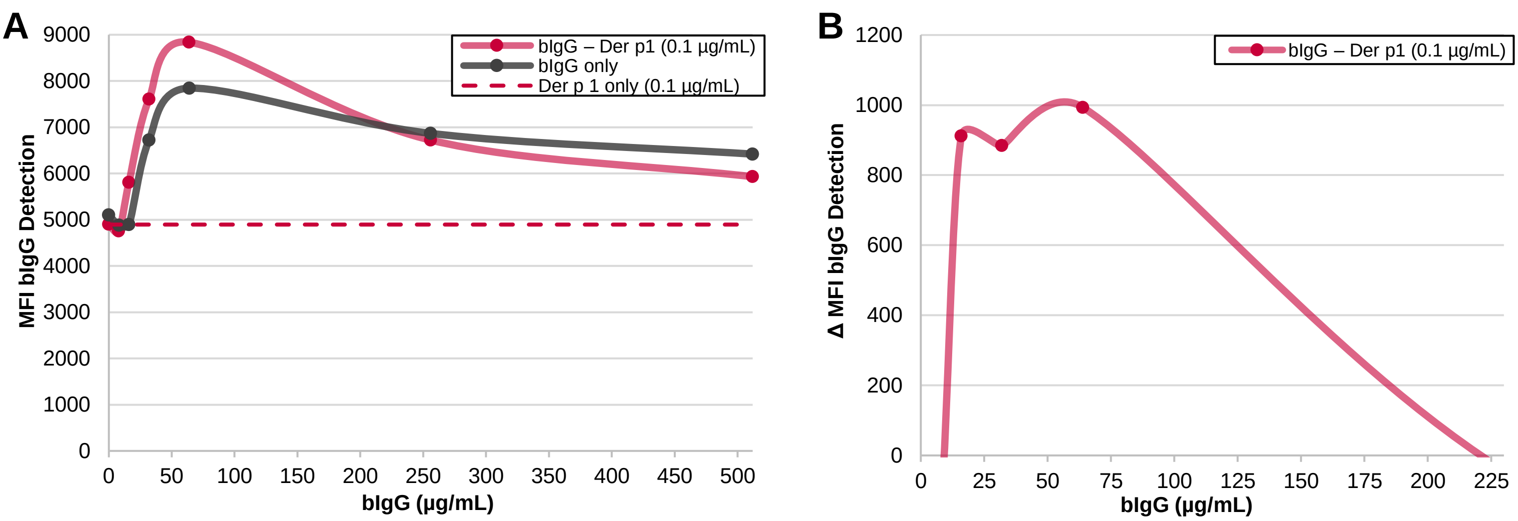

Figure 4. **Median fluorescence intensity (MFI) curves of the bIgG detection signal on $CD14^+$ monocytes.** Human PBMCs were exposed to bIgG only (0–512 µg/mL), Der p1 protein only (0.1 µg/mL), and the bIgG – Der p1 ICs in one representative donor (donor 1). The detection curves were derived from the bIgG MFI signal gated on $CD14^+$ monocytes (**A**). The delta MFI (ΔMFI) curve was obtained by subtracting the bIgG only MFI signal from the bIgG – Der p1 MFI signal (**B**).

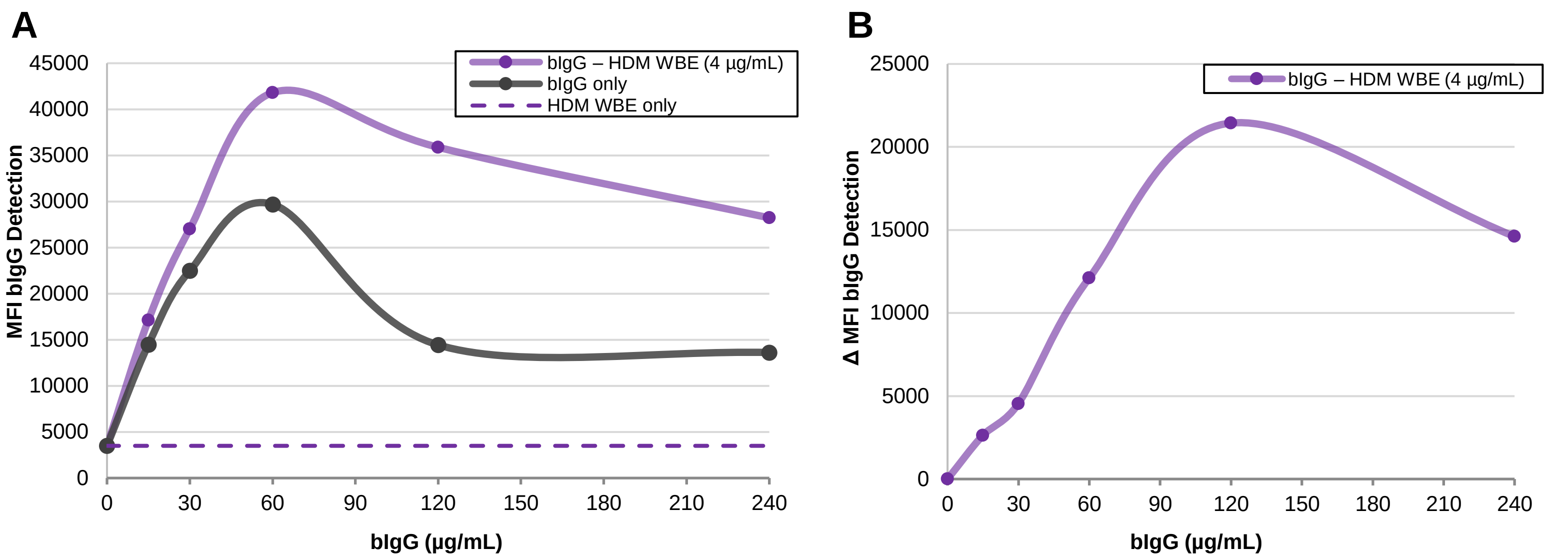

Figure 5. **MFI curves of the bIgG detection signal on CD14⁺ monocytes.** Human PBMCs were exposed to bIgG only (0–240 µg/mL), house dust mite whole body extract (HDM WBE) only (4 µg/mL), and the bIgG – HDM WBE ICs in one representative donor (donor 4). The detection curves were derived from the bIgG MFI signal gated on CD14⁺ monocytes (**A**). The delta MFI (ΔMFI) curve was obtained by subtracting the bIgG only MFI signal from the bIgG – HDM WBE MFI signal (**B**).

3.2 HDM Whole Body Extract Enhances Immune Complex Formation

To enhance IC formation, HDM WBE was also tested, leveraging its broad allergen diversity to maximize allergen-specific bIgG availability and signal resolution. The bIgG–HDM WBE condition showed a greatly increased MFI compared to the bIgG-only curve across several bIgG concentrations (Figure 5A), indicating strong IC formation and binding to CD14⁺ human monocytes of a representative donor. The ΔMFI peak for the bIgG–HDM WBE condition increased >20-fold compared to the bIgG–Der p1 condition (Figure 4B, 5B). The optimal bIgG–HDM WBE ratio was observed at 120 µg/mL bIgG to 4 µg/mL HDM WBE, owing to a marked decrease in background bIgG-only MFI (Figure 5B). This result was replicated in two additional donors (Figure S3). However, unlike bIgG–Der p1 ICs, no binding was observed for bIgG–HDM WBE ICs to CD19⁺ B cells (Figure S4A).

3.3 FcγRIIa Inhibition Abolishes Immune Complex Binding

To demonstrate the binding of bIgG–HDM WBE ICs to the monocyte FcγRIIa, a monoclonal blocking antibody was used to inhibit FcγRIIa engagement. PBMCs were pre-incubated with an α-FcγRIIa blocking antibody, followed by incubations with the bIgG–HDM WBE ICs, bIgG only, or HDM WBE only. The blocking of FcγRIIa effectively abolished the binding of both ICs and bIgG, as evidenced by a reduction in (Δ)MFI to a near-background level, comparable to the HDM WBE only MFI (Figure 6, 7, S5). Moreover, CD19⁺ B cells served as a control, as this population expresses the FcγRIIb isoform instead of FcγRIIa, the receptor targeted by the blocking antibody. As expected, incubation with α-FcγRIIa had no effect on MFI curves in CD19⁺ B cells (Figure S4).

3.4 The Model of Trained Immunity

Having determined the optimal antibody–antigen ratios for the formation of large ICs, their potential to induce innate immune training in CD14⁺ monocytes was investigated. Using an established

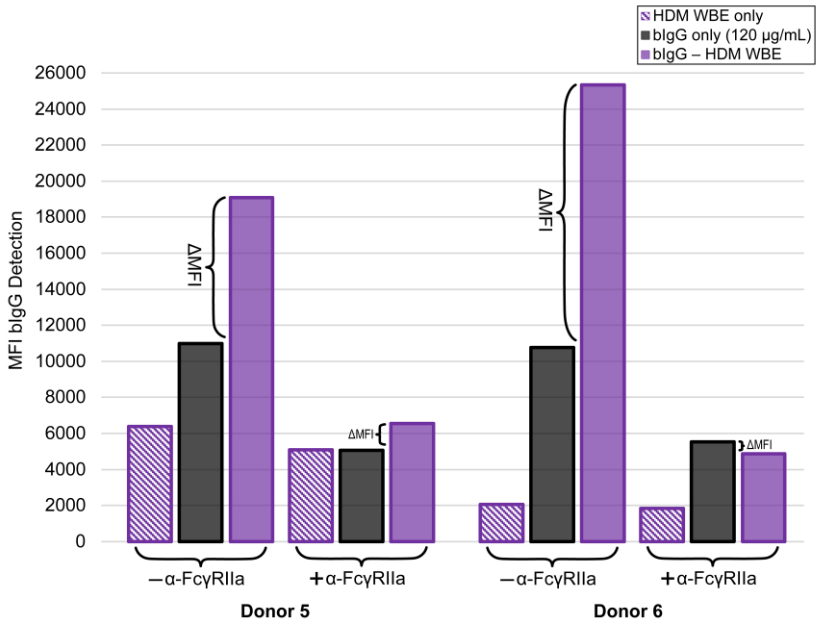

Figure 6. **MFI of the bIgG detection signal at a single bIgG concentration (120 µg/mL) on CD14⁺ monocytes with (+) or without (–) FcγRIIa blocking in two donors.** PBMCs were pre-incubated with (+) and without (–) an α-FcγRIIa blocking antibody, followed by incubations with the bIgG–HDM WBE ICs, bIgG only (120 µg/mL), or HDM WBE only (4 µg/mL) in two donors (donor 5 & 6). The MFI values were derived from the bIgG MFI signal gated on CD14⁺ monocytes.

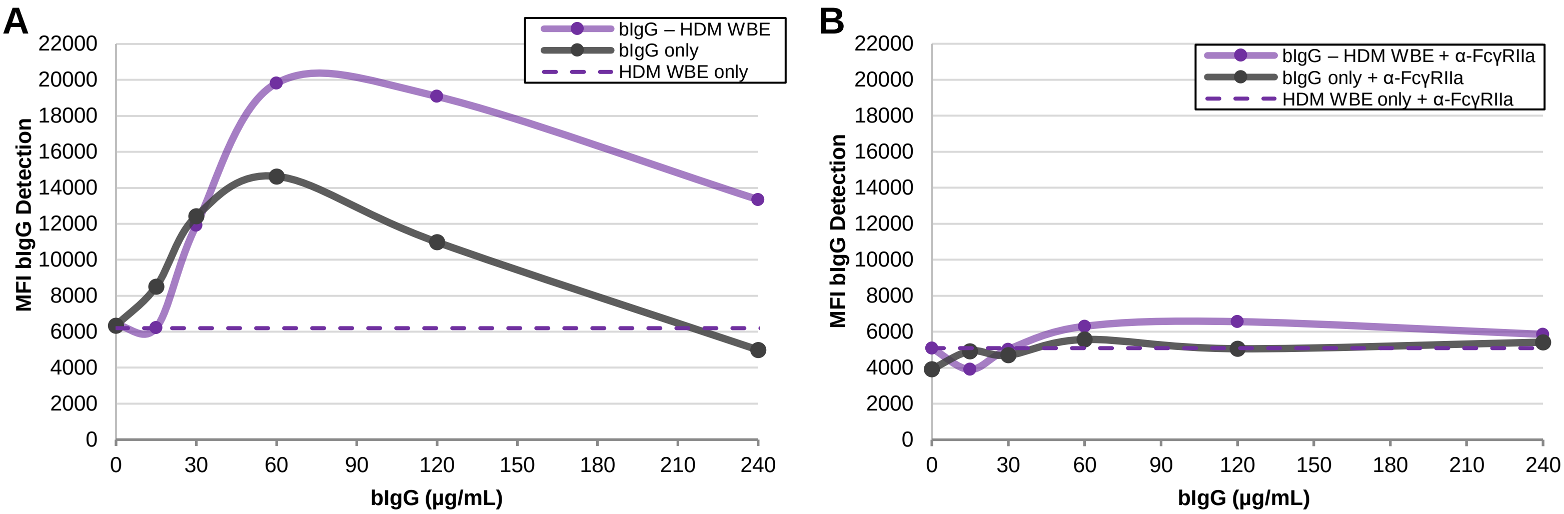

Figure 7. **Median fluorescence intensity (MFI) curves of the bIgG detection signal on CD14⁺ monocytes (A) and on CD14⁺ monocytes of which the FcγRIIa (CD32a) was blocked using α-FcγRIIa (B).** PBMCs were incubated with and without α-CD32a prior to being exposed to bIgG only (0–240 µg/mL), house dust mite whole body extract (HDM WBE) only (4 µg/mL), and the bIgG – HDM WBE ICs in one representative donor (donor 5). The detection curves were derived from the bIgG MFI signal gated on CD14⁺ monocytes.

in vitro model, CD14⁺ monocytes were exposed to the ICs or their single constituents for 24 hours, followed by washing and a 6-day resting period during which the monocytes differentiated into macrophages (Figure S8). On day 7, the macrophages were stimulated with TLR4 (LPS) or TLR7/8 (R848) ligands for 24 hrs. The production of TNF-α, IL-6, and IL-10 was subsequently measured in the culture supernatants

3.5 Validating the Model of Trained Immunity

To validate this model of trained immunity, curdlan (5 µg/mL) and aggregated bIgG (200 µg/mL) were employed as positive controls, while a high-dose LPS (0.5 µg/mL) served as a tolerance-inducing control. RPMI⁺ was used as the negative control (untrained cells) and cytokine production was expressed as fold change relative to the untrained cells. Each training condition was followed by LPS or R848 stimulation, with RPMI⁺ serving as an unstimulated control. None of the training controls induced a significantly different cytokine production compared to the untrained cells, due to the high donor variability (Figure 8). For curdlan-trained cells, no differences in TNF-α and IL-6 production were observed relative to the untrained cells, with minimal variation among donors (Figure 8A-D). However, a small, non-significant increase in TNF-α upon R848 stimulation was noted (Figure 8B). Aggregated bIgG induced high TNF-α and IL-6 fold changes with large variability among the donors after LPS or R848 stimulation, but the low median values mainly indicated no induction of training (Figure 8A-D). Similarly, the cells exposed to high-dose LPS also showed high donor variability in TNF-α and IL-6 cytokine production; however, the decreased median fold changes of high-dose LPS hinted at tolerance (Figure 8A-D). IL-10 production in LPS- and aggregated bIgG-trained cells was reduced following both LPS and R848 stimulation, but this decrease did not reach significance (Figure 8E,F). Finally, elevated cytokine background levels for high-dose LPS and aggregated bIgG were noted for the RPMI$^+$ stimulation control (Figure S6A-C).

3.6 The Training Potential of bIgG–HDM Immune Complexes

The training potential of the four optimal ratios for IC formation was evaluated in the above model: Der p1 (0.1 µg/mL) with bIgG (30 or 60 µg/mL), and HDM WBE (4 µg/mL) with bIgG (60 or 120 µg/mL). The optimization of IC formation described in the above sections was time-intensive, which limited the number of biological replicates to 2–3 donors per condition. Elevated background signals were observed in the RPMI$^+$ stimulation control group for bIgG only (30 µg/mL), and two IC conditions (bIgG 60 µg/mL – Der p1 0.1 µg/mL / HDM WBE 4 µg/mL), indicating a potentially increased background of cytokine production (Figure S6D-I). Importantly, the small sample size precludes definitive interpretations; however, the results presented below offer tentative and highly cautious observations that may suggest a potential training effect of these ICs.

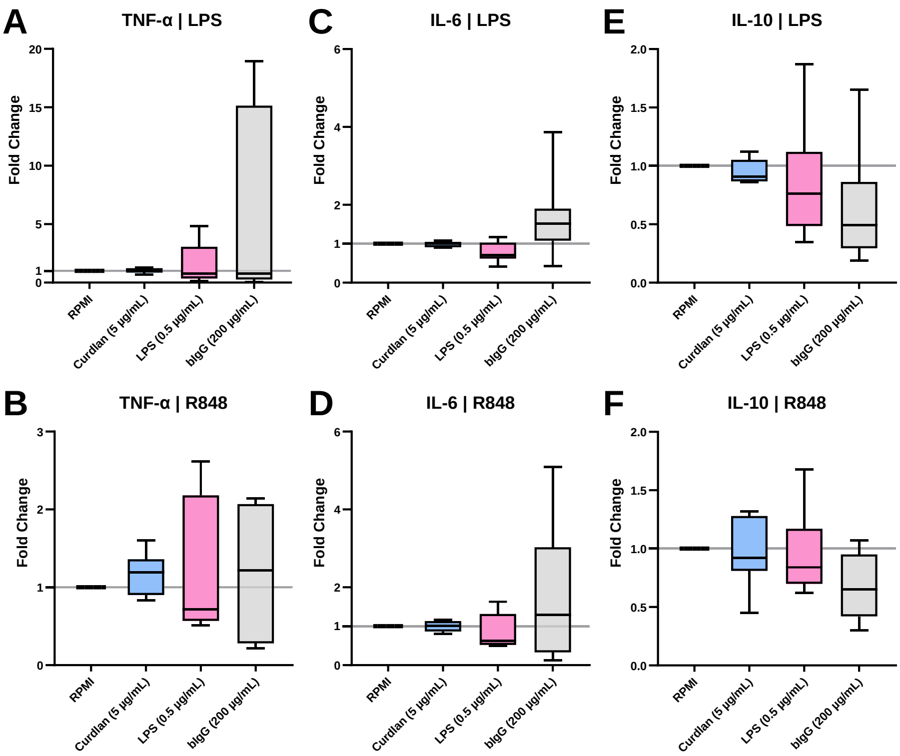

Figure 8. **The fold changes in TNF-α (A,B), IL-6 (C,D), and IL-10 (E,F) production of the cells trained with Curdlan (5 µg/mL), LPS (0.5 µg/mL), and aggregated bIgG (200 µg/mL) relative to the RPMI$^+$ control group.** The graph title denotes the stimuli used on day 7 (**A,C,E:** LPS and **B,D,F:** R848). The average (min–max range) of cytokines in the RPMI controls of all 7 donors tested were, as organized by graph: **A:** 2546 (202–7658 pg/mL), **B:** 11729 (2293–30784 pg/mL), **C:** 2928 (142–6754 pg/mL), **D:** 9301 (1195–13822 pg/mL), **E:** 773 (56–1943 pg/mL), and **F:** 605 (60–1214 pg/mL). The Box and Whisker Plots represent 50% of the data, and the line within the box indicates the median value, while the upper and lower whiskers denote the upper and lower 25% of the data, respectively.

bIgG – Der p1 ICs

For bIgG (30 µg/mL) – Der p1 (0.1 µg/mL) ICs, cells from one donor showed increased TNF-α and IL-6 fold changes compared to the bIgG-only condition, accompanied by a slight decrease in IL-10 (Figure $9_{[left]}$; donor 8). In contrast, the second donor tested showed minimal deviations from the untrained control and did not exceed bIgG-only fold changes (Figure $9_{[left]}$; donor 9). More variation was observed for bIgG (60 µg/mL) – Der p1 (0.1 µg/mL) ICs. Donor 10 exhibited increased TNF-α and IL-6 production upon LPS or R848 stimulation; however, the increase relative to bIgG-only was higher for TNF-α and not for IL-6 (Figure 9A-$D_{[right]}$; donor 10). Donors 7 and 11, generally showed less pronounced TNF-α and IL-6 fold changes compared to the control group. Both donors also demonstrated a decrease in IL-10 when stimulated with LPS or R848 (Figure 9E,$F_{[right]}$).

bIgG – HDM WBE ICs

The same donors (donors 10 and 11) were also exposed to bIgG–HDM WBE ICs (bIgG 60 µg/mL – HDM WBE 4 µg/mL). Again, donor 10 responded with pronounced increases in TNF-α and IL-6 production relative to the control group upon LPS or R848 stimulation. As with the previous observation, donor 11 generally showed no notable changes compared to the control (Figure 10A-$D_{[left]}$).

Moreover, a decreasing trend in IL-10 production was observed for donor 11 upon R848 stimulation, similar to their response when exposed to bIgG – Der p1 ICs (compare donor 11 in Figure 9E,$F_{[right]}$ with Figure10E,$F_{[left]}$). Likewise, donor 10 showed elevated IL-10 levels when exposed to the IC condition upon R848 stimulation, but not for the bIgG-only condition, comparable to their response to bIgG – Der p1 ICs (compare donor 10 in Figure 9$F_{[right]}$ with Figure10$F_{[left]}$).

Finally, the IC condition with the highest MFI signal, and a comparatively low bIgG background, was tested (bIgG 120 µg/mL – HDM WBE 4 µg/mL). Results were inconsistent or showed no change in TNF-α or IL-6 production (Figure 10A-$D_{[right]}$). Notably, donor 12 showed elevated IL-6 levels upon LPS stimulation, and to a lesser extent when stimulated with R848 (Figure 10C,$D_{[right]}$). In line with some of the above observations, IL-10 production was decreased in R848-stimulated cells (Figure 10$F_{[right]}$). The fold change values for TNF-α, IL-6, and IL-10 production for each of the conditions can be found in supplementary table S1.

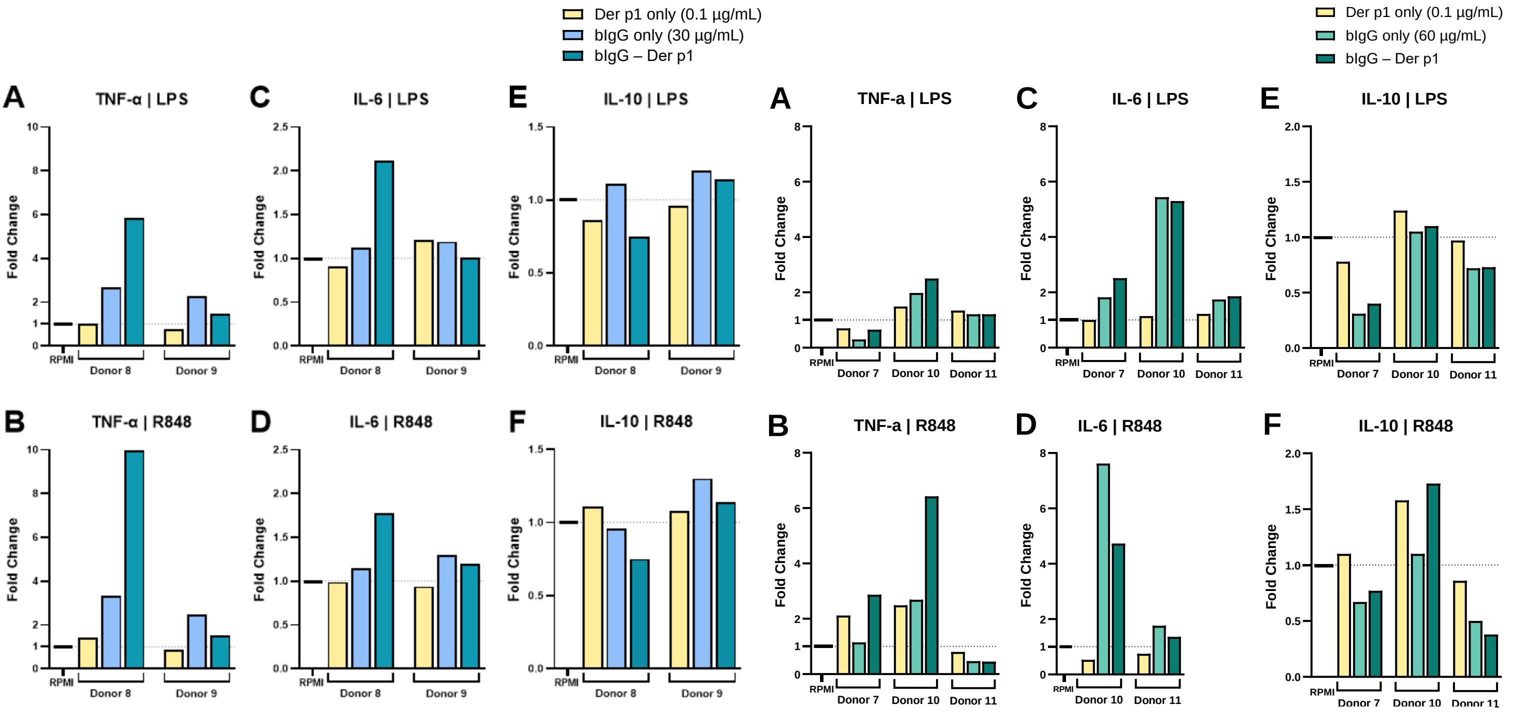

Figure 9. **The fold changes in TNF-α (A,B), IL-6 (C,D), and IL-10 (E,F) production of the cells trained with bIgG only (30 [blue color scheme, left] or 60 [green color scheme, right] µg/mL), Der p1 only (0.1 µg/mL), and their corresponding bIgG–Der p1 ICs relative to the RPMI+ control group.** The graph title denotes the stimuli used on day 7 (**A,C,E:** LPS and **B,D,F:** R848). The average cytokines levels in the RPMI controls of the donors tested were, as organized in order per graph: leftmost **A:** 1067 pg/mL, **B:** 3227 pg/mL, **C:** 4729 pg/mL, **D:** 10108 pg/mL, **E:** 1381 pg/mL, and **F:** 584 pg/mL. Rightmost **A:** 5860 pg/mL, **B:** 13717 pg/mL, **C:** 3578 pg/mL, **D:** 7539 pg/mL, **E:** 606 pg/mL, and **F:** 481 pg/mL. The bar charts indicate the fold change value per donor due to a low sample size per condition.

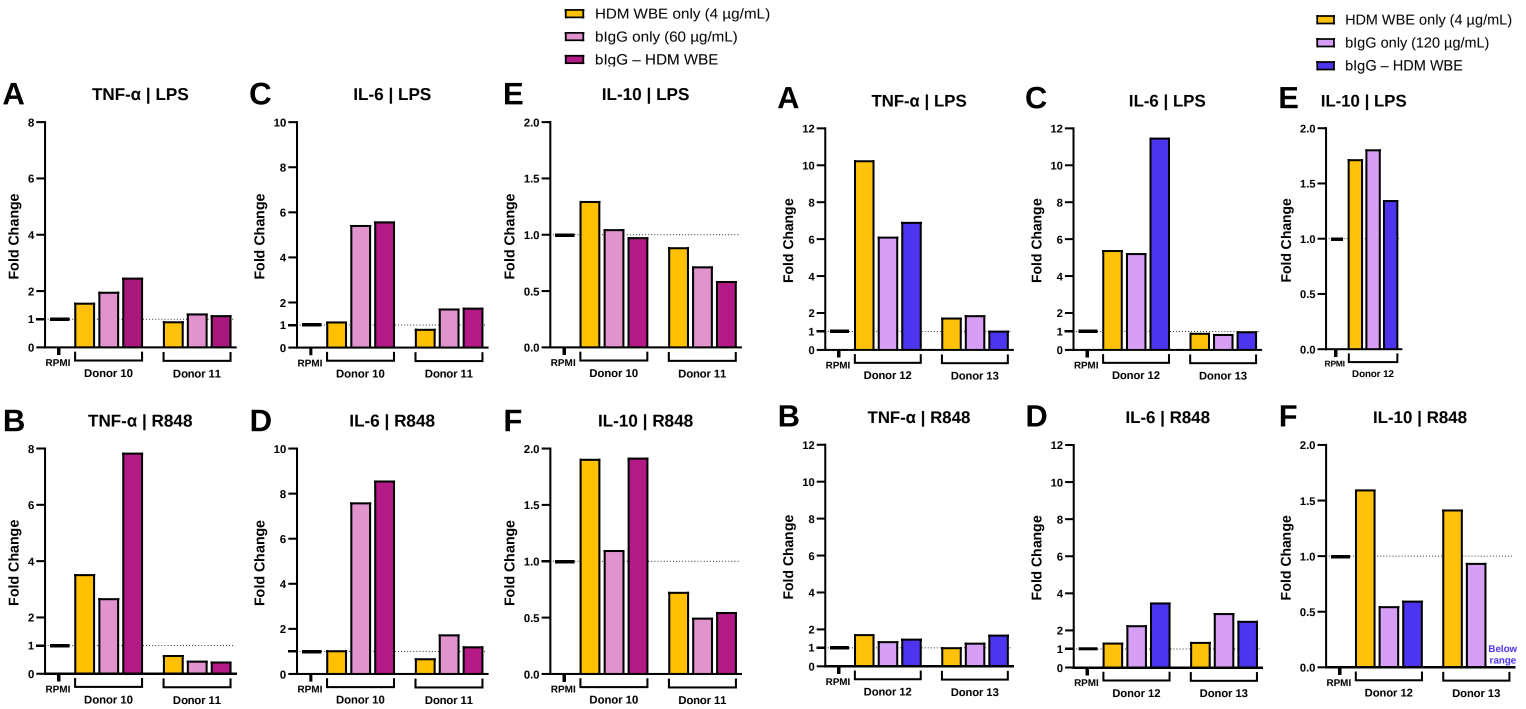

Figure 10. **The fold changes in TNF-α (A,B), IL-6 (C,D), and IL-10 (E,F) production of the cells trained with bIgG only (60 [magenta color scheme, left] or 120 [purple color scheme, right] µg/mL), HDM WBE only (4 µg/mL), and their corresponding bIgG–HDM WBE ICs relative to the RPMI+ control group.** The graph title denotes the stimuli used on day 7 (**A,C,E:** LPS and **B,D,F:** R848). The average cytokines levels in the RPMI controls of the donors tested were, as organized in order per graph: leftmost **A:** 6323 pg/mL, **B:** 18071 pg/mL, **C:** 1990 pg/mL, **D:** 7539 pg/mL, **E:** 355 pg/mL, and **F:** **481** pg/mL. Rightmost **A:** 302 pg/mL, **B:** 18141 pg/mL, **C:** 153 pg/mL, **D:** 10258 pg/mL, **E:** 60 pg/mL, and **F:** 542 pg/mL. The bar charts indicate the fold change value per donor due to a low sample size per condition.

4. Discussion

This study demonstrates that bIgG forms ICs with HDM allergens, particularly when using the WBE rather than isolated Der p1 or Der p2. These ICs bind to human monocytes via FcγRIIa, as evidenced by FcγRIIa-blocking experiments. However, the capacity of these ICs to induce trained immunity or tolerance in monocytes remains inconclusive due to limitations in sample size and high donor variability.

4.1 The Formation and Binding of bIgG–HDM Immune Complexes

The IC binding experiments yielded bell-shaped ΔMFI curves, in line with classic antibody–antigen lattice formation principles, where maximal IC formation occurs near molar equivalence. However, ICs formed from the combination of bIgG and single allergen Der p1 exhibited relatively low ΔMFI values, suggesting limited IC formation. A likely explanation for this is due to the polyclonal nature of the bIgG stock, which is isolated from the colostrum of a single cow and provides a heterologous mixture of IgG molecules specific to many different epitopes and antigens. The formation of ICs relies on the specific binding of bIgG to Der p1, however the presence of these Der p1-specific bIgG is, in this case, dependent on the exposure of the cow to HDM. In fact, cows are unlikely to be naturally exposed to HDM as these mites prefer an indoors microclimate and mainly feed on human skin scales (Bergmann, 2022).In contrast, cows are exposed to storage mites (SM), which mainly belong to the Acaridae and Glycyphagidae families and are highly present in straw, hay, and animal stables (Solarz et al., 2022). Since HDM and SM allergens share structural homology (Bessot & Pauli, 2011), SM-specific bIgG might cross-react with corresponding HDM allergens to form ICs. However, current evidence remains inconclusive, and the observed reactivity may simply represent co-sensitization rather than true cross-reactivity (Cuevas et al., 2022; Huppertz et al., 2020; Saridomichelakis et al., 2008; Sidenius et al., 2001; Zhang et al., 2012).

To address this limitation, a more established system was tested using whole-body HDM extract, which contains a variety of different allergens (Collins et al., 1991; Knol et al., 2024). The presence of more allergen types is expected to allow the polyclonal bIgG stock to target a broader range of allergens, potentially enhancing signal strength. In line with this hypothesis, the bIgG–HDM ICs bound strongly to $CD14^+$ monocytes, with a >20-fold higher ΔMFI peak than the bIgG–Der p1 condition. In contrast to using single allergens, a WBE may give rise to multiple ΔMFI peaks as it contains various HDM allergen types. To better understand these optima, the WBE mixture was incubated with increasing bIgG concentrations up to 960 µg/mL, but no additional ΔMFI peaks were observed in this range (Figure S3C,D). Despite the strong binding of bIgG–HDM WBE ICs, the specific HDM allergens contributing to this interaction remain undefined. Given the variability in allergen composition among different HDM WBEs (Casset et al., 2012), immunoblotting should be employed for further characterization of the HDM allergens targeted by bIgG.

Additionally, since the bIgG stock is isolated from the colostrum of a single cow, variations in allergen specificity between bIgG stocks may arise because mite exposure differs among cows. For instance, cows that spend more time in stables, particularly stables with high mite presence, are likely to have greater exposure and thus higher levels of allergen-specific bIgG compared to cows that are primarily outdoors (Virtanen et al., 2009). In this study, the bIgG stocks from three different cows were tested, but no consistent differences in IC formation were observed. Finally, next to the IC conditions, the bIgG-only condition also exhibited dose-dependent binding, however this could not be due to IC formation as no antigens were present. Instead, bIgG may form aggregates, particularly when stored in frozen storage for extended periods (Jain et al., 2021), that are known to bind FcγRIIa on monocytes (Hartog et al., 2014; Nederend et al., 2020; Porbahaie et al., 2022). This was addressed in these experiments by centrifuging the bIgG stock solution before use, a method shown to minimize aggregates and potentially reduce background MFI (Porbahaie et al., 2022).

Unlike monocytes, which express activating FcγRIIa, B cells solely express the inhibitory FcγRIIb (Bournazos et al., 2020). The balance between activating and inhibitory signals modulates immune functions, including the IgG production of B cells, maturation of antigen presenting cells (APC), and antigen processing (Wu et al., 2021). Data from this study indicated that $CD19^+$ B cells bound ICs formed with Der p1 or Der p2 allergens, but no IC binding was detected with the allergens present in the HDM WBE. This may be due to differences in the concentrations of Der p1 and Der p2 within the HDM WBE compared to their individual stocks, resulting in non-equivalent molar ratios that may have prevented IC formation. Nevertheless, strong bIgG–HDM WBE IC binding was observed in monocytes, and the high degree of homology (96%) between the extracellular regions of both FcγRII isoforms suggests comparable binding specificities (Rankin et al., 2006). Patel et al. (2019) reviewed numerous factors impacting FcγR-mediated mechanisms, including genetic variation, IC size, antigen properties, and post-translation modifications of FcγRs. Given the complexity of factors involved, additional data is required to fully understand B cell binding of bIgG–HDM WBE immune complexes.

4.2 bIgG–HDM Immune Complexes Engage Human Monocyte FcγRIIa

The specific blocking of FcyRIIa abolished the binding of both the bIgG–HDM ICs and the bIgG-only conditions, implicating their binding to FcγRIIa on $CD14^+$ monocytes. This is supported by the lack of reduction in MFI in FcγRIIb-expressing $CD19^+$ B cells under identical blocking conditions. While the formation and binding of bIgG–HDM ICs has not been shown before, binding of bIgG only to FcγRIIa is in line with previous studies (Hartog et al., 2014; Kramski et al., 2012; Nederend et al., 2020; Porbahaie et al., 2022). Contrary to the findings of this study, Den Hartog et al. (2014), using an α-FcyRIIa blocking antibody of the same clone, and Kramski et al. (2012) employing α-FcyRI/II/III, could only partially block the binding of bIgG to FcyRIIa in neutrophils and monocytes (30-60% reduction, resp.). Due to this incomplete blocking, a potential role for FcRn in bIgG binding was suggested by Porbahaie et al. (2022). While bIgG has been shown to bind FcRn to limited degree (Ober et al., 2001), the inhibited binding of bIgG only and the ICs as observed in this study, suggests that the monocyte FcyRIIa is the primary mediator of bIgG–HDM IC binding.

4.3 Inconsistent Training of bIgG–HDM ICs in a Model Requiring Optimization

Despite confirming IC binding, this study provides only early, limited observations and did not resolve whether bIgG–HDM ICs induce trained immunity or tolerance. The initial, exploratory analysis found that, while restimulation elicited sporadic donor-specific increases in TNF-α/IL-6 production, along with decreases in IL-10, levels, definitive trends were limited by a high donor variability, small sample sizes, elevated baseline cytokine levels, and inconsistent responses to the training controls. Indeed, a significant variability in training among donors has been noted before (Domínguez-Andrés et al., 2021).

Notably, the binding and crosslinking of multiple FcγRIIa receptors is critical for initiating ITAM-mediated signaling and downstream effector functions (Bournazos & Ravetch, 2017). However, allergen extracts like HDM WBE, may also contain nonallergenic materials and contaminants (e.g., microbial ligands, endotoxins) with inherent immunomodulatory or toxic effects that would confound the cytokine production of cells exposed to bIgG–HDM ICs (Jacquet, 2011; Valenta et al., 2018).

Furthermore, single HDM allergens may exhibit standalone immunomodulatory properties, independent of FcγRIIa. For instance, Der p2 structurally mimics the TLR4 co-receptor MD-2, enabling it to bind LPS, even at trace levels, and promote TLR4-dependent airway inflammation (Jacquet, 2011). Similarly, the protease activity of Der p1 disrupts epithelial barriers, potentially amplifying innate immune activation (Jacquet, 2011). In line with these allergen effects, the HDM WBE-only condition showed increased TNF-α, IL-6, and IL-10 production in some of the donors tested.

Importantly, Casset et al. (2012) found that commercially available HDM WBEs exhibit significant variability in allergen composition and concentration, often lacking key allergens and yielding inconsistent allergenic activity, which may lead to differences in bIgG–HDM ICs formation, binding, and their training potential across WBEs. These inconsistencies, combined with possible contamination in the WBE and the standalone effects of HDM allergens, may complicate the evaluation of IC-specific effects in immune training.

Moreover, the increased production of cytokines in several RPMI$^+$ control groups and the lack of consistent responses towards positive controls (e.g., curdlan, LPS) further underscores methodological limitations, such as a suboptimal training protocol. For instance, the elevated cytokine production in untrained cells may stem from pre-activation due to mechanical stress during cell handling (Evers et al., 2022), donor-specific factors (e.g., recent infections, baseline inflammation), or contaminants such as LPS (Chaiwut & Kasinrerk, 2022). Similarly, a reduced cell viability or cellular stress may exacerbate this by releasing damage-associated molecular patterns (DAMPs) from dying cells, activating innate immune cells (Ito, 2014; Naqvi et al., 2022).

Additionally, a low purity (i.e., <90%) isolation of monocytes enables immune crosstalk with other leukocytes, including lymphocytes (Charron et al., 2015; Mueller et al., 2007), influencing cytokine production and misrepresenting training results. In this study, the monocyte purity in the trained immunity experiments was approximately 70-80%. While the resting and washing of the cells typically improves purity (Zhou et al., 2012), residual lymphocyte contamination may have persisted, potentially contributing to increased cytokine background levels.

Taken together, while it is known that cytokine production may vary considerably among donors, individual allergens or contaminants, as well as cellular stress and the potential for immune crosstalk in low-purity monocyte cultures, may have inherent immunomodulatory effects that would obscure IC-specific training effects. Accounting for these confounding variables through model optimization will clarify the role of bIgG–HDM ICs in trained immunity.

4.4 Recommendations for the Training Model

To strengthen the validity of the trained immunity model, several optimizations are recommended to address the limitations identified in this study.

Firstly, Increasing the sample size will mitigate donor variability by reducing the standard error of the sampling distribution, as described by the central limit theorem (Kwak & Kim, 2017). Minimizing background cytokine production requires appropriate handling of the cells to prevent pre-activation and cellular stress. While this study supplemented cultures with 10% human serum to promote viability and differentiation, Domínguez-Andrés et al. (2021) recommends titrating human serum (0%–10%) to avoid serum-mediated interactions with stimuli. Additionally, integrating an LDH cytotoxicity assay will enable the normalization of cytokine measurements to cell viability and density, improving the reliability of results. Given the inconsistent training effect observed with curdlan, whole glucan particle, another well-characterized Dectin-1 agonist, may serve as a more reliable inducer of trained immunity (Braian et al., 2023; Moerings et al., 2021; Porbahaie et al., 2022). Cross-validating agonists across suppliers and batches will further control for reagent variability. Commercially available HDM WBEs may not contain all major HDM allergens and could introduce contaminants such as microbial ligands. To improve specificity, HDM allergens should be purified and the optimal single-allergen–bIgG ratios for IC formation should be established. Combining these single allergens at defined optimal ratios would create a contamination-free HDM allergen mixture containing all major allergens. Finally, the low-purity monocyte cultures may have allowed immune crosstalk with residual leukocytes, which could have influenced cytokine production, potentially masking the effects of training. The recent acquisition of a magnetic cell separator will achieve high monocyte purity (>95%), ensuring that the observed training effects are specific to individual stimuli in monocytes.

Together, the above optimizations will enhance the validity of the trained immunity model and resolve the training potential of bIgG–HDM ICs.

4.5 Further Research

Optimizing the trained immunity model will enable the evaluation of the training potential of bIgG–HDM ICs. Hypothetically, if a training or tolerogenic effect is demonstrated, the mechanism may be explored further by analyzing key epigenetic modifications associated with trained immunity, specifically histone acetylation at lysine 27 (H3K27Ac) and methylation at lysine 4 (H3K4me1/me3) (van der Heijden et al., 2018; Vuscan et al., 2024). Furthermore, epigenetic modifications and metabolic changes are closely linked, and together they drive the induction of trained immunity (Ferreira et al., 2024). The metabolic changes associated with training are characterized by focusing on pathways such as glycolysis, oxidative phosphorylation, the tricarboxylic acid cycle, and lipid metabolism.

The potential implications of this research are significant. If bIgG–HDM ICs are shown to induce trained immunity, these studies could provide insights into how the inclusion of bIgG in early-life diets may contribute to preventing allergic asthma or even affect ongoing asthma (Ulfman et al., 2018). Similarly, maternal vaccination with HDM allergens may promote tolerance to these asthma-associated allergens in the breastfed child (Mosconi et al., 2010; Verhasselt, 2015; Verhasselt et al., 2008).

Assuming a training/tolerizing effect, it will also be interesting to determine whether bIgG–HDM ICs can induce trained immunity or tolerance in monocytes derived from patients with existing allergic asthma. Hypothetically, these ICs might reprogram monocytes and possibly attenuate chronic airway inflammation (Wang et al., 2019). While the possibility of leveraging trained immunity or tolerance to reprogram monocytes from a pathological to a more balanced phenotype in allergic asthma patients presents an intriguing avenue, much more research is needed to further explore this hypothesis.

If bIgG–HDM ICs do not induce trained immunity or tolerance, other bioactive components of bovine milk could be explored for their potential contribution to the observed protection against allergic asthma.

5. Conclusion

In conclusion, this study demonstrates that bIgG forms large ICs with HDM allergens that bind to CD14+ monocytes via FcγRIIa. However, IC formation was limited with single allergens (Der p1 or p2), likely due to the polyclonal nature of the bIgG stock favoring multivalent interactions with a multi-allergen HDM extract. While bIgG–Der p1 ICs showed moderate binding, bIgG–HDM WBE ICs exhibited a >20-fold increase in FcγRIIa engagement in CD14+ monocytes. Crucially, FcγRIIa blocking abolished IC binding, suggesting a central role of FcγRIIa in binding and mediating potential IC effects.

The capacity of bIgG–HDM ICs to induce trained immunity remains unresolved. The initial, exploratory analysis found that, while restimulation elicited sporadic donor-specific increases in TNF-α/IL-6 production, along with decreases in IL-10, levels, definitive trends were limited by a high donor variability, small sample sizes, elevated baseline cytokine levels, and inconsistent responses to the training controls. Further optimization of the training model will address these limitations.

If a training or tolerogenic effect is demonstrated, future research should investigate key epigenetic markers and the metabolic reprogramming driving this response. Findings could reveal how early-life dietary bIgG or maternal HDM vaccination may contribute to preventing allergic asthma. This approach will clarify the role of bIgG–HDM ICs in trained immunity and potentially strengthen mechanistic links to the epidemiological association between raw milk consumption and asthma protection.

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Supplementary Data

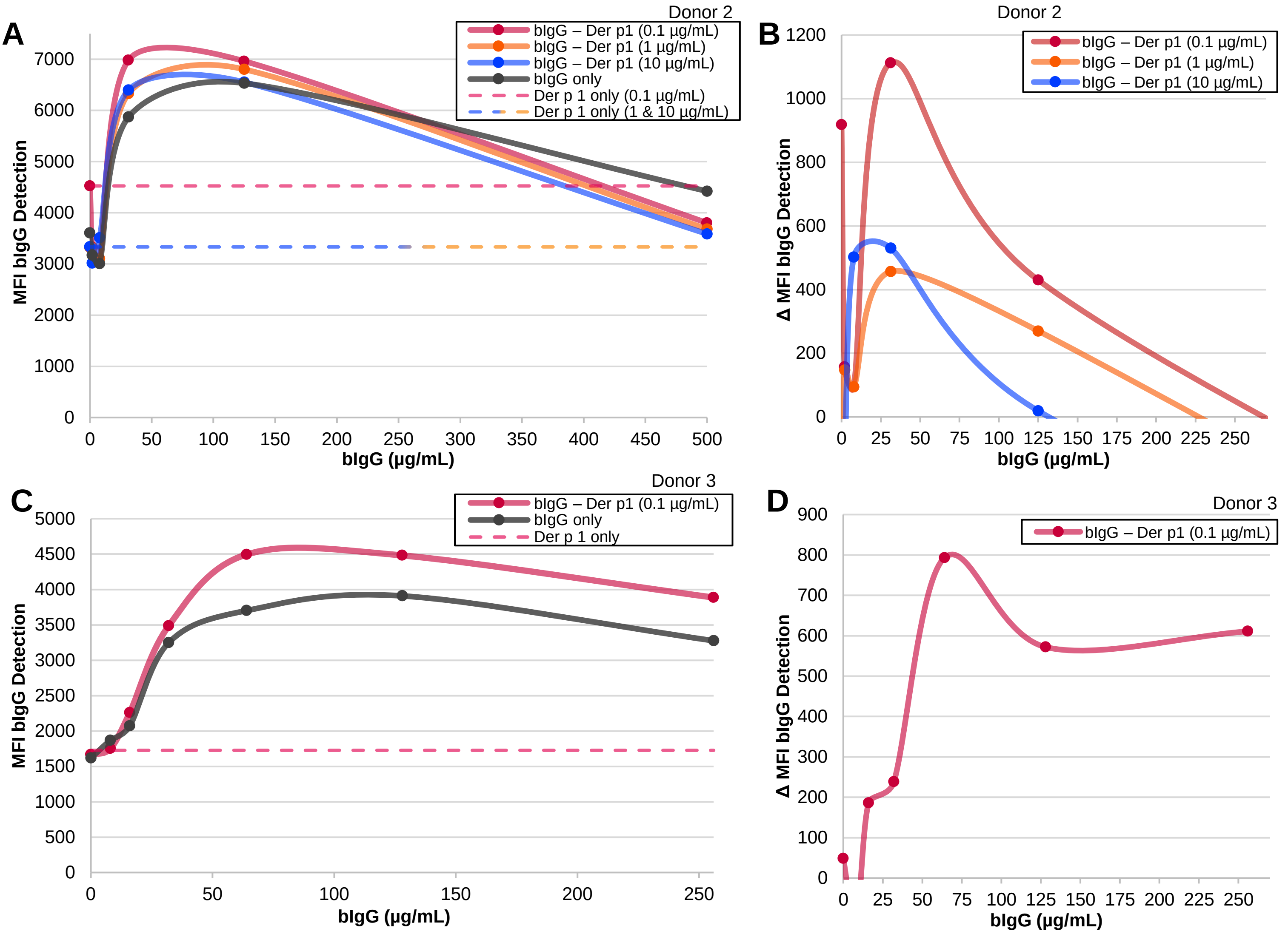

Figure S1. **MFI curves of the bIgG detection signal on CD14⁺ monocytes from two additional donors.** PBMCs were exposed to bIgG only (0–512 µg/mL), Der p 1 protein only (0.1, 1, and 10 µg/mL), and the corresponding bIgG – Der p 1 ICs in two representative donors 2 & 3. The data was derived from the bIgG MFI signal gated on CD14⁺ monocytes (**A&C**). The "Der p1 only (1 & 10 µg/mL)" curve represents two separate curves for each concentration. The delta MFI (ΔMFI) curve was obtained by substracting the bIgG only MFI from the bIgG – Der p 1 MFI (**B&D**).

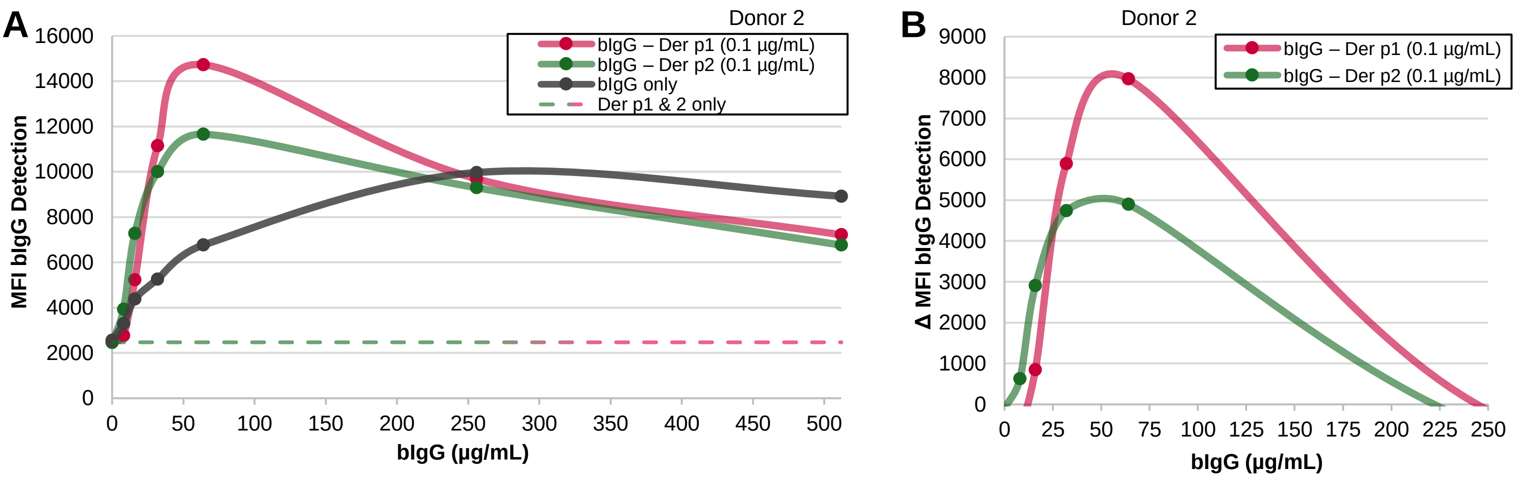

Figure S2. **MFI curves of the bIgG detection signal on CD19⁺ B cells.** PBMCs were exposed to bIgG only (0–512 µg/mL), Der p 1 protein only (0.1 µg/mL), Der p 2 protein only (0.1 µg/mL), and the corresponding bIgG – Der p 1 and bIgG – Der p 2 ICs in a representative donor 2. The detection curves were derived from the bIgG MFI signal gated on CD19⁺ B cells (**A**), the "Der p1 & 2 only" curve represents two separate curves of the 'allergen only' MFI. The delta MFI (ΔMFI) curves were obtained by substracting the bIgG only MFI signal from the bIgG – Der p 1 and bIgG – Der p 2 MFI signal separately (**B**).

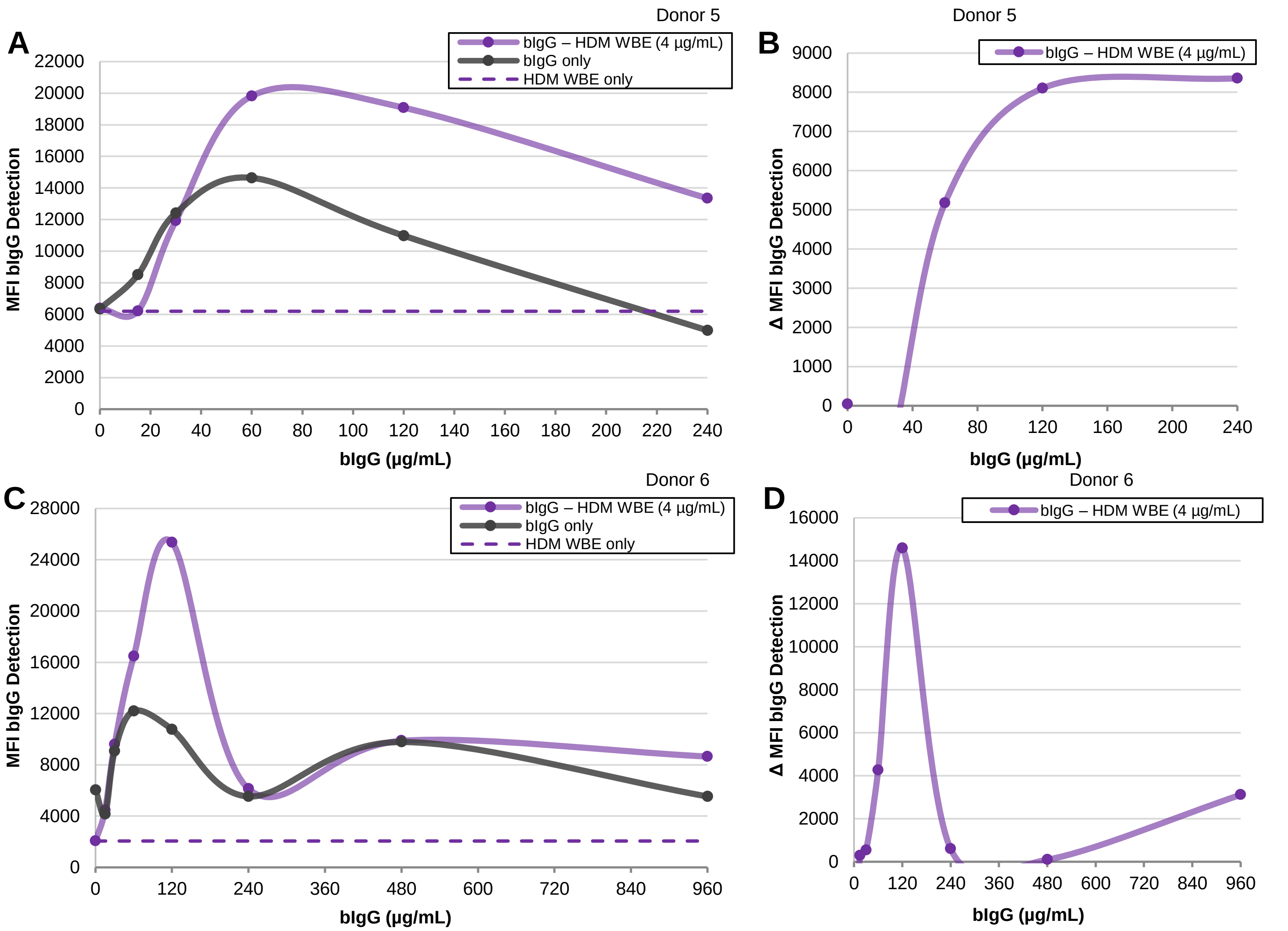

Figure S3. **MFI curves of the bIgG detection signal on CD14⁺ monocytes from two additional donors.** PBMCs were exposed to bIgG only (0–960 µg/mL), house dust mite whole body extract (HDM WBE) only (4 µg/mL), and the corresponding bIgG – HDM WBE ICs in two additional representative donors (donor 5 & 6). The detection curves were derived from the bIgG MFI signal gated on CD14⁺ monocytes (**A&C**). The delta MFI (ΔMFI) curve was obtained by substracting the bIgG only MFI signal from the bIgG – HDM WBE MFI signal (**B&D**).

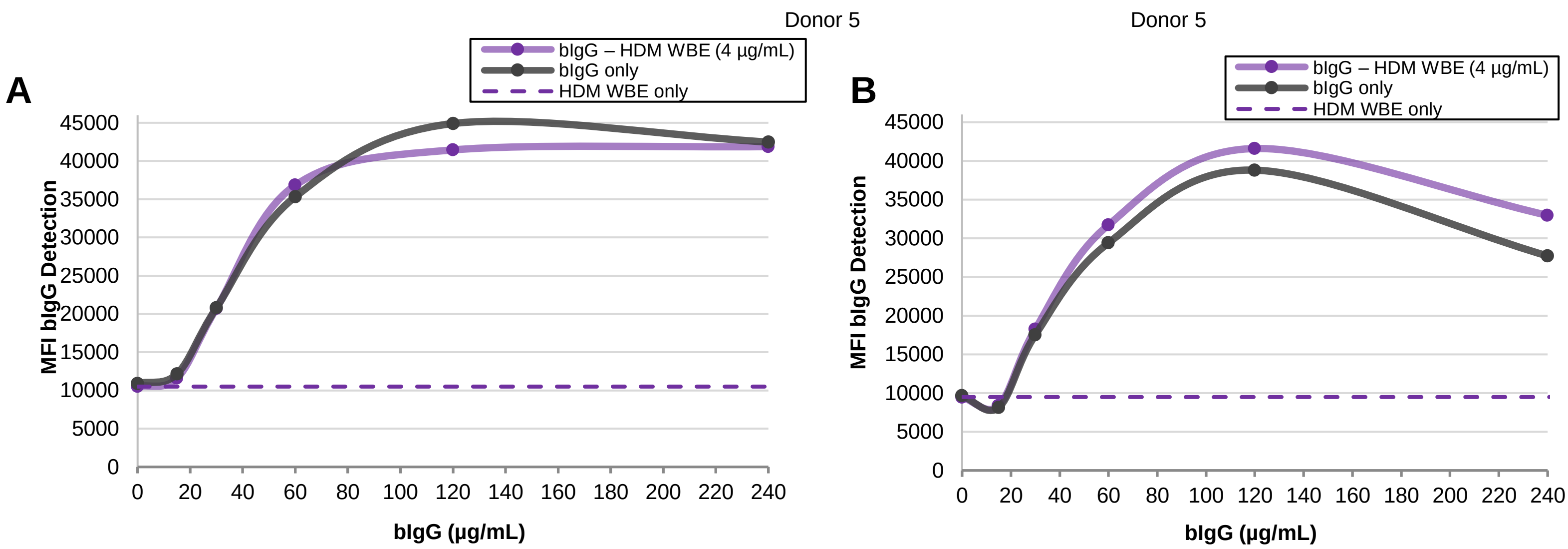

Figure S4. **MFI curves of the bIgG detection signal on regular (A) and CD32a-blocked (B) CD19⁺ B cells.** PBMCs were incubated with and without α-CD32a prior to being exposed to bIgG only (0–240 µg/mL), house dust mite whole body extract (HDM WBE) only (4 µg/mL), and the corresponding bIgG – HDM WBE ICs in donor 5. The detection curves were derived from the bIgG MFI signal gated on CD19⁺ B cells.

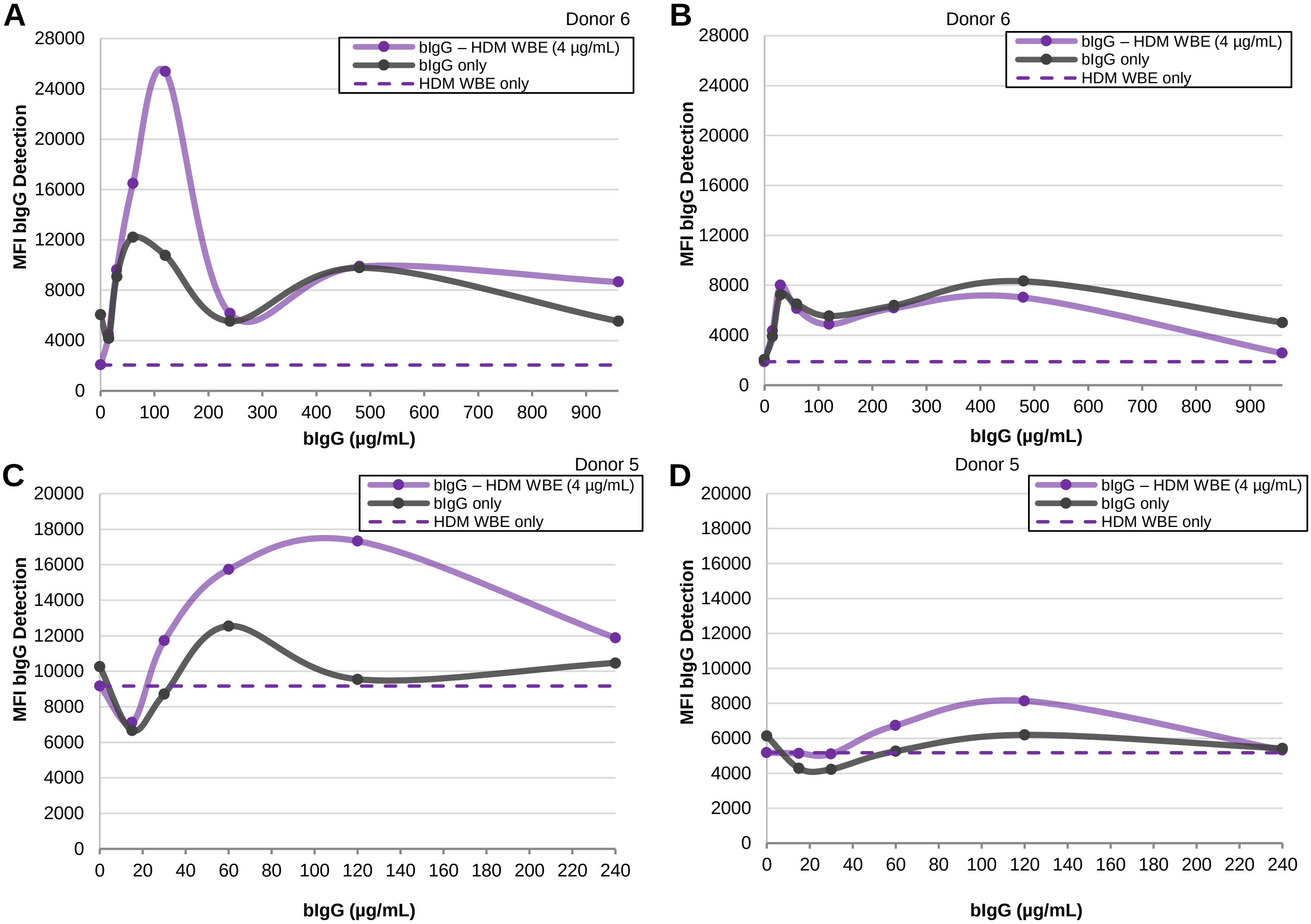

Figure S5. **MFI curves of the bIgG detection signal on regular (A&C) and CD32a-blocked (B&D) CD14⁺ monocytes.** PBMCs were incubated with and without α-CD32a prior to being exposed to bIgG only (0–960 µg/mL), house dust mite whole body extract (HDM WBE) only (4 µg/mL), and the bIgG – HDM WBE ICs in two donors (donor 5 & 6). PBMCs obtained from donor 5 were used for two separate IC binding experiments using different bIgG stocks (bIgG-3 for Figure 3; and bIgG-1 for Figure S5C-D). The detection curves were derived from the bIgG MFI signal gated on CD14⁺ monocytes.

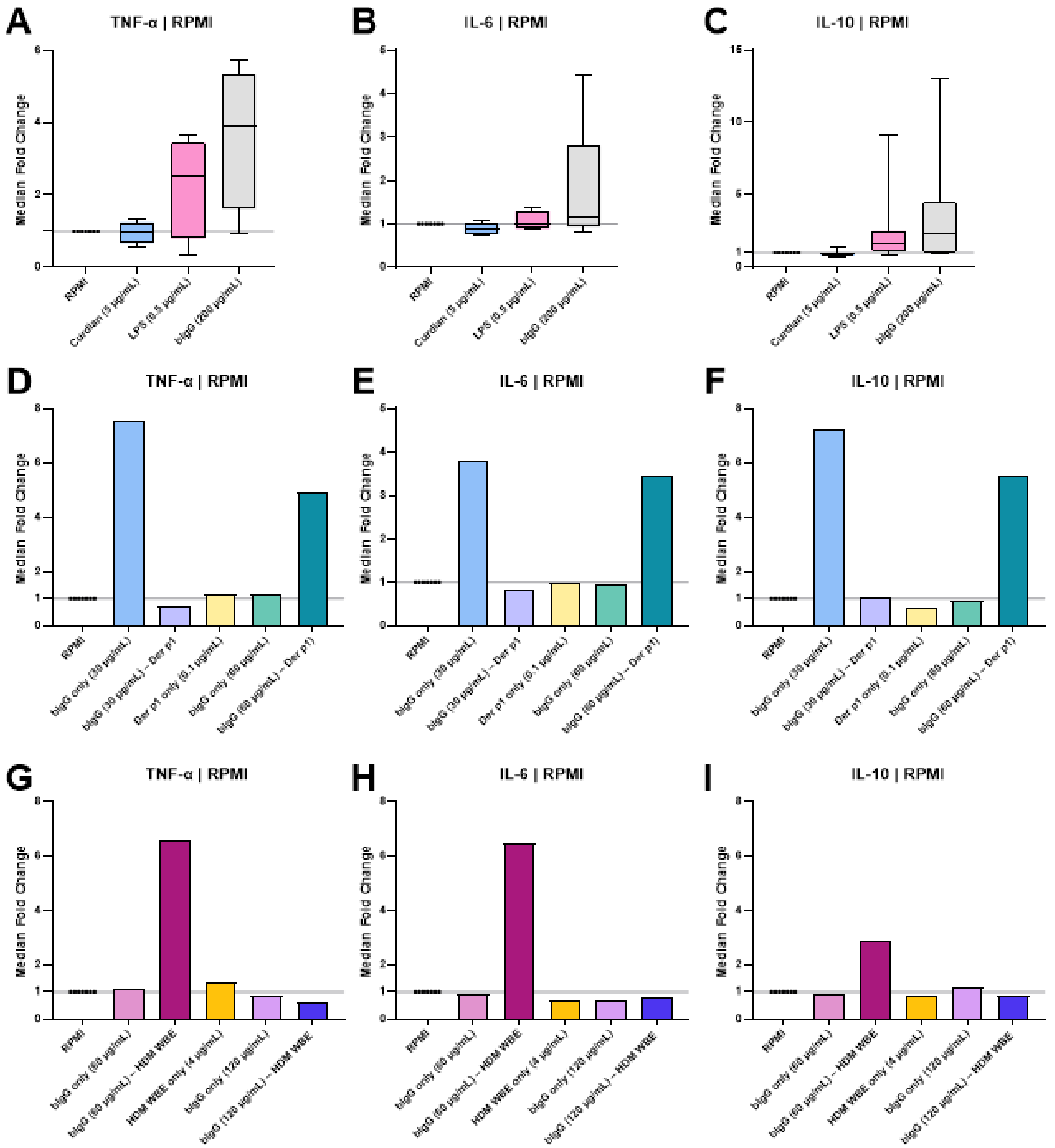

Figure S6. **The fold changes in TNF-α (A,D,G), IL-6 (B,E,H), and IL-10 (C,F,I) production of the cells trained with Curdlan (5 µg/mL), LPS (0.5 µg/mL), aggregated bIgG (200 µg/mL), bIgG only (30 µg/mL), bIgG–Der p1, Der p1 only (0.1 µg/mL), bIgG only (60 µg/mL), bIgG–Der p1, HDM WBE only (4 µg/mL) and BIgG–HDM WBE relative to the RPMI⁺ control group when not stimulated on day 7.** The average (min–max range) of cytokines in the RPMI controls of all the donors tested were, as organized by graph: **A,D,G:** 357 (14–896 pg/mL), **B,E,H:** 175 (20–297 pg/mL), **C,F,I:** 48 (8–89 pg/mL). The Box and Whisker Plots (**A-C**) represent 50% of the data, and the line within the box indicates the median value, while the upper and lower whiskers denote the upper and lower 25% of the data, respectively. The bar charts (**D-I**) indicate median values because a low number of donors (2-3) were used per condition.

Table S1. **The fold changes in TNF-α, IL-6, and IL-10 production of the cells trained with various stimuli (see column headings) relative to the RPMI⁺ control group.**

TNF-a	Donor	RPMI	Curdlan (5 μg/mL)	LPS (0.5 μg/mL)	bIgG (200 μg/mL)	bIgG only (30 μg/mL)	bIgG only (60 μg/mL)	Der p1 only (0.1 μg/mL)	bIgG (30 μg/mL) – Der p1	bIgG (60 μg/mL) – Der p1)	bIgG only (120 μg/mL)	HDM WBE only (4 μg/mL)	bIgG (60 μg/mL) – HDM WBE	bIgG (120 μg/mL) – HDM WBE
RPMI	Donor 1	1	0.57	0.34	0.93	7.56			0.74					
	Donor 2	1	1.33	2.84	4.17		1.78	2.07		9.63		7.44	13.04	
	Donor 3	1	0.96	2.18	3.65		0.52	0.26		0.25		0.24	0.16	
	Donor 4	1	0.99	3.65	5.71						0.89	1.35		0.61
LPS	Donor 1	1	0.85	0.35	0.07		0.3	0.7		0.65				
	Donor 2	1	1.01	0.12	0.37	2.68		1.01	5.84					
	Donor 3	1	0.67	0.36	0.26	2.26		0.77	1.48					
	Donor 4	1	1.3	1.12	1.16		1.98	1.49		2.5		1.59	2.48	
	Donor 5	1	1.1	0.76	0.78		1.21	1.34		1.21		0.93	1.15	
	Donor 6	1	1.08	3.07	15.14						6.14	10.28		6.94
	Donor 7	1	1.23	4.86	18.96						1.89	1.76		1.05
R848	Donor 1	1	0.99	2.18	0.22		1.15	2.12		2.87				
	Donor 2	1	1.29	0.71	1.22	3.33		1.42	9.97					
	Donor 3	1	1.36	0.51	0.6	2.47		0.87	1.52					
	Donor 4	1	1.19	2.62	2.07		2.69	2.49		6.43		3.54	7.86	
	Donor 5	1	0.9	0.57	0.28		0.47	0.8		0.45		0.67	0.44	
	Donor 6	1	1.6	0.93	1.9						1.37	1.75		1.5
	Donor 7	1	0.83	0.72	2.14						1.29	1.04		1.72

IL-6	Donor	RPMI	Curdlan (5 μg/mL)	LPS (0.5 μg/mL)	bIgG (200 μg/mL)	bIgG only (30 μg/mL)	bIgG only (60 μg/mL)	Der p1 only (0.1 μg/mL)	bIgG (30 μg/mL) – Der p1	bIgG (60 μg/mL) – Der p1)	bIgG only (120 μg/mL)	HDM WBE only (4 μg/mL)	bIgG (60 μg/mL) – HDM WBE	bIgG (120 μg/mL) – HDM WBE
RPMI	Donor 1	1	0.73	1.38	4.43	3.82		1	0.86					
	Donor 2	1	1.06	1	1.14		0.97	1.24		6.13		1.25	12.22	
	Donor 3	1	1	1.21	1.04		0.92	0.87		0.79		0.68	0.74	
	Donor 4	1	0.78	0.88	0.81						0.63	0.54		0.92
	Donor 5	1	0.88	0.9	1.16						0.82	0.75		0.7
	Donor 6	1												
LPS	Donor 1	1	0.9	0.82	3.86		1.82	1		2.51				
	Donor 2	1	1.03	0.66	1.9	1.12		0.91	2.11					
	Donor 3	1	0.98	1.17	1.66	1.19		1.21	1.01					
	Donor 4	1	1.02	0.62	1.52		5.44	1.14		5.3		1.16	5.6	
	Donor 5	1	0.91	0.41	0.43		1.74	1.22		1.86		0.84	1.78	
	Donor 6	1	1.01	1.03	1.34						5.25	5.41		11.5
	Donor 7	1	1.08	0.71	1.08						0.87	0.93		1.01
R848	Donor 1	1	1.02	1.21	5.09	1.15		0.99	1.78					
	Donor 2	1	1.17	1.63	2.34	1.3		0.94	1.2					
	Donor 3	1	0.8	0.69	2.16		7.62	0.53		4.73		1.06	8.59	

	Donor 4	1	0.89	0.53	0.12		1.76	0.75		1.36		0.7	1.23	
	Donor 5	1	1.12	0.56	0.43						2.29	1.35		3.51
	Donor 6	1	1	0.5	0.4						2.94	1.39		2.52

IL-10	Donor	RPMI	Curdlan (5 µg/mL)	LPS (0.5 µg/mL)	bIgG (200 µg/mL)	bIgG only (30 µg/mL)	bIgG only (60 µg/mL)	Der p1 only (0.1 µg/mL)	bIgG (30 µg/mL) – Der p1	bIgG (60 µg/mL) – Der p1)	bIgG only (120 µg/mL)	HDM WBE only (4 µg/mL)	bIgG (60 µg/mL) – HDM WBE	bIgG (120 µg/mL) – HDM WBE
RPMI	Donor 1	1	0.7	9.13	13.01		6.06	0.62		7.73				
	Donor 2	1	1.33	2.33	3.66	1.64		1.13	1.31					
	Donor 3	1	0.95	0.87	4.52	12.86		0.61	0.75					
	Donor 4	1	0.8	2.53	1.9		0.93	0.7		5.51		5.64	5.18	
	Donor 5	1	0.95	1.56	1.02		0.83	0.73		0.72		0.53	0.63	
	Donor 6	1	0.87	1.11	0.94						0.96	0.83		1
	Donor 7	1	0.88	1.42	2.29						1.45	0.87		0.77
LPS	Donor 1	1	0.86	0.35	0.45		0.31	0.78		0.4				
	Donor 2	1	0.87	0.53	0.19	1.11		0.86	0.94					
	Donor 3	1	0.91	0.71	0.6	1.2		0.96	1.09					
	Donor 4	1	1.12	0.87	0.54		1.05	1.24		1.1		1.3	0.98	
	Donor 5	1	0.91	0.82	0.33		0.72	0.97		0.73		0.89	0.59	
	Donor 6	1	1.03	1.87	1.65						1.81	1.72		1.35
R848	Donor 1	1	1.28	0.62	0.87		0.67	1.1		0.77				
	Donor 2	1	0.83	0.72	0.42	0.96		1.11	0.75					
	Donor 3	1	0.45	0.84	0.95	1.3		1.08	1.14					
	Donor 4	1	1.08	1.68	0.65		1.1	1.58		1.73		1.91	1.92	
	Donor 5	1	0.92	0.7	0.3		0.5	0.86		0.38		0.73	0.55	
	Donor 6	1	1.32	1.17	0.43						0.55	1.6		0.6
	Donor 7	1	0.81	1.11	1.07						0.94	1.41		

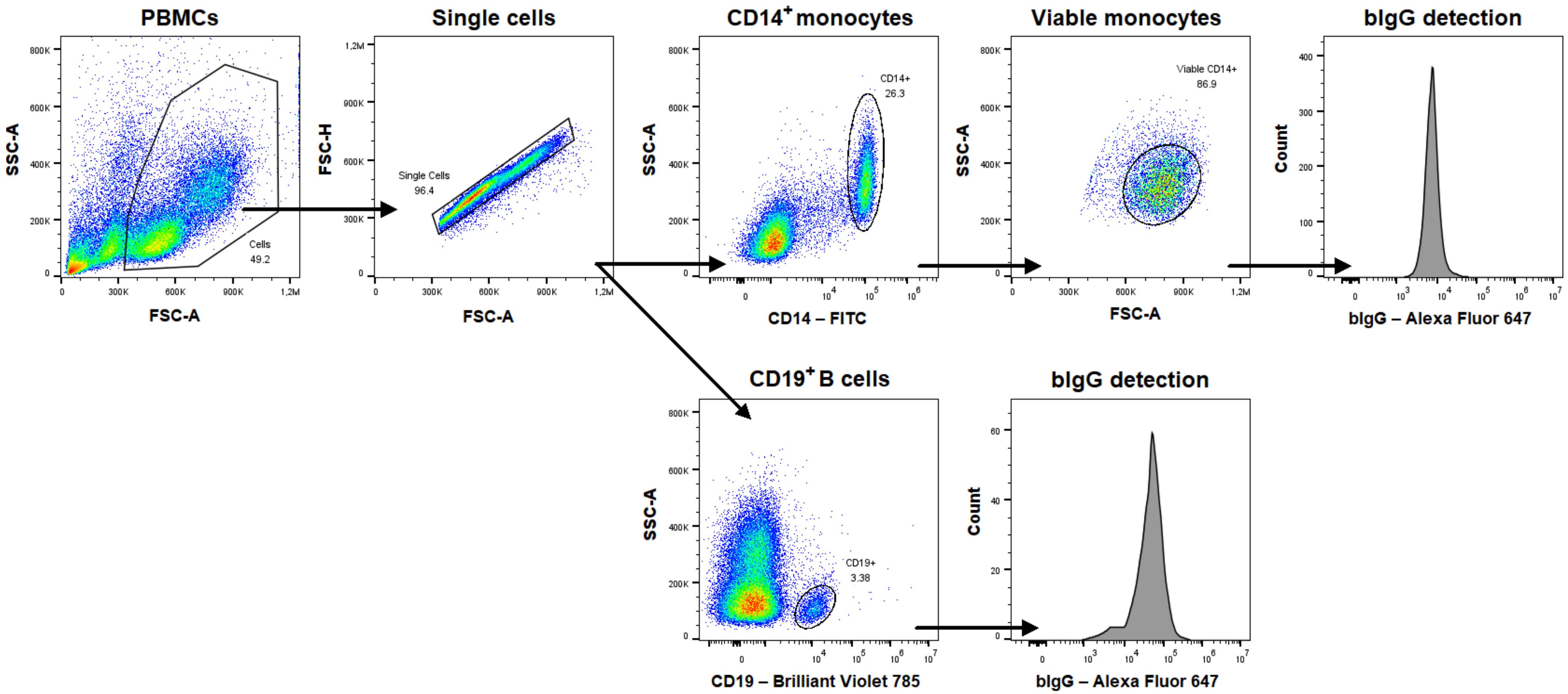

Figure S7. **The gating strategy to quantify bound bIgG on CD14+ monocytes and CD19+ B cells.**

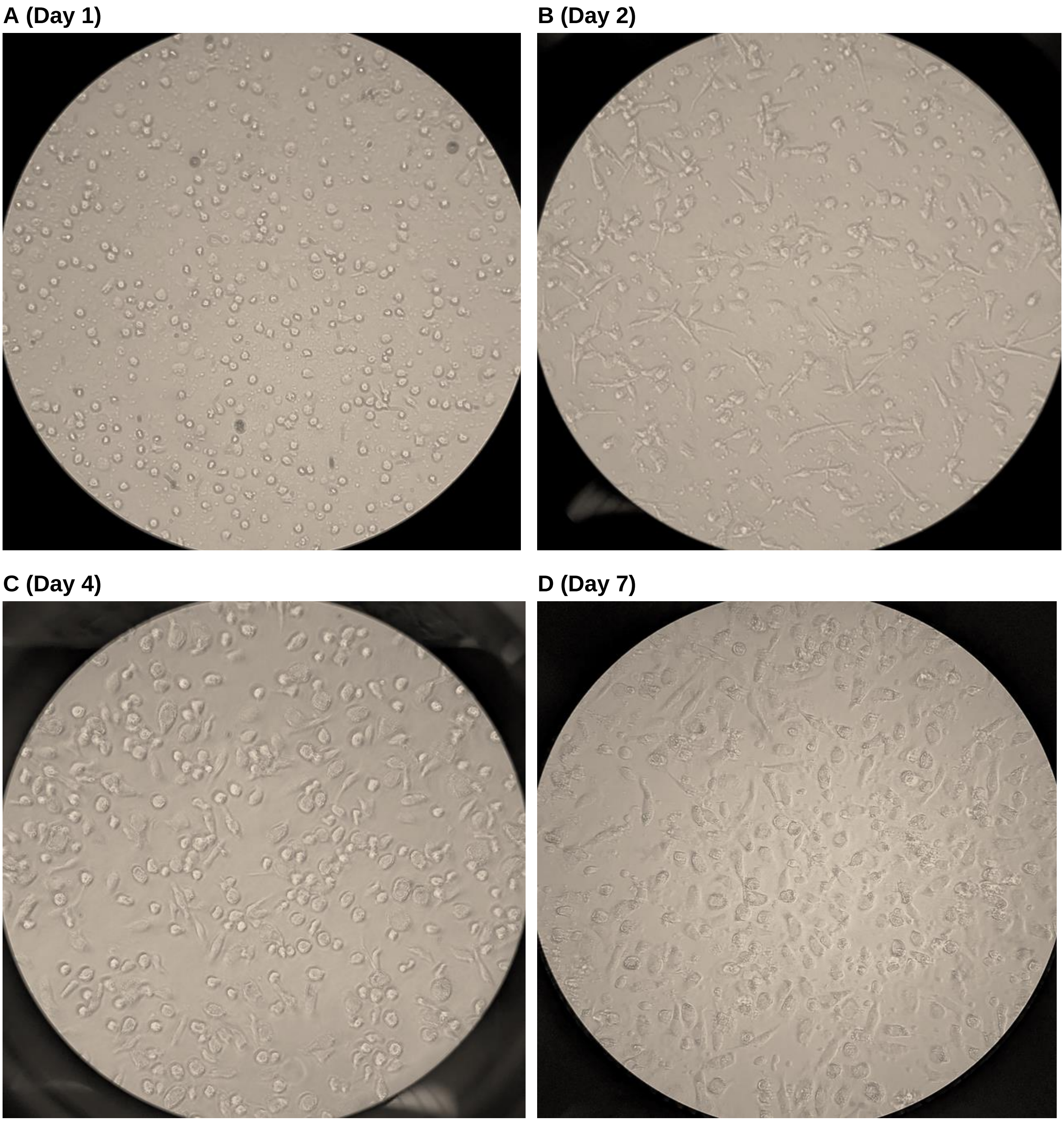

Figure S8. **Differentiation of monocytes into macrophages during the training protocol.** Representative microscopy images of stimulated $CD14^+$ monocytes cultured in $RPMI^+$ with 10% human serum on day 1, 2, 4, and 7. The human serum contains factors such as GM-CSF that drive differentiation into macrophages. Microscopy images confirmed small, mainly round monocytes on day 1. On the following days, the cells exhibited progressive morphological changes consistent with macrophage differentiation, including increased cell size and granularity, associated with ‘fried-egg’ like M1-like macrophages, and stretched, spindle-like cells representing M2-like macrophages (Italiani & Boraschi, 2014).

Human PBMC isolation and monocyte (-) selection from buffy coats using autoMACS Neo

Monocyte negative selection protocol (manual magnetic labeling)

- Required reagents:
    - Pan Monocyte Isolation Kit (#130-096-537)
    - MACS separation buffer (#130-091-221)

- Notes before start:
    - Store the MACS Chill Racks in the refrigerator until the coolant is switched to a solid state.
    - Adjust all Buffer and samples to room temperature before use.
    - Volumes given below are for **10^7 cells**. Adjust the volume based on the cell count.

1. Prepare cells (PBMCs) and determine cell number.
2. Resuspend the cell pellet in **30 µL** of separation buffer per 10^7 total cells.
3. Add **10 µL** of FcR Blocking Reagent per 10^7 total cells.
4. Add **10 µL** of the Biotin-Antibody Cocktail per 10^7 total cells.
5. Mix well and incubate for 5 minutes in the refrigerator (2–8 °C).
6. Add **30 µL** of the separation buffer per 10^7 total cells.
7. Add **20 µL** of the Anti-Biotin MicroBeads per 10^7 total cells.
8. Mix well and incubate for 10 minutes in the refrigerator (2–8 °C).
9. Place the sample(s) and empty 15mL tubes in the Chill Rack.
    - position A = sample
    - position B = unlabeled (negative) fraction
    - position C = labeled (positive) fraction
10. Prepare and prime the instrument.
11. Place the 15mL Chill Rack on the MACS MiniSampler S.
12. Select the same Chill Rack in the Experiment tab. An experiment is created automatically.
13. Tap to select sample positions and name the sample(s).
14. Assign the reagent by scanning the barcode or manually selecting the **Pan monocyte isolation kit**.
15. Tap Labelling in the reagent placement dialog and select **Manual**.
16. Enter the sample volume into the Volume submenu and press Enter.
17. Select Run and collect enriched monocytes at position B (negative fraction).

Figure S9. **Negative selection protocol for isolating monocytes using the autoMACS and manual magnetic labeling.**