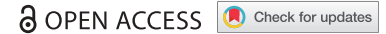


REPORTS



## Important roles of CD32 in promoting suppression of IL-4 induced immune responses by a novel anti-IL-4R $\alpha$ therapeutic antibody

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### ABSTRACT

Asthma is characterized by airway hyperresponsiveness and inflammation, as well as underlying structural changes to the airways. Interleukin-4 (IL-4) is a key T-helper type 2 (Th2) cytokine that plays important roles in the pathogenesis of atopic and eosinophilic asthma. We developed a novel humanized anti-IL-4R $\alpha$  antibody that can potently inhibit IL-4/IL-13-mediated TF-1 cell proliferation. Using monocytes isolated from human peripheral blood mononuclear cells (PBMCs), we revealed a critical role of CD32 in modulating the immune responses of monocytes in response to blockade of IL-4R $\alpha$  signaling pathway. We, therefore, devised a new strategy to increase the efficacy of the anti-IL-4R $\alpha$  monoclonal antibody for the treatment of asthma and other atopic diseases by co-engaging CD32 and IL-4R $\alpha$  on monocytic cells by choosing IgG classes or Fc mutations with higher affinities for CD32. The antibody with selectively enhanced affinity for CD32A displayed superior suppression of IL-4-induced monocytes' activities, including the down-regulation of CD23 expression. Intriguingly, further analysis demonstrated that both CD32A and CD32B contributed to the enhancement of antibody-mediated suppression of CD23 expression from monocytes in response to blockade of IL-4R $\alpha$  signaling. Furthermore, inhibition of IgE secretion from human PBMC by the antibody variants further suggests that the complex allergic inflammation mediated by IL-4/IL-4R $\alpha$  signaling might result from a global network where multiple cell types that express multiple Fc $\gamma$ Rs are all involved, of which CD32, especially CD32A, is a key mediator. In this respect, our study provides new insights into designing therapeutic antibodies for targeting Th2 cytokine-mediated allergic pathogenesis.

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### Introduction

Asthma is characterized by airway hyper-responsiveness (AHR) and inflammation, as well as underlying structural changes to the airways. Asthma affects an estimated 241 million children and adults worldwide, of which approximately 5–10% is severe asthma.<sup>1,2</sup>


Interleukin-4 (IL-4) and interleukin-13 (IL-13) are major T-helper type 2 (Th2) cytokines that play important roles in the pathogenesis of atopic and eosinophilic asthma. IL-4 plays a key role in regulating naïve T cells to develop into Th2-like cells capable of producing cytokines such as IL-4, IL-13, and IL-5. IL-4 also determines the class switch of human B cells to expression of IgE and IgG4 and mouse B cells to IgE and IgG1. In addition, IL-4 upregulates major histocompatibility complex class II (MHCII) and CD23 expression, as well as the expression of the IL-4 receptor on B cells and myeloid lineage cells, such as monocytic cells.<sup>3</sup> IL-4 receptors (IL-4R) are present in a broad spectrum of tissues, including hematopoietic, endothelial, epithelial, muscle, fibroblast, hepatocyte, and brain tissues. The receptor consists of a 140 kDa IL-4R $\alpha$  chain that binds IL-4 with high affinity (KD 20 to 300 pM); this chain is shared with IL-13. There are two types of receptor complexes centered on IL-4R $\alpha$ , type I: IL-4R $\alpha$ / $\gamma$ c and type

II: IL-4R $\alpha$ /IL-13R $\alpha$ 1. The binding of IL-4 to IL-4R $\alpha$ , in the type I complex, induces dimerization with the common  $\gamma$ c chain ( $\gamma$ c), which then activates JAK1 and JAK3. Activated JAKs result in the phosphorylation and activation of STAT-6. Phosphorylated STAT-6 dimers translocate to the nucleus and activate the transcription of genes, including CD23 and MHCII in B cells, IL-4, and IL-13 in T cells, and eotaxin in fibroblasts.<sup>3</sup>

Multiple lines of evidence support the role of IL-4/IL-13 pathway in promoting asthma and the atopic syndrome. In animal models, blockade of the IL-4/IL-13 pathway through the use of antibodies or antagonists of IL-4/IL-13 and IL-4R $\alpha$  have been shown to reduce AHR, airway inflammation, mucus overproduction, eosinophil recruitment, and airway remodeling.<sup>1</sup> In clinical studies, patients on dupilumab, an anti-IL-4R $\alpha$  monoclonal antibody (mAb) that targets IL-4R $\alpha$ , thereby inhibiting the binding of both IL-4 and IL-13, showed an 87% reduction of the asthma exacerbation, improved forced expiratory volume in 1 s (FEV<sub>1</sub>) and improvements in quality of life.<sup>2,4</sup> Taken together, the results suggest that targeting the IL-4/IL-13 signaling pathway, especially with antibodies, could provide additional therapeutic means to treat asthma and other atopic diseases.

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The cytotoxic functions mediated by the Fc domain of therapeutic antibodies via binding to Fc $\alpha$  receptors, for instance, antibody-dependent cell-mediated cytotoxicity, and antibody-dependent cellular phagocytosis, are crucial for their therapeutic efficacies.<sup>5-8</sup> Four human subclasses of IgG (IgG1, IgG2, IgG3, IgG4) are produced in response to various antigens. Correspondingly, there are two classes of Fc $\gamma$ Rs exhibiting opposite effector functions: 1) the activating receptors (hFc $\gamma$ RI, hFc $\gamma$ RIIA, hFc $\gamma$ RIIC, Fc $\gamma$ RIIIA, and Fc $\gamma$ RIIIB), which possess immunoreceptor tyrosine-based activation motifs (ITAMs) in their intracytoplasmic domain, and 2) the inhibitory receptor (hFc $\gamma$ RIIB), which possesses an immunoreceptor tyrosine-based inhibitory motif (ITIM).<sup>9</sup> Most cells express several Fc $\gamma$ Rs and the effector responses mediated by different IgG subclasses are the sum of their differential affinities for activating or inhibitory hFc $\gamma$ Rs.<sup>9-12</sup>

Human monocytic cells, including monocytes, monocyte-derived macrophages and dendritic cells (DCs) express all three major Fc $\gamma$ Rs, CD64 (Fc $\gamma$ RI), CD32 (Fc $\gamma$ RII), and CD16 (Fc $\gamma$ RIII). These cells constitute an important component of both normal and asthmatic airways, and play a number of critical roles in the regulation of asthmatic airway inflammation.<sup>13,14</sup> CD32A is the most abundant and prevalent activating Fc $\gamma$ R on monocytes and macrophages.<sup>12,15</sup> Indeed, CD32A was found to promote secretion of several pro-inflammatory cytokines including tumor necrosis factor, IL-1 $\beta$ , and IL-8 from monocytes/macrophages isolated from patients with rheumatoid arthritis.<sup>16</sup> By contrast, CD32B, the inhibitory counterpart of CD32A, is expressed at relatively low levels on monocytes and macrophages. As the only Fc $\gamma$ R expressed on B cells, the inhibitory functions of CD32B on B cell activities are well documented.<sup>17-19</sup> However, CD32B is expressed and inducible by IL-4 on human monocytes and can inhibit CD32A-mediated macrophage activation.<sup>20,21</sup> Furthermore, it was shown that co-expression of CD32A and CD32B on monocyte-derived DCs reveals a homeostatic checkpoint for inducing tolerance or

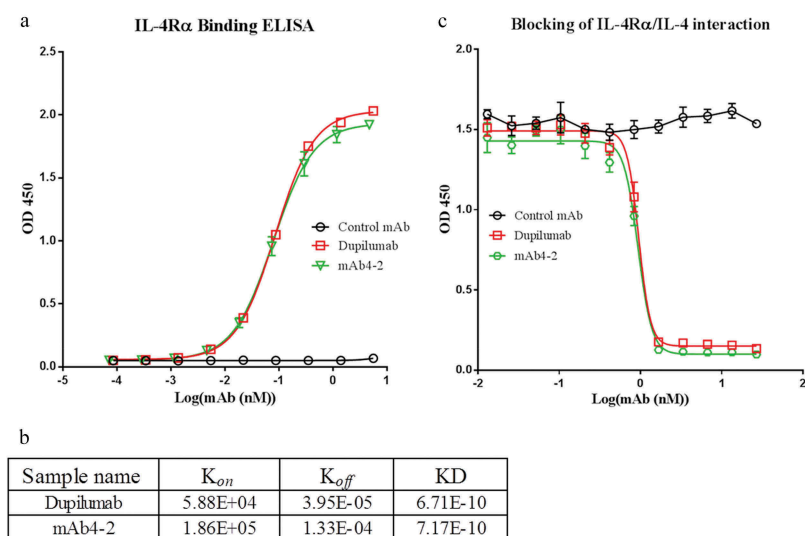
immunity by immune complexes (ICs).<sup>22</sup> These studies suggest that CD32 might be crucial in modulating the allergic immune responses in asthma via regulating monocytes activities.

In this study, we reveal a critical role of CD32, especially CD32A, in modulating the immune responses of monocytes in response to blockade of IL-4Ra signaling pathway by a novel anti-IL-4 $\alpha$  antibody. Based on this finding, we devised a new strategy to increase the efficacy of the anti-IL-4Ra mAb for treatment of asthma and other atopic diseases by co-engaging CD32 and IL-4Ra on monocytic cells by choosing IgG subclasses and Fc mutations with higher affinities for CD32. Both CD32A and CD32B contributed to the enhancement of antibody-mediated suppression of CD23 expression from monocytes in response to blockade of IL-4Ra signaling. Indeed, inhibition of IgE secretion from human peripheral blood mononuclear cells (PBMCs) by the antibody variants further suggest that the complex allergic inflammation mediated by IL-4/IL-4Ra signaling might result from a global network of multiple cell types that express multiple Fc $\gamma$ Rs, of which CD32, especially CD32A, is a key mediator. In this respect, our study provides insights into designing therapeutic antibodies for targeting Th2 cytokine-mediated allergic pathogenesis.

## Results

### Characterization of the humanized anti-hIL-4Ra mAb

Mouse mAbs against IL-4Ra were obtained by screening a large panel of hybridomas created from IL-4Ra immunized mice. Clone mAb4-2 exhibiting the highest affinity for IL-4Ra extracellular domains (ECDs) was selected and subsequently humanized. The humanized mAb4-2 (referred as mAb4-2) showed a binding affinity for IL-4Ra ECDs comparable to that of dupilumab (Figure 1(a)). Biacore further confirmed that the equilibrium dissociation constant (KD) of both antibodies are



**Figure 1.** Characterization of the anti-IL-4Ra monoclonal antibody clone mAb4-2. (a) Binding abilities of mAb4-2 to IL-4Ra were measured by ELISA. IL-4Ra antigen was coated on the plate. Antibodies, mAb4-2, dupilumab, and control mAb were serially diluted and added to the plate; (b) the equilibrium dissociation constants (KD) of dupilumab and mAb4-2 for IL-4Ra were measured by Biacore; (c) The ability of mAb4-2 to block the interaction of IL-4Ra and IL-4 was assayed by competition ELISA. mAb4-2, dupilumab, and control mAb were serially diluted and pre-incubated with IL-4Ra. The sample mixtures were then added to the plate coated with IL-4.

within  $10^{-10}$ M (Figure 1(b)). Furthermore, the ability of mAb4-2 to block the interaction of IL-4 with IL-4R $\alpha$  was also found to be comparable to that of dupilumab (Figure 1(c)).

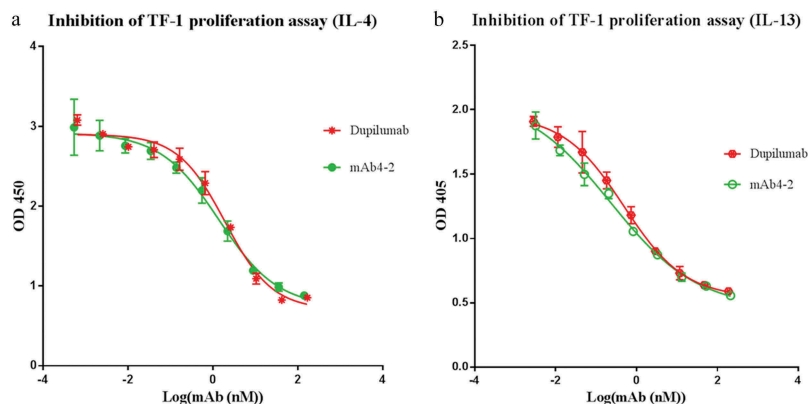
Next, we tested the bioactivity of mAb4-2 in IL-4-dependent TF-1 cell proliferation assays. Addition of mAb4-2 effectively inhibited IL-4-mediated TF-1 cell proliferation with an  $IC_{50}$  of 1.38nM, which is on par with dupilumab, whose  $IC_{50}$  is 1.93 nM (Figure 2(a)). Given that dupilumab can inhibit the binding of IL-13 to the receptor complexes by binding to IL-4R $\alpha$ , we stimulated TF-1 cell proliferation using IL-13 instead of IL-4 and tested the ability of mAb4-2 to inhibit IL-13 dependent cell growth. As expected, mAb4-2 also effectively inhibited IL-13-mediated TF-1 proliferation at an  $IC_{50}$  of 0.23 nM, compared to that of dupilumab ( $IC_{50}$ : 0.49 nM). These data demonstrate that mAb4-2, like dupilumab, is a powerful dual blocker that simultaneously inhibits IL-4- and IL-13-mediated cell proliferation (Figure 2(b)).

In addition to bioactivities, biophysical and chemical analysis also demonstrated that mAb4-2 has favorable properties as a drug candidate, including purity, thermal stability, and pharmacokinetics (Fig S1A, B, C). Therefore, mAb4-2 was chosen for further characterization and optimization.

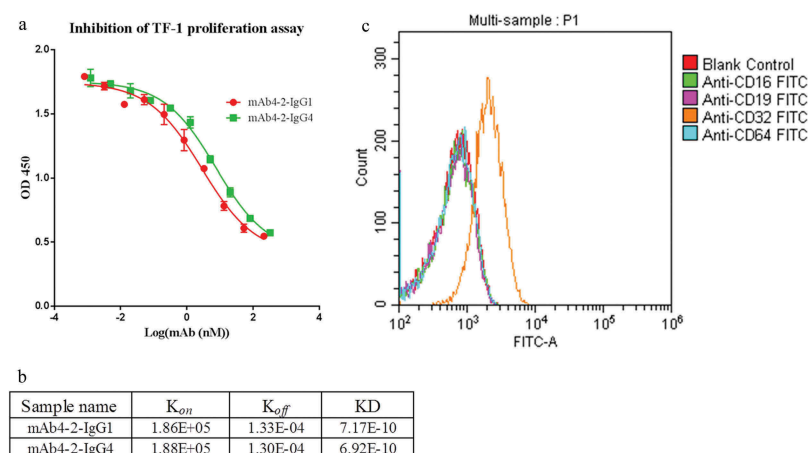
### Characterization of Fc $\gamma$ R $\alpha$ s on TF-1 cells that interact with mAb4-2 antibody variants of various IgG subclasses

During our initial optimization for the mAb4-2 backbone structure, we discovered that mAb4-2 of an IgG1 subclass (referred as mAb4-2-IgG1) showed a fourfold increase of potency ( $IC_{50}$ : 3.8 nM) in inhibiting IL-4-mediated TF-1 cell proliferation compared with that of mAb4-2 of an IgG4 subclass ( $IC_{50}$ : 13.3nM; referred as mAb4-2-IgG4) (Figure 3(a)). The difference of activities of two IgG subclasses was surprising, as Biacore showed that switching Fc subclasses of mAb4-2 did not change the binding affinities of the two antibodies (Figure 3(b)). The Biacore result led us to think that the Fc effects on inhibition of cell proliferation might be due to the interaction of IgG subclasses with Fc $\gamma$ R on TF-1 cells.

To test this hypothesis, we first made an N297A mutant (mAb4-2-IgG1-N297A) that abolishes the ability of IgG1 binding to Fc $\gamma$ R $\alpha$ s owing to inhibition of glycosylation,<sup>9</sup> while maintaining its affinity for the IL-4R $\alpha$  ECD intact. Proliferation assay demonstrated that the potency of mAb4-2-IgG1 was significantly impaired by the N297 mutation (Fig S2), suggesting that the interaction of the antibody Fc domain with Fc $\gamma$ R $\alpha$ s



**Figure 2.** MAb4-2 possesses abilities to inhibit TF-1 cell proliferation mediated by either IL-4 or IL-13. (a) mAb4-2 inhibited IL-4-induced TF-1 proliferation in a dose-dependent manner. Dupilumab, a marketed anti-IL-4R $\alpha$  mAb, serves as a control mAb; (b) mAb4-2 inhibited IL-13-induced TF-1 proliferation in a dose-dependent manner. Dupilumab serves as a control mAb.



**Figure 3.** The mAb4-2 potency of suppressing TF-1 proliferation depends upon the antibody's Fc. (a) after serial dilution, mAb4-2-IgG1 and mAb4-2-IgG4, the antibody with either IgG1 or IgG4 heavy chain were added to TF-1 cells in the presence of IL-4; (b) the equilibrium dissociation constants (KD) of mAb4-2-IgG1 and mAb4-2-IgG4 for IL-4R $\alpha$  were obtained using Biacore; (c) Fc $\gamma$ R $\alpha$ s on the surface of TF-1 cells were identified by FACS using FITC-labeled anti-CD16, anti-CD32, and anti-CD64 specific antibodies. FITC-labeled anti-CD19 serves as negative control.

does significantly affect the antibody's activity. To further identify which receptors are involved in these events, we investigated the Fc gamma receptors expressed on TF-1 cells by fluorescence-activated cell sorting (FACS). We found that TF-1 cells only express CD32 (FcγRII), but not CD16 and CD64 (Figure 3(c)), suggesting that CD32 is the receptor with which the Fc domain of mAb4-2 antibodies interacts. Because the commercial anti-CD32 antibody cannot distinguish CD32A (FcγRIIA) and CD32B (FcγRIIB) isoforms, we expressed in-house anti-CD32A and anti-CD32B antibodies, which showed high selectivity for CD32 isoforms expressed on TF-1 cells. FACS conducted with the two antibodies confirmed that CD32A is more abundant than CD32B on TF-1 cells, thus showing that CD32A is the major FcγR in TF-1 cells with which anti-IL-4 antibodies interact (Fig S3A, B).

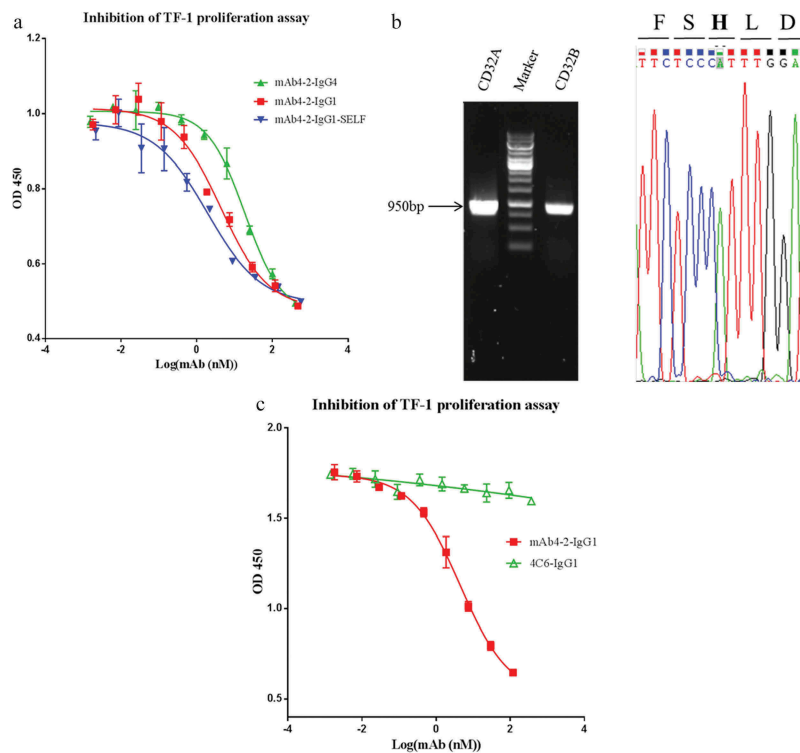
### CD32 (FcγRII) provides additional inhibitory signals for suppressing TF-1 cell proliferation in response to blockade of anti-IL-4Ra signaling

To confirm that the affinity of IgG subclasses for CD32 plays a role in modulating the antibody activities, we introduced S267E/L328F (SELF) double mutations on the Fc portion of mAb4-2-IgG1, named mAb4-2-IgG1-SELF.<sup>19</sup> We then tested this antibody's ability to bind TF-1 cells engineered to express different CD32 constructs. In agreement with the previous study, mAb-IgG1-SELF whose affinity for CD32B is enhanced by up to 430-fold showed increased binding to TF-1 cells

overexpressing CD32B, as well as cells overexpressing CD32A-R<sub>131</sub>, but not cells overexpressing CD32A-H<sub>131</sub> (Fig S4).

Next, the potency of mAb4-2 variants in inhibition of TF-1 cell proliferation was assessed in response to IL-4. As shown earlier, mAb4-2-IgG1 showed a fourfold increase of potency in inhibiting IL-4-mediated TF-1 cell proliferation relative to the IgG4 counterpart, suggesting that CD32A, which, unlike CD32B, has a tenfold higher affinity for IgG1 relative to IgG4, played a key role in the event, in agreement with its dominating expression pattern on TF-1 cells. Furthermore, mAb4-2-IgG1-SELF showed another fourfold increase in potency (IC<sub>50</sub>: 0.9 nM) relative to that of mAb4-2-IgG1. In contrast, IgG1-SELF, a non-targeting antibody with the S267E/L328F mutations, showed no effects on IL-4-induced TF-1 cell proliferation, indicating that the increased affinity for CD32A (IgG1/IgG4 > 10-fold) as well as for CD32B (IgG1-SELF/IgG1 > 430-fold) facilitated the inhibitory function of the antibodies following the ligation of the antibody with IL-4Ra (Figure 4(a)).

Given CD32A has two isoforms, CD32A-R<sub>131</sub> and CD32A-H<sub>131</sub>, we next identified which isoform was expressed on TF-1 cells by genotyping. Interestingly, we found that CD32A-H<sub>131</sub> and CD32B mRNAs were both transcribed in TF-1 cells, although the amount of CD32B mRNA was about half of that of CD32A mRNA (Figure 4(b)). This result confirmed our FACS finding in that CD32A is abundantly expressed from TF-1 cells, while CD32B is expressed to



**Figure 4.** CD32 plays an important role in modulating antibody-mediated inhibition of TF-1 proliferation in response to blockade of IL-4Ra. (a) mAb4-2-IgG4, mAb4-2-IgG1, and mAb4-2-IgG1-SELF<sup>19</sup> were added to TF-1 cells in the presence of IL-4, following serial dilution; (b) an agarose gel showed the full-length CD32A and CD32B cDNA amplified by PCR using specific primers, following the reverse transcription from the extracted mRNA of TF-1 cells (left). Left lane: CD32A cDNA; middle Lane: molecular weight ladder; right Lane: CD32B cDNA; the sequencing result of CD32A cDNA (right), "A", the single-nucleotide polymorphism (SNP), was highlighted, which results in H<sub>131</sub> (bold) in the amino acid sequence; (c) a surrogate antibody of mAb4-2-IgG1, 4C6-IgG1, which can bind to IL-4Ra, but cannot block the signaling of IL-4Ra, was added to TF-1 cells in the presence of IL-4 after serial dilution. mAb4-2-IgG1 was used in the same assay as a positive control.

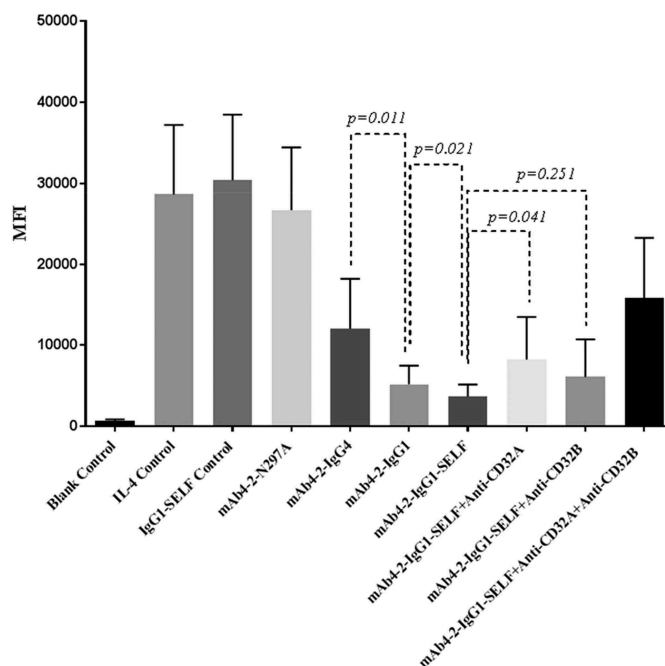
a much lesser degree. The fact that the IgG1 antibody introduced with the SELF mutation increased the inhibitory potency of the antibody compared to the parental antibody supports the role of CD32B in this event. Since TF-1 expresses CD32A-H<sub>131</sub>, an isotype whose affinity for SELF mutations is not enhanced, the increased potency by the SELF mutant can be only explained by its binding to CD32B. Taken together, this observation demonstrated that CD32A, possibly in conjunction with CD32B, plays critical roles in modulating the inhibitory functions of the anti-IL-4 antibodies.

Intriguingly, a surrogate of mAb4-2-IgG1, 4C6, which exhibits the same affinity for IL-4R $\alpha$  but cannot block IL-4R $\alpha$  signal transduction (Fig S5A, B), failed to inhibit IL-4R signaling (Figure 4(c)). This finding suggests that blockade of the IL-4R $\alpha$  by the mAb4-2 antibody is a prerequisite for CD32A that is ligated with the antibody Fc to send out additional inhibitory signals.

### CD32, especially CD32A, plays critical roles in reinforcing the antibody-mediated suppression of IL-4-induced CD23 expression on CD14+ monocyte

The expression level of CD23, the IgE low-affinity receptor II, on peripheral blood monocytes is IL-4 dependent<sup>23</sup> and appears to be associated with asthma severity and exacerbation.<sup>24</sup> We then tested mAb4-2 variants for their ability to block IL-4-induced CD23 expression of CD14+ monocytes from human PBMC. As predicted, mAb4-2-IgG4 showed a potent activity to inhibit CD23 expression from CD14+ monocytes, when compared to controls. Interestingly, when mAb4-2-IgG1 was used, the ability of blocking IL-4-induced CD23 expression was significantly improved (Figure 5). Since the affinity of CD32A for the IgG1 subclass is tenfold higher than that of the IgG4 counterpart, we reasoned that CD32A plays an important role in this event. In agreement with this finding, CD32A is the most abundant Fc $\gamma$ R expressed on monocytes as well as monocyte-derived macrophages and dendritic cells.<sup>12</sup>

Next, we wanted to test if enhancing the binding affinity of the antibody for CD32 can further improve the inhibition of CD23 expression from CD14+ monocytes. As seen previously, the addition of mAb4-2-IgG1-SELF showed increased inhibition relative to mAb4-2-IgG1, however, to a lesser degree ( $p=0.02$ ) when compared to that of mAb4-2-IgG1 from the IgG4 counterpart ( $p=0.01$ ) (Figure 5). To confirm that CD32A indeed contributed to the enhancement of the inhibitory activity of the antibody, we, therefore, examined IL-4-induced CD23 expression on isolated blood monocytes in the presence of anti-IL-4R $\alpha$  antibodies and the two anti-CD32A- and CD32B-specific antibodies. Addition of anti-CD32A antibody into samples in the presence of mAb4-2-IgG1-SELF significantly increased the CD23 expression relative to that of mAb4-2-IgG1-SELF treatment alone ( $p < .05$ ). By contrast, the addition of anti-CD32B antibody into samples in the presence of mAb4-2-IgG1-SELF did not significantly increase the CD23 expression relative to that of mAb4-2-IgG1-SELF treatment alone ( $p > 0.05$ ). However, anti-CD32A antibody could not fully rescue the CD23 expression to the level comparable to that of mAb4-2-IgG4 treatment. Interestingly,

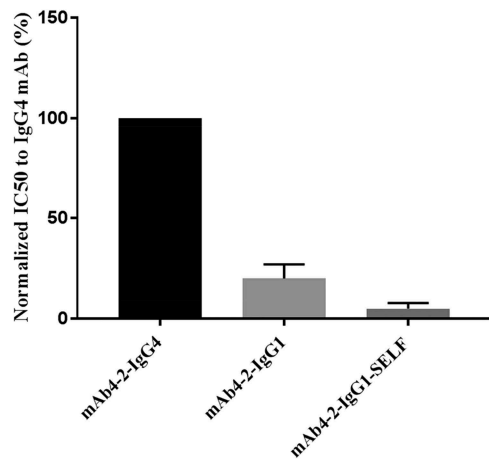


**Figure 5.** CD32A, together with CD32B, plays a key role in modulating antibody-mediated suppression of CD23 expression on monocytes in response to IL-4R $\alpha$  blockade. Monocytes were isolated from six donor's PBMCs. The level of CD23 expression from the blood monocytes treated with a variety of reagents in the presence of IL-4 was accessed by FACS. IL-4 control: cells treated with IL-4 in the absence of antibodies; IgG1-SELF control: an irrelevant IgG1 mAb with the SELF mutation; mAb4-2-N297A: an aglycosylation form of mAb4-2, owing to the point mutation of N297; mAb4-2-IgG4: the IgG4 mAb4-2; mAb4-2-IgG1: the IgG1 mAb4-2; mAb4-2-IgG1-SELF: the IgG1 mAb4-2 with the SELF mutation; mAb4-2-IgG1-SELF+Anti-CD32A: monocytes were treated with mAb4-2-IgG1-SELF and anti-CD32A antibody simultaneously; mAb4-2-IgG1-SELF+Anti-CD32B: monocytes were treated with mAb4-2-IgG1-SELF and anti-CD32B antibody simultaneously; mAb4-2-IgG1-SELF+Anti-CD32A+Anti-CD32B: three antibodies, mAb4-2-IgG1-SELF, anti-CD32A, and anti-CD32B were added to monocyte samples simultaneously.

adding both anti-CD32A and anti-CD32B together fully rescued the expression of CD23 compared to that of adding mAb4-2-IgG4 alone (Figure 5). Taken together, these results demonstrated that, although CD32A appeared to be more important for mAb4-2 antibody variants mediated suppression of CD23 expression than CD32B, both CD32 isotypes worked coordinately in aiding antibodies to deliver extra inhibitory signals in monocytes in addition to blockade of IL-4R $\alpha$ .

### Both CD32A and CD32B play critical roles in inhibition of IL-4-induced IgE secretion from PBMC

As IgE levels in asthma patients are associated with increased frequency of exacerbations under the control of IL-4,<sup>25</sup> we tested mAb4-2 antibodies for suppression of IL-4-induced IgE secretion using human PBMC. mAb4-2-IgG1 antibody showed a nearly fivefold stronger suppression of IL-4-mediated IgE secretion from PBMC relative to the IgG4 counterpart, once again suggesting that CD32A contributed to the mAb4-2 antibody-mediated suppression of IgE secretion. Introduction of SELF mutations provided an extra fourfold inhibition relative to the IgG1 counterpart (Figure 6). Since IgE is secreted from B cells that only express CD32B, this result



**Figure 6.** Both CD32A and CD32B play important roles in modulating antibody-mediated suppression of IgE secretion from human PBMCs. The IC50 of each antibody, mAb4-2-IgG4, mAb4-2-IgG1, and mAb4-2-IgG1-SELF in the suppression of IgE secretion from blood B cells were measured in the presence of IL-4. The calculated IC50 for each antibody was normalized to and expressed as a percentage of that of mAb4-2-IgG4. The lower the percentage, the higher the potency is relative to the IgG4 antibody.

implies that CD32B is also involved in modulating the antibody activity in the suppression of IgE secretion. Interestingly, the roles of CD32A in this event may reflect a more complex networking amongst different immune cells, since CD32A is not expressed on B cells but on nonlymphoid cells.

## Discussion

In this study, we developed a humanized anti-IL-4R $\alpha$  mAb, mAb4-2, which can efficiently block IL-4/IL-13-mediated cell signaling in a Th2 cytokine-mediated proliferation model. Using this molecule, we studied the effects of the interaction of the Fc portion of the antibody with Fc $\gamma$ Rs on the effector cells on modulating IL-4-induced immune responses. We found that the binding affinity of the antibody Fc for CD32, especially the activating CD32A, has profound effects on modulating the immune responses mediated by IL-4/IL-4R $\alpha$  signaling.

IL-4 has a variety of effects in various tissues. In keeping with the broad range of actions of this cytokine, IL-4 receptors are also expressed in a broad spectrum of tissues. Analysis using IL-4R $\alpha$  variants demonstrated that IL-4R $\alpha$  chain cytoplasmic region regulates two distinct activities. One is required for activation of proliferative pathways, and the other is required for IL-4-induced expression of proteins such as CD23 and IgE.<sup>3</sup> In the TF-1 cell proliferation model, we investigated the effectiveness of mAb4-2 antibody variants in inhibiting the IL-4-dependent proliferation of this cell line. The following variants, mAb4-2-IgG1-SELF > mAb4-2-IgG1 > mAb4-2-IgG4, exhibited increased suppression of TF-1 cell proliferation in a manner that depended on the affinity for Fc $\gamma$ R. Further analysis demonstrated that TF-1 cells express CD32 only, indicating that the enhanced potency is due to improved binding of the Fc portion of the antibody to CD32. FACS analysis using anti-CD32A and anti-CD32B specific antibodies as well as genotyping showed that CD32A is more abundantly expressed on TF-1 cells than CD32B.

Consistent with this observation, mAb4-2-IgG1 exhibited much stronger inhibitory activity relative to mAb4-2-IgG4, which may be explained by the difference of binding affinity of the two Fc subclasses for CD32A, but not for CD32B.<sup>11</sup> In support of the CD32B expression pattern, the SELF mutations significantly improved the potency of both IgG1 and IgG4 due to its increased affinity for CD32B (although SELF exhibits strong affinity for CD32A-R131, this variant is not expressed in the cells tested). Our studies demonstrated that CD32, both CD32A and CD32B, plays a key role in modulating the activities of anti-IL-4 antibodies in suppressing the IL-4-induced TF-1 proliferation.

Bruhns et al.<sup>11</sup> have conducted a comprehensive comparison of the affinities of human Fc $\gamma$ Rs for human IgG subclasses, where they showed that IgG1 and IgG4 have similar affinities for CD32B ( $K_A = 2 \times 10^5 \text{ M}^{-1}$ ), whereas, compared to IgG4, IgG1 binds tenfold stronger to CD32A. These data are in alignment with our results, and suggest that CD32A might be the major Fc $\gamma$ RII that regulates the activities of anti-IL-4R $\alpha$  antibodies for the following reasons: 1) IgG1 subclass significantly improved the antibody potency when compared to the IgG4 counterpart, and the increased potency was due to the stronger affinity between IgG1 and CD32A; 2) CD32A proteins are much more abundant and prevalent on the surface of TF-1 cells relative to CD32B proteins; and 3) although the differences in affinities between the IgG1/IgG4 antibodies and between IgG1-SELF/IgG1 antibodies are 10-fold and 430-fold for CD32A and CD32B, respectively, the two pairs show the same 4-fold increase in potency.

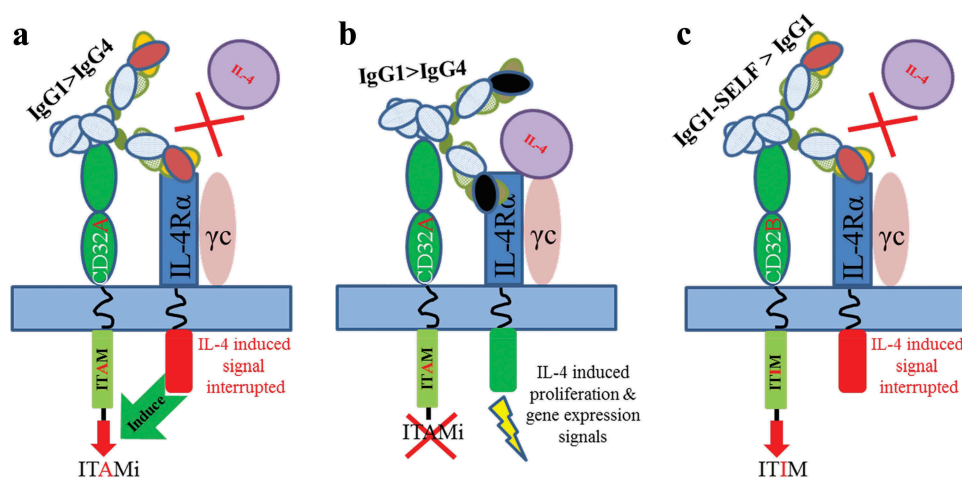
Since CD32A is an activating receptor, the inhibitory activity associated with this receptor was initially surprising to us. We, therefore, set out to investigate the phenomenon further by using a surrogate of mAb4-2, 4C6, which has the same affinity for IL-4R $\alpha$  but cannot block IL-4R $\alpha$  signal transduction. We found that 4C6 failed to block IL-4-induced TF-1 proliferation. This finding raises a possibility that CD32A is only activated in response to the blockade of IL-4R $\alpha$  pathways, and that cross-linking the IL-4R $\alpha$  with CD32A by the antibody positively upregulates the Fc $\gamma$ R activity to reinforce the inhibitory signals of IL-4R $\alpha$  blockade. Indeed, the Elson's group<sup>26</sup> demonstrated that CD32A was involved in regulation of the inhibitory function of an anti-TLR4 antibody. The not-yet-well-defined phenomenon, known as inhibitory ITAM (ITAMi), in terms of activating Fc $\gamma$ Rs sending out inhibitory signals, relies on a so-called "scorpion effect", where an antibody interacts simultaneously with a target and an Fc $\gamma$ R on the same cell in *cis*.<sup>12</sup> Although ITAMi remains to be further elucidated, recent research from the Monteiro lab,<sup>27</sup> where they showed that tyrosine 304 within CD32A ITAM is critical for ITAMi signaling, may shed light on the mechanism. However, in our study, we demonstrated that blocking the targeted receptor by antibodies is a prerequisite for CD32A-mediated ITAMi signaling. Whether or not the process is target specific, i.e., specific for IL-4R $\alpha$ , remains to be further characterized. More importantly, it is plausible that the status of the targeted receptor either activated or inhibited by an antagonist or an agonist may dictate the CD32A activating or inhibitory responses, respectively (Figure 7).

Upregulation of CD23 expression, the low-affinity IgE receptor IgERII, is one of the hallmarks of IL-4-induced immune responses and is believed to have important roles in the pathogenesis of asthma.<sup>28</sup> Thus, we assessed the activity of our antibodies in suppressing the upregulation of CD23 expression using isolated monocytes from human PBMC. We discovered that antibodies of IgG1 subclass rendered much stronger suppression than did antibodies of IgG4 subclass, indicating that an FcγR that has a much stronger affinity for IgG1 than IgG4 might contribute to this effect. Therefore, the data from this ex-vivo model aligned reasonably well with our in-vitro cell assays. Since CD32A is the most abundant FcγR on the monocytic cells, we reasoned that CD32A also plays a crucial role in modulating the expression of CD23 on blood monocytes alongside the mAb4-2 antibodies. In line with this finding, using an anti-CD32A antibody, we demonstrated that CD32A is indeed responsible for enhancing the inhibitory function of the mAb4-2 antibodies, since the addition of anti-CD32A antibody partially rescued the suppression of CD23 expression rendered by mAb4-2-IgG1-SELF and mAb4-2-IgG1, respectively. Although adding anti-CD32B antibody did not rescue the CD23 expression from the suppression by the SELF mutant ( $p > .05$ ), addition of both CD32A and CD32B completely rescue the CD23 expression to levels comparable to that of mAb4-2-IgG4 from the SELF mutant. The importance of CD32A in the regulation of CD23 on monocytes may simply reflect the expression level of the receptor when compared to CD32B. Further studies need to be done to tease out the roles of the two CD32 isoforms in this event.

The above result, however, cannot rule out the possibility that FcγRs on monocytes, other than CD32, can also bind to the Fc portion of the antibodies, and thus transmit intracellular signals accordingly. In fact, the inhibition of mAb4-2-IgG4 alone relative to controls might reflect the blockade of IL-4Ra plus the net inhibitory signals from all FcγRs bound to the IgG4 Fc. In the case of CD23 expression, the extra impact of FcγRs on the antibody potency above the IgG4

antibody appears to be the sum of activities of both FcγRIIs, of which CD32A was more dominant. Therefore, the observed activity might be the combination of the affinities and densities of all FcγRs together, in that FcγRs on monocytes, both activating and inactivating, contribute to the reinforcement of the inhibitory signals in response to the inhibition of IL-4Ra signaling.

In the case of IgE secretion, our anti-IL-4Ra antibodies displayed a strong inhibitory ability in a manner that appears to associate with the affinity for CD32A, since the IgG1 antibody provides much stronger inhibition than the IgG4 counterpart. Moreover, introducing SELF mutations to the IgG1 antibody further increased the inhibitory potency of the parental IgG1 antibody. These results indicate that, in addition to CD32A, CD32B may also modulate an antibody's ability to suppress IgE secretion, given that CD32B is the only FcγR expressed on B cells.<sup>29</sup> Recently, CD32C, whose extracellular region is homologous to CD32B while the cytoplasmic tail is homologous to CD32A with the presence of an ITAM, was found to be expressed on B cells in people who possess an open reading frame (ORF) allele, and could counterbalance the negative feedback of CD32B.<sup>30</sup> As expected, CD32C and CD32B indeed have similar binding affinities for both IgG1 and IgG4, given the high homology of their extracellular domains.<sup>11</sup> Thus, the net negative signaling observed in this event might reflect the possibilities that: 1) none or few hPBMCs that we tested have the functional CD32C expressed, as less than 15% of healthy individuals have the ORF allele; 2) CD32B may be more abundantly expressed on B cells compared to CD32C with the ORF allele; 3) more intriguingly, it is plausible that CD32C, when engaged with anti-IL-4Ra antibodies, might execute ITAMi signaling as CD32A did. However, the fact that CD32A has a role in this event may reflect a more complex situation, where the amount of soluble IgE in PBMCs can be affected by multiple factors and immune cells. Indeed, IL-4 simultaneously induces IgE production and CD23 expression.



**Figure 7.** Schematic of the model of IL-4Ra blockade dependent ITAMi. (a) mAb4-2 binds to IL-4Ra and blocks the interaction of IL-4 with IL-4Ra while cross-linking the CD32A (the binding affinity  $\square$  IgG1 > IgG4). The disruption of the IL-4-mediated signaling allows IL-4Ra to signal the Fc bound CD32A, resulting in activation of the ITAMi signaling; (b) 4C6 binds to IL-4Ra but cannot block the interaction of IL-4 with IL-4Ra, while cross-linking the CD32A. The undisturbed IL-4/IL-4Ra signaling does not send out signals to the Fc-bound CD32A, and thus no ITAMi is activated; (c) In the case of CD32B, mAb4-2 binds to IL-4Ra and blocks the interaction of IL-4 with IL-4Ra, while cross-linking the CD32B (the binding affinity  $\square$  IgG1-SELF > IgG1), resulting in activation of the ITIM signaling.

Multiple lines of evidence demonstrated that IgE production from B cells is negatively regulated by CD23.<sup>28</sup> There are two isoforms of CD23, CD23a and CD23b. CD23a is exclusively expressed on human B cells, whereas CD23b is upregulated by IL-4 on a variety of hematopoietic cells, including B cells and monocytes. Using CD23 transgenic mice, Payet-Jamroz M et al. demonstrated that CD23 expressed on non-lymphoid cells provided B cells with signals that resulted in the decrease of IgE production.<sup>31</sup> More recently, the Chan lab showed that signaling through CD23 on B cells differs from that of the monocytic lineage cells.<sup>32</sup> They proposed that CD23 plays roles in the regulation of IgE synthesis in human B cells, whereas CD23 may regulate the phagocytosis of IgE and allergen in monocytes. These data suggest that, as a principal mediator of most type I allergic reactions, IgE production is tightly controlled by comprehensive cross-talk between B cells and surrounding non-lymphoid cells, in which CD23 plays key roles in response to IL-4. Indeed, conflicting observations were also seen for the roles of CD23 and IgE in the regulation of inflammatory responses in asthma, probably due to the complexity of the disease environment. The Borish lab showed that individuals with asthma displayed a much higher level of CD23 expression on monocytes relative to that of normal individuals,<sup>24</sup> whereas the Weiss' group demonstrated that the elevated IgE levels seen in asthmatic patients were associated with reduced CD23 expression.<sup>25</sup> Therefore, the amount of IgE observed in our studies was most likely a net result from the manipulation of the global signaling of IL-4R $\alpha$ , activating and inhibitory Fc $\gamma$ Rs on B cells and monocytes by anti-IL-4R $\alpha$  antibodies.

Nevertheless, we revealed an important role of the activating CD32, in particular, CD32A, in modulating anti-IL-4R $\alpha$  antibody-mediated suppression of IL-4-induced inflammatory responses. Cross-linking of IL-4R $\alpha$  and CD32A by antibodies allows CD32A to send out extra inhibitory signals in response to IL-4R $\alpha$  blockade. It appears that the situation of the targeted receptor affected by antibodies tells CD32A what to do, since the blockade of IL-4R $\alpha$  is prerequisite for activating CD32A-mediated ITAMi signaling. Furthermore, in a Th2 cytokine-mediated allergic inflammatory environment, monocytes, as a key modulator of allergic inflammation, express various Fc $\gamma$ Rs, of which CD32A is the most abundant and prevalent. It is plausible that such similar regulations could also be seen in IL-13-mediated immune responses, as IL-13 not only shares IL-4R $\alpha$  in its receptor complexes but also shares some of the effector functions with IL-4, including regulation of IgE production and CD23 expression.<sup>33</sup> Further studies need to be done to verify this hypothesis. Therefore, design and application of therapeutic antibodies to intervene Th2 cytokine-mediated atopic diseases, including asthma, should take CD32A activities into consideration, as well as the contribution of other activating and inhibitory Fc $\gamma$ Rs, in modulating the inflammatory responses in addition to blockade of receptors.

## Materials and methods

### Cell culture

TF-1 cells, a factor – dependent human erythroleukemic cell line, were purchased from the American Type Culture

Collection (ATCC® CRL-2003™). TF-1 cells were cultured in RPMI 1640 medium (Gibco™), supplemented with 10% heat-inactivated fetal bovine serum (Gibco™), 1% GlutaMAX™ Supplement (Gibco™), 1% Penicillin-Streptomycin (Gibco™) and 5 ng/ml recombinant human GM-CSF (Amoytop Biotech) in a humidified CO<sub>2</sub> incubator at 37°C. PBMCs were separated by a Histopaque-1077 (Sigma) gradient method using blood from healthy donors (after informed consent, Changhai Hospital, Shanghai, China) and cultured in RPMI 1640 complete medium. HEK293E, purchased from the NRC Biotechnology Research Institute, were cultured in freestyle 293 medium (Life technologies) with 1% Penicillin-Streptomycin.

### Protein expression and purification

Constructs expressing antibody variants were generated using pTT5 vector (NRC Biotechnology Research Institute). The resulting expression vectors were transiently transfected into HEK293E cells using 1  $\mu$ g/mL 25-kDa linear polyethylenimine (Polysciences, Inc.). One day after transfection, valproic acid (Sigma) was added to cell culture at a final concentration of 3 mM. On day 2 post-transfection, medium comprising 10% GlutaMAX, 10% 400 g/L glucose and 80% freestyle 293 medium was added to the cell culture at 10% of the total volume. Conditioned medium was collected 5–6 days after transient transfection. Antibodies in the culture media were purified by MabSelect SuRe (GE) affinity columns using an Akta Avant 25 fast protein liquid chromatography (FPLC) purification system. After equilibrating the column with buffer A (25 mM sodium phosphate, 150 mM sodium chloride, pH = 7.0), the culture media containing anti-IL-4R antibodies were applied to the column, which was then eluted with buffer B (100 mM sodium citrate, pH = 3.5) to collect the desired proteins. The eluted antibodies were further polished by gel filtration on a Superdex™ 200 column (GE) if necessary.

### Humanization of mAb4-2 and antibody engineering

Humanization of mAb4-2 was conducted by grafting complementarity determining regions of the parental murine anti-human IL-4R $\alpha$  antibody (Clone mAb4-2) into human heavy and light-chain frameworks as described previously.<sup>34</sup> PCR site-directed mutagenesis was used to generate IgG variants of the humanized mAb4-2 following the manufacturer's instructions (Cat#R010B, Takara). The sequences of all variants were verified by sequencing at Genewiz (Shuzou, China). MAbs capable of discriminating the human inhibitory Fc $\gamma$ -receptor IIB (CD32B) from the activating Fc $\gamma$ -receptor IIA (CD32A) were prepared in-house. The sequence information of 2B6 (specific for CD32B and designated Anti-CD32B) and IV.3 (specific for CD32A and designated Anti-CD32A) were extracted from US20160159908<sup>35</sup> and US20160145336,<sup>36</sup> respectively. Gene synthesis and sequencing were all performed by Genewiz (Shuzou, China). Genes for all antibodies were subcloned into the mammalian expression vector pTT5 and transiently expressed from HEK293E cells. Antibodies in the cell culture media were purified with protein A affinity columns (Mabselect, GE) by FPLC.



### Enzyme-linked immunosorbent assay

Ninety-six-well plates (Cat#9018, Costar) were coated with 10 ng/well of FLAG-tagged IL-4R $\alpha$  (produced in-house) overnight. The plates were washed with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBS-T), blocked for 1 h with PBS containing 1% bovine serum albumin (BSA) and incubated with serial dilutions of the purified anti-IL-4R $\alpha$  antibodies for 1 h at room temperature. The plates were washed with PBS-T for 5 min three times and incubated with horseradish peroxidase (HRP)-conjugated goat-anti-human IgG (Fc-specific and highly cross-adsorbed) antibody (Cat#SAB3701283, Sigma) for 1 h at room temperature. The plates were washed, and the reaction developed with chromogenic substrates for 2-3 min. The plates were then read on a SpectraMax 190 reader (Molecular Devices) at 450 nm. The same protocol was used for competition ELISA, except that the plates were coated with 200 ng/well of IL-4 followed by incubation of serial dilutions of the purified antibodies in the presence of 200 ng/ml biotinylated IL-4R $\alpha$ . HRP-Streptavidin was then used to detect bound competing IL-4R $\alpha$ .

### TF-1 cell proliferation assay

Prior to use, TF-1 cells were harvested in logarithmic phase and washed 2 times in PBS. Cells were resuspended in the assay medium (the growth medium without GM-CSF) and added to a 96-well cell culture plate at  $1 \times 10^5$  cells/150  $\mu$ l/well, and placed for an hour in a humidified chamber at 37°C and 5% CO<sub>2</sub>. After 1 h, serial dilutions of anti-IL-4R $\alpha$  antibodies were added to each well in the presence of 80 ng/ml rhIL-4 (Cat#204-IL-050, R&D systems) or rhIL-13 (Cat#213-ILB-005, R&D systems) for a final reaction volume of 200  $\mu$ l, and the plates were incubated for 96 h at 37°C and 5% CO<sub>2</sub>. At 90 h, CCK-8 reagent (Cat#CK04, Dojindo) was added as per manufacturer's instructions. The plates were read at 405/450 nm on a SpectraMax 190 reader (Molecular Devices). The IC<sub>50</sub> values were calculated using GraphPad Prism 6 software (GraphPad Software).

### Flow cytometric analysis of CD23 expression on monocytes

PBMCs were separated from the blood of healthy donors by the Histopaque-1077 (Sigma) gradient method. Isolated PBMCs in complete RPMI-1640 medium were added to round-bottom 96-well plates (Cat#3799, Costar®) at  $2 \times 10^5$  cells/150  $\mu$ l/well. Fifty microliters of serial dilutions of anti-IL-4R $\alpha$  antibodies were added to each well in the presence of 80 ng/ml rhIL-4 for a final reaction volume of 200  $\mu$ l, and the plates were incubated for 48 h at 37°C and 5% CO<sub>2</sub>. After 2 days, PBMCs were stained with phycoerythrin mouse anti-human CD23 antibody (Cat#555711, BD biosciences), and recombinant human IgG1 (in-house) was added to block non-specific binding to Fc receptors at 1mg/ml. After fixation with 4% paraformaldehyde for 10 min at room temperature, PBMCs were analyzed using CytoFLEX Cytometer System (Beckman Coulter). Monocytes were identified based on forward scatter/side scatter, as well as CD14 staining.

In the case of identification of Fc $\gamma$ R<sub>s</sub> expressed from TF-1 cells, TF-1 cells were incubated with fluorescein isothiocyanate-(FITC)-conjugated mAbs (FITC-isotype control, Cat#555748; FITC-CD19, Cat#555412; FITC-CD16, Cat#555406; FITC-CD32, Cat#555448; FITC-CD64, Cat#555527; all from BD Biosciences) for 1 h at room temperature in PBS containing 1% BSA. Flow cytometry was performed as described above.

### ELISA analysis of IgE secretion from PBMCs

PBMCs in complete RPMI-1640 medium were added to round-bottom 96-well plates (Cat#3799, Costar®) at  $2 \times 10^5$  cells/150  $\mu$ l/well. Fifty microliters of serial dilutions of anti-IL-4R $\alpha$  antibodies were added to each well in the presence of 80 ng/ml rhIL-4 for a final reaction volume of 200  $\mu$ l. The plates were incubated for 14 days at 37°C and 5% CO<sub>2</sub>. After 14 days, cell culture supernatants were collected and IgE concentrations were determined by a sandwich ELISA. Briefly, IgE in the culture medium was captured by anti-human IgE antibody (Cat#I6284-1MG, Sigma) coated plates after 1 h incubation at room temperature. The plates were washed and incubated with biotinylated anti-Human IgE antibody (A18803, Thermo Fisher Scientific) for 1 h at room temperature. The plates were washed and incubated with HRP-conjugated streptavidin (Cat#554066, BD Biosciences) for 1 h at room temperature. The plates were washed, and the reaction developed with the addition of chromogenic substrates for 2-3 min. The plates were then read on a SpectraMax 190 reader (Molecular Devices) at 450 nm. IgE levels from the culture medium were quantitated with an IgE standard curve.

### Binding affinity analysis by surface plasmon resonance (SPR)

The interaction of antibody variants with IL-4R $\alpha$  was monitored using Biacore T200 (GE Healthcare). Briefly, antibody variants were captured by protein A/G (Cat#21186, Thermo Scientific) immobilized on the CM5 sensor chip (Cat#BR-1005-30, GE Healthcare). Varying concentrations of IL-4R $\alpha$  were injected at a flow rate of 30  $\mu$ l/min. Sensorgrams were obtained at each concentration, and kinetic analysis was performed using Biacore T200 Evaluation Software (GE Healthcare).

### CD32A genotyping of TF-1 cell line

mRNA of TF-1 cells was extracted, and complementary DNA was synthesized using PrimeScript<sup>TM</sup> 1st Strand cDNA Synthesis Kit (Takara). The cDNA of CD32A was amplified by PCR using specific primers (Forward primer: 5'-CACTGGACGTTGGC ACAGTGCTGGG -3, reverse primer: 5'-ATGACCACATGGC ATAACGTTACTC -3). The CD32A genotype of TF-1 cell line was determined by sequencing the PCR product (Genewiz).

### Abbreviations

AHR	airway hyper-responsiveness
DCs	dendritic cells
FPLC	fast protein liquid chromatography
FACS	fluorescence-activated cell sorting
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif

ICs	immune complexes
IL-4	interleukin-4
IL-4R	IL-4 receptor
IL-13	interleukin-13
ITAMi	inhibitory ITAM
MHCII	major histocompatibility complex class II
mAb	monoclonal antibody
ORF	open reading frame
PBMCs	peripheral blood mononuclear cells
Th2	T-helper type 2.

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## Disclosure of Potential Conflicts of Interest

None. All authors are employees of 3sBio when the study was carried out.

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