MEDI-563, a humanized anti–IL-5 receptor α mAb with enhanced antibody-dependent cell-mediated cytotoxicity function

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Background: Peripheral blood eosinophilia and lung mucosal eosinophil infiltration are hallmarks of bronchial asthma. IL-5 is a critical cytokine for eosinophil maturation, survival, and mobilization. Attempts to target eosinophils for the treatment of asthma by means of IL-5 neutralization have only resulted in partial removal of airway eosinophils, and this warrants the development of more effective interventions to further explore the role of eosinophils in the clinical expression of asthma. Objective: We sought to develop a novel humanized anti–IL-5 receptor α (IL-5Rα) mAb with enhanced effector function (MEDI-563) that potently depletes circulating and tissue-resident eosinophils and basophils for the treatment of asthma. Methods: We used surface plasmon resonance to determine the binding affinity of MEDI-563 to FcγRIIIa. Primary human eosinophils and basophils were used to demonstrate antibody-dependent cell-mediated cytotoxicity. The binding epitope of MEDI-563 on IL-5Rα was determined by using site-directed mutagenesis. The consequences of MEDI-563 administration on peripheral blood and bone marrow eosinophil depletion was investigated in nonhuman primates. Results: MEDI-563 binds to an epitope on IL-5Rα that is in close proximity to the IL-5 binding site, and it inhibits IL-5–mediated cell proliferation. MEDI-563 potently induces antibody-dependent cell-mediated cytotoxicity of both eosinophils (half-maximal effective concentration = 0.9 pmol/L) and basophils (half-maximal effective concentration = 0.5 pmol/L) *in vitro*. In nonhuman primates MEDI-563 depletes blood eosinophils and eosinophil precursors in the bone marrow. Conclusions: MEDI-563 might provide a novel approach for the treatment of asthma through active antibody-dependent cell-mediated depletion of eosinophils and basophils rather than through passive removal of IL-5. (J Allergy Clin Immunol 2010;125:1344-53.)

Key words: *Asthma, eosinophil, antibody-dependent cell-mediated cytotoxicity, FcγRIIIα, basophil, IL-5, IL-5 receptor, monoclonal antibody*

Activated eosinophils are the cellular source of granule-associated basic proteins,[1] reactive oxygen species,[2] and lipid mediators,[3] which collectively can damage surrounding cells and induce airway hyperresponsiveness and mucus hypersecretion.[4,5] IL-5 is the principal cytokine mediating eosinophil mobilization, maturation, activation, and survival.[6] In human subjects IL-5 receptor (IL-5R) is expressed exclusively on eosinophil and basophil progenitors in the bone marrow (BM) and on mature eosinophils and basophils.[7-10] Indeed, neutralization of IL-5 in murine[11] and nonhuman primate[12] models of asthma resulted in reduction of eosinophil counts, which was associated with improved lung pathology. Furthermore, increased numbers of eosinophils in the airways and peripheral blood of subjects with asthma have been shown to correlate with asthma severity.[13]

These findings prompted the development of IL-5–neutralizing monoclonal antibodies (mAbs). In initial clinical trials IL-5 neutralization in subjects with mild-to-moderate asthma resulted in almost complete depletion of circulating and sputum eosinophil counts, but it did not improve lung function.[14,15] These observations have recently been corroborated in subjects with severe refractory asthma,[16,17] yet IL-5 neutralization by mepolizumab in those trials significantly reduced exacerbation frequency, improved Asthma Quality of Life Questionnaire scores, and allowed prednisone sparing, demonstrating for the first time a causal role for eosinophils in asthma exacerbations.[16,17] Interestingly, although mepolizumab significantly reduced circulating and sputum eosinophil counts, its effect on reducing mucosal eosinophilia was only partial at best and did not reach significance, even after prolonged high-dose exposure (12 months, 750 mg/mo).[16,18] Therefore more powerful means of depleting eosinophils in lung tissue are needed to further explore the contribution of eosinophils to asthma pathology.

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Abbreviations used

ADCC: Antibody-dependent cell-mediated cytotoxicity
APC: Allophycocyanin
BM: Bone marrow
BMMNC: Bone marrow mononuclear cell
EC_{50}: Half-maximal effective concentration
ECP: Eosinophil cationic protein
EDN: Eosinophil-derived neurotoxin
ELISA: Enzyme linked immunosorbent assay
FcγR: Fcγreceptor
IL-5R: IL-5 receptor
mAb: Monoclonal antibody
NK: Natural killer

Here we describe the development of MEDI-563, a novel humanized afucosylated monoclonal antibody IgG1κ mAb specific for the human IL-5Rα.[19] MEDI-563 binds to a conformationally distinct epitope within domain 1 of IL-5Rα, a region previously implicated in IL-5 binding.[20] Afucosylation of the oligosaccharide core of human IgG1 has previously been shown to result in a 5- to 50-fold higher affinity to human FcγRIIIa, the main activating Fcγ receptor (FcγR) expressed on natural killer (NK) cells, macrophages, and neutrophils.[21,22] Afucosylation enhances the interaction of MEDI-563 with FcγRIIIa and heightens antibody-dependent cell-mediated cytotoxicity (ADCC) functions by more than 1,000-fold over the parental antibody. These MEDI-563 properties might result in a more complete removal of airway eosinophils and basophils and subsequently result in greater reductions of asthma exacerbations and possibly improvements in other clinical expressions of asthma.

METHODS

Epitope mapping of MEDI-563

Extracellular IL-5Rα knockout mutants were engineered by substituting regions of full-length human IL-5Rα with corresponding segments of murine IL-5Rα and *vice versa* for knock-in mutants. Mutants were transiently expressed in HEK293F cells. HEK293F transfectants were incubated with 1 μg/mL MEDI-563 for 1 hour on ice in phosphate-buffered saline (PBS). After washing, cells were incubated with goat anti-human IgG–fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories, West Grove, Pa) and then analyzed with the LSRII flow cytometer (BD Biosciences, San Jose, Calif). Expression levels of swap mutants were monitored with either goat anti-human IL-5Rα polyclonal antibody (GeneTex, San Antonio, Tex) or goat anti-mouse IL-5Rα polyclonal antibody (R&D Systems, Minneapolis, Minn).

ADCC assays

ADCC assays were performed with autologous NK cells as effector cells, as indicated. 10E4 NK cells and 10E5 bone marrow mononuclear cell (BMMNCs; approximately 1% IL-5Rα$^+$ cells) were added to each well in 96-well, flat-bottom microtiter plates. Serial dilutions of MEDI-563, parent αIL-5Rα mAb, or afucosylated hIgG1 isotype control were added. After 18 hours of incubation at 37°C, the assays were stopped. The number of IL-5Rα$^+$ cells was determined by means of flow cytometry with anti–IL-5Rα mAb KM 1257 (10 μg/mL) and phycoerythrin-labeled goat anti-mouse IgG F(ab′)2 (Jackson ImmunoResearch Labs). For ADCC assays with peripheral blood–derived eosinophils or basophils, 5× 10E4 NK cells and 10E4 eosinophils or basophils were coincubated in 96-well flat-bottom plates in the presence of serial dilutions of MEDI-563 or parent αIL-5Rα mAb for 22 hours. Assays were stopped by putting plates on ice and replacing the culture medium with PBS/BSA to which Annexin V Alexa 647 at 1:500 dilution was added. Cells were analyzed on a flow cytometer (LSRII, BD Biosciences) and the percentage of Annexin V–positive eosinophils/basophils was measured. Eosinophils were identified based on their granularity (high side scatter) and basophils based on FcεRIα expression. ADCC was determined by gating on Annexin V–positive target cells. The recovery of target cells was quantitative (approximately 20% of the total cell number). Supernatants were collected at the end of ADCC assays to measure eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) levels by using an enzyme linked immunosorbent assay (ELISA). Total ECP and EDN levels were determined by means of eosinophil lysis with 1% Triton X-100 (100% degranulation), and a mixture of the cytokines RANTES (13 nmol/L), eotaxin (12 nmol/L), and IL-33 (6 nmol/L) was used as a positive control.

Administration of MEDI-563 in nonhuman primates

Thirty-four female and male cynomolgus monkeys were administered vehicle (n = 10) or MEDI-563 at 0.1 mg/kg (n = 4), 1 mg/kg (n = 6), 10 mg/kg (n = 4), or 30 mg/kg (n = 10) intravenously once on days 1, 22, 43, and 64. Six monkeys each from the vehicle, 1 mg/kg, and 30 mg/kg groups and 4 monkeys each from the 0.1 mg/kg and 10 mg/kg groups were killed and necropsied on day 67 (terminal necropsy), and the remaining monkeys were killed and necropsied on day 85 (recovery necropsies). BM smears were taken on the days of the necropsy. Eosinophil and neutrophil precursors (myeloblast and promyeloblast stages) were enumerated based on morphologic appearance. Blood was drawn on days −10 and −3 (baseline) and on days 4, 25, 46, and 64 during MEDI-563 administration, and eosinophils were enumerated with an Advia 120 hematology analyzer (Siemens, Deerfield, Ill). See the Methods section in this article's Online Repository at www.jacionline.org for additional information on reagents, proteins, and antibodies; cell lines and primary cells; measurements of kinetic rates and binding constants; Affymetrix gene array analysis; CTLL-2 proliferation assay; flow cytometry; ELISA; and immunohistochemistry.

RESULTS

MEDI-563 interaction with IL-5Rα

MEDI-563 bound to recombinant human and cynomolgus monkey IL-5Rα extracellular domains with a dissociation constant of 11 and 42 pmol/L, respectively, whereas the F(ab) fragment bound with an approximately 100-fold lower affinity, as assessed by means of surface plasmon resonance (Table I). Consistent with the specific expression of IL-5Rα on human eosinophils and basophils among a large variety of hematopoietic cell types (Fig 1, *A*), MEDI-563 exclusively stained peripheral blood eosinophils and basophils from healthy subjects (Fig 1, *B*). We consistently found that eosinophils expressed about a 3-fold higher level of IL-5Rα compared with basophils, as quantified based on median fluorescence intensity (Fig 1, *B*). In addition, MEDI-563 identified a small but specific fraction (approximately 0.9%) of BMMNCs that most likely represented the eosinophil/basophil lineage precursors (Fig 1, *B*). To further characterize the binding affinity of MEDI-563 to cell-surface IL-5Rα on peripheral blood eosinophils, we used flow cytometry in the presence of increasing concentrations of mAb. To overcome the high-background staining from the enhanced MEDI-563 interaction with FcγRIII expressed on human and cynomolgus monkey eosinophils,[23,24] we used the fucosylated parent αIL-5Rα mAb, which only differs from MEDI-563 in its lower binding affinity for FcγRIII (Table II). Parent αIL-5Rα stained human and cynomolgus monkey peripheral blood eosinophils with a half-maximal effective concentration (EC_{50}) of 26 and 40 pmol/L, respectively (Fig 1, *C*), values comparable with the MEDI-563 binding affinities to the extracellular receptor domains (Table I). Both mAbs inhibited IL-5–induced proliferation of CTLL-2 cells

TABLE I. Kinetic rate/binding constants of MEDI-563 and MEDI-563 F(ab) to human and cynomolgus monkey IL-5Rα

	MEDI-563			MEDI-563 F(ab)		
	K_{on} (1/ms × 10^5) ± SEM	K_{off} (1/s × 10^{-3}) ± SEM	K_D (nmol/L) ± SEM	K_{on} (1/ms × 10^5) ± SEM	K_{off} (1/s × 10^{-3}) ± SEM	K_D (nmol/L) ± SEM
Human IL-5Rα	43.6 ± 0.5	0.048 ± 0.02	0.011 ± 0.005	15.8 ± 2.6	1.92 ± 0.01	1.26 ± 1.0
Cyno IL-5Rα	252 ± 141	0.818 ± 0.301	0.042 ± 0.035	15.7 ± 2.1	31.8 ± 1.25	20.5 ± 1.95

Cyno, Cynomolgus monkey; K_D, dissociation constant K_{off}, off rate; K_{on}, on rate; *SEM*, standard error of the mean.

transfected with recombinant human IL-5Rαβ with identical potencies (half maximal inhibitory concentration = 0.3 nmol/L; Fig 1, *D*).

Mapping the binding epitope of MEDI-563 on IL-5Rα

The lack of MEDI-563 binding to the murine IL-5Rα was exploited as a means to identify the human IL-5Rα receptor epitope recognized by MEDI-563. Extracellular human IL-5Rα domains 1 (D1), 2 (D2), and 3 (D3) were replaced with the corresponding murine IL-5Rα domain sequences to create knockout variants or *vice versa* to create knock-in variants (Fig 2, *A*). The expression levels of all variants were monitored with anti-human or anti-mouse IL-5Rα polyclonal antibodies by using flow cytometry. In each instance MEDI-563 bound only to the constructs containing human IL-5Rα D1 (Fig 2, *A*). An alignment of human, cynomolgus monkey, and murine IL-5Rα D1 amino acid sequences identified differences between the receptors, and these areas were targeted to further characterize the binding epitope of MEDI-563 (Fig 2, *B*). Only swap mutants encoding human segment B were recognized by MEDI-563, thus identifying the region containing the binding epitope (Fig 2, *C*). Further refinement of the epitope was possible by swapping the amino acids in segment B not conserved between the human and murine sequences. Substituting amino acids N40, N42, Q46, D56, and E58 in the human IL-5Rα segment B with the corresponding murine residues had no effect on the MEDI-563 binding to human IL-5Rα. However, a single amino acid change to isoleucine at position 61 (I61) was sufficient to confer the MEDI-563 binding to murine IL-5Rα. Conversely, MEDI-563 binding to human IL-5Rα was obliterated by replacing I61 with the murine lysine residue at position 61 (K61), ultimately identifying amino acid I61 from the human IL-5Rα as the critical residue for MEDI-563 binding (Fig 2, *D*). Furthermore, segment B (amino acids 40-61) in D1 (including I61) is 100% conserved between human subjects and cynomolgus monkeys, thus explaining the cross-reactivity of MEDI-563 with cynomolgus monkey IL-5Rα (Fig 2, *B*).

MEDI-563 mediates eosinophil apoptosis *in vitro* through enhanced ADCC

The absence of the monosaccharide fucose on the oligosaccharide core of the human IgG1 has previously been shown to result in an enhanced binding affinity to human FcγRIIIa and subsequently an enhanced ADCC. Indeed, when tested by means of surface plasmon resonance with soluble human FcγR domains, the binding affinity of MEDI-563 for human FcγRIIIa was increased 6-fold compared with the fucosylated parental anti–IL-5Rα mAb (parent αIL-5Rα mAb) but was similar for all other FcγRs tested (Table II). We next investigated the potency of MEDI-563 to mediate eosinophil and basophil apoptosis *in vitro*. In the presence, but not absence, of autologous NK effector cells, MEDI-563 induced eosinophil and basophil apoptosis, as assessed by means of Annexin V staining, with EC_{50} values of 0.9 and 0.5 pmol/L, respectively (Fig 3, *A*). However, when the fucosylated parental αIL-5Rα mAb was used at concentrations 1,000-fold higher than the MEDI-563 EC_{50}, it did not induce target-cell apoptosis above background levels. (Fig 3, *A*), although its binding affinity for IL-5Rα (data not shown) and its potency to inhibit IL-5–induced cell proliferation (Fig 1, *D*) were indistinguishable from those of MEDI-563. MEDI-563 also depleted human IL-5Rα$^+$ BMMNCs when cocultured with NK effector cells, whereas an irrelevant afucosylated isotype control mAb was ineffective (Fig 3, *B*). In contrast to stimulation with a mixture of cytokines (RANTES, eotaxin, and IL-33), eosinophil apoptosis induced by MEDI-563 was not associated with the release of EDN or ECP, indicating a lack of significant eosinophil degranulation (Fig 3, *C*). Taken together, these data clearly indicate an enhanced MEDI-563 ADCC potency to deplete IL-5Rα–expressing eosinophils, basophils, and BMMNCs *in vitro* as a result of fucose deficiency.

Depletion of eosinophils in BM and peripheral blood of nonhuman primates

We have shown that MEDI-563 binds to human and cynomolgus monkey IL-5Rα on eosinophils with similar potency (Fig 1, *C*) and to human and cynomolgus monkey FcγRIIIa with a 6- and 8-fold higher affinity, respectively, compared with the parent αIL-5Rα mAb (Table II). Thus investigating the mechanism and potency of MEDI-563 to deplete eosinophils in cynomolgus monkeys proved to be a useful tool in predicting its effects in human subjects. Four MEDI-563 (0.1, 1, 10, and 30 mg/kg) or vehicle intravenous doses were administered to cynomolgus monkeys once every 3 weeks for 12 weeks. Peripheral blood eosinophils and BM eosinophil precursors were measured at different time points after drug administration. Blood eosinophil counts decreased close to the limit of detection after the first administration of MEDI-563 at all dose levels investigated and remained undetectable for the rest of the study (Fig 4, *A*). Similarly, eosinophil precursors in the BM, as assessed by histologic methods, were reduced 80% or greater in all the MEDI-563 doses 3 days after the last administration (terminal necropsy) and remained undetectable until 18 days after the last dose in the 30 mg/kg group (recovery necropsy; Fig 4, *B*). The profound effect observed with MEDI-563 in the BM was specific for the eosinophil lineage because numbers of neutrophil precursors (myeloblast and promyelocyte stages) remained unchanged (Fig 4, *C*). In conclusion, the pharmacologic efficacy and acceptable safety characteristics exhibited by MEDI-563 warrant its further investigation in subjects with asthma.

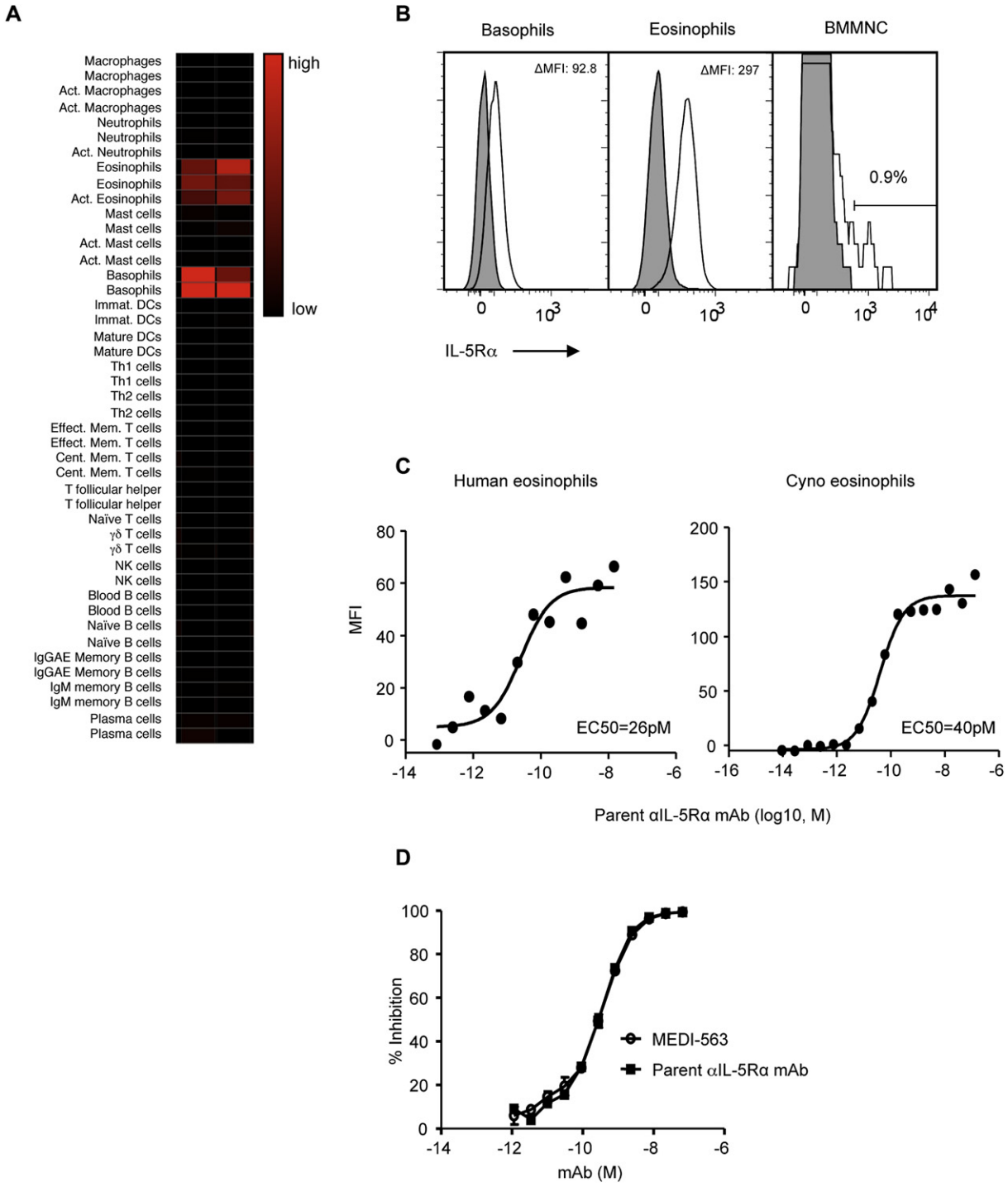

FIG 1. Interaction of MEDI-563 with IL-5Rα. **A**, *IL5RA* mRNA expression on human immune cells. **B**, Human IL-5Rα expression on basophils (n = 2), eosinophils (n = 3), and BMMNCs (n = 4) using MEDI-563 F(ab′)$_2$ or an irrelevant control antibody *(shaded areas)*. **C**, Binding of parent αIL-5Rα mAb to human and cynomolgus monkey *(Cyno)* eosinophils. *MFI*, Mean fluorescence intensity. **D**, Inhibition of IL-5–induced CTLL-2 cell proliferation (± SD). Shown are representative experiments. *n*, Number of experiments performed.

IL-5Rα expression in lung tissue of subjects with mild asthma

To gain better insight into the ability of MEDI-563 to bind to lung tissue–resident eosinophils, we performed immunohistochemistry studies on biopsy specimens from subjects with mild atopic asthma. Mast cells were frequently present in both bronchi (49 ± 8 cells/mm^2) and peripheral tissue (62 ± 15 cells/mm^2) along with tissue eosinophilia (bronchi, 168 ± 90 cells/mm^2; peripheral

TABLE II. Binding affinities of MEDI-563 and parent αIL-5Rα mAb to human and cynomolgus monkey Fcγ receptors assessed by means of surface plasmon resonance*

	Human FcγRI, K_D (nmol/L) ± SEM	Human FcγRIIa, K_D (nmol/L) ± SEM	Human FcγRIIb, K_D (nmol/L) ± SEM	Human FcγRIIIa(V), K_D (nmol/L), ± SEM	Cyno FcγRI, K_D (nmol/L)	Cyno FcγRIIa, K_D (nmol/L)	Cyno FcγRIIb, K_D (nmol/L)	Cyno FcγRIIIa, K_D (nmol/L) ± SEM
MEDI-563	18.5± 2.5	1,280 ± 10	4,580 ± 1,150	45.5 ± 0.5	1	3,170	2,070	23 ± 3
Parent αIL-5Rα mAb	18	1,170	3,890	275.5 ± 0.5	1	2,970	1,720	195.5 ± 10.5

Cyno, Cynomolgus monkeys; K_D, dissociation constant; *SEM*, standard error of the mean.
*Note the 6- and 8-fold higher affinity of MEDI-563 for human and cynomolgus monkey FcγRIIIa, respectively, without affecting the affinity for other Fcγ receptors.

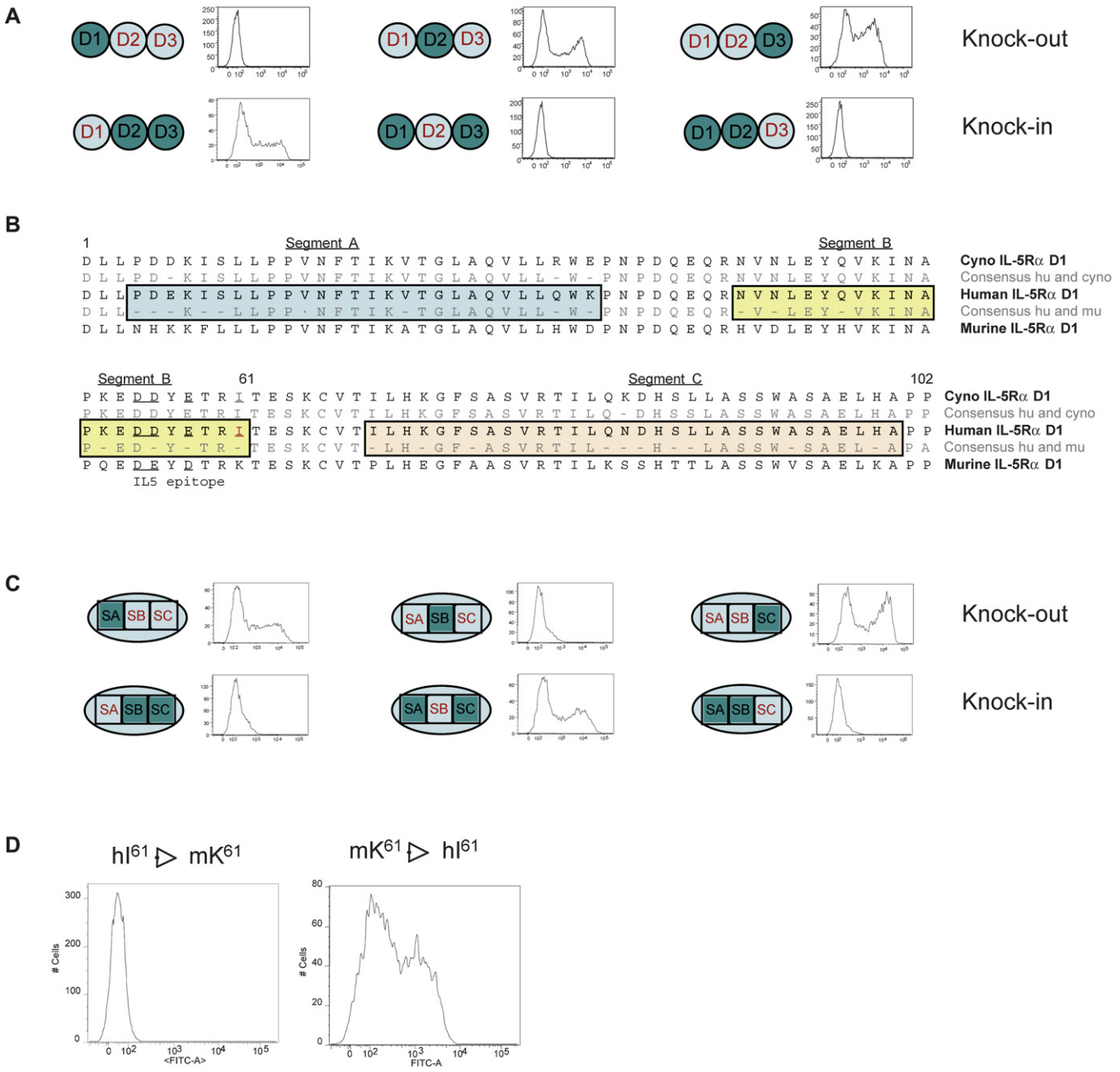

FIG 2. Identification of the MEDI-563 binding epitope to human IL-5Rα. **A**, MEDI-563 binding to extracellular chimeric human IL-5Rα *(light green)* and murine IL-5Rα *(dark green)* variants. *D*, Domain. **B**, Sequence alignment of murine, human, and cynomolgus monkey *(Cyno)* IL-5Rα D1 (differences are shown as *dashes*; segments A *[SA]*, B *[SB]*, and C *[SC]* are boxed). **C**, MEDI-563 binding to IL-5Rα segment variants. **D**, Isoleucin 61 (I61) of human IL-5Rα is the critical residue for MEDI-563 binding. I61 is shown in *red* in Fig 2, *B*.

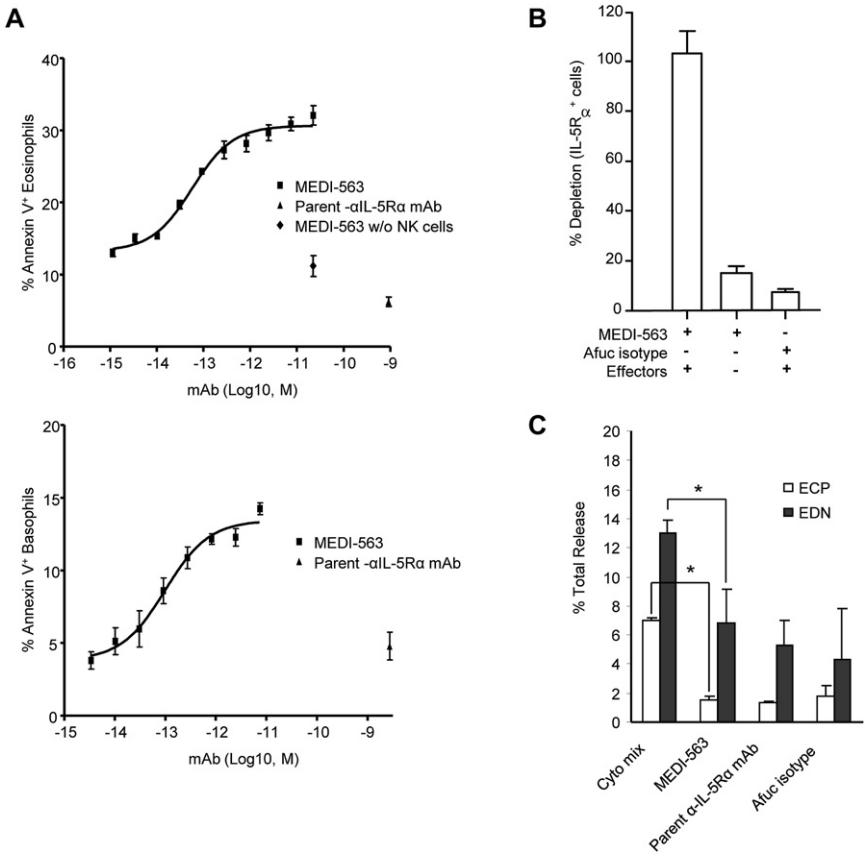

FIG 3. Enhanced ADCC of MEDI-563. **A**, MEDI-563–mediated ADCC of human eosinophils (EC_{50} = 0.9 pmol/L, n = 5) and basophils (EC_{50} = 0.5 pmol/L, n = 3). **B**, Depletion of human IL-5Rα$^+$ BMMNCs by MEDI-563 *in vitro* (n = 2). **C**, ADCC of eosinophils is not associated with ECP and EDN release (n = 2). *Afuc isotype*, Afucosylated isotype antibody control. Total ECP and EDN release induced by Triton X-100 was set to 100%. *Cyto mix*, RANTES plus eotaxin plus IL-33. Shown are representative experiments. *n*, Number of experiments performed. *Significant as determined by using the Student *t* test ($P < .05$).

tissue, 227 ± 59 cells/mm^2). In both bronchial and peripheral tissue biopsy specimens, scattered cells displayed an intense immunoreactivity with MEDI-563 (Fig 5, *A* and *B*). Generally, the staining had a characteristic surface membrane pattern (Fig 5, *A*, inset). No staining was found with isotype-matched control antibodies or in control subjects lacking primary antibodies. Furthermore, nonspecific binding of MEDI-563 to Fc receptors on eosinophils was ruled out because F(ab′)$_2$ fragments of MEDI-563 produced a similarly intense immunoreactivity. Parallel identification of eosinophils and MEDI-563 staining confirmed a widespread and uniform MEDI-563–positive immunoreactivity on tissue eosinophils at all airway levels (Fig 5, *C*). Double staining for IL-5Rα and mast cell tryptase was performed to test whether MEDI-563 bound to mast cells (Fig 5, *D-F*). In short, our analysis failed to detect any colocalization, suggesting that airway mast cells do not express IL-5Rα.

In conclusion, the present study demonstrates the capacity of MEDI-563 to recognize tissue eosinophils in both central and peripheral airways of subjects with asthma. Although single intravenous administrations of MEDI-563 in an initial phase I study of subjects with mild asthma resulted in persistent peripheral blood eosinopenia lasting up to 3 months,[25] it remains to be seen whether MEDI-563 can also efficiently deplete lung tissue eosinophils.

DISCUSSION

IL-5 blockade in subjects with asthma has failed to improve parameters of lung function in response to allergen challenge despite rapid and near-complete depletion of eosinophils from peripheral blood and sputum.[14-17] However, a causal link between reduced eosinophil numbers and a reduction in exacerbation frequency and prednisone requirement has recently been demonstrated in subjects with severe refractory eosinophilic asthma.[16,17] Interestingly, results from 2 independent clinical trials demonstrated that eosinophil depletion from the airways only reached 50% to 60% compared with eosinophil counts before drug administration, even with repeated doses of the anti–IL-5 antibody mepolizumab.[16,18] Although IL-5 is considered to be essential for eosinophil differentiation and mobilization, other cytokines, including IL-3 and GM-CSF, are also important for eosinophil survival. This might provide an explanation for the eosinophil persistence during IL-5 blockade.[26,27] Therefore a strategy that depletes lung eosinophils and basophils more effectively might result in a greater reduction in exacerbation frequency and provide better asthma control.

The restricted expression of IL-5Rα on human eosinophils and basophils and their progenitors in the BM renders IL-5Rα an ideal target for specific cell depletion. Compared with IL-5–neutralization strategies, our anti–IL-5Rα mAb, MEDI-563, might

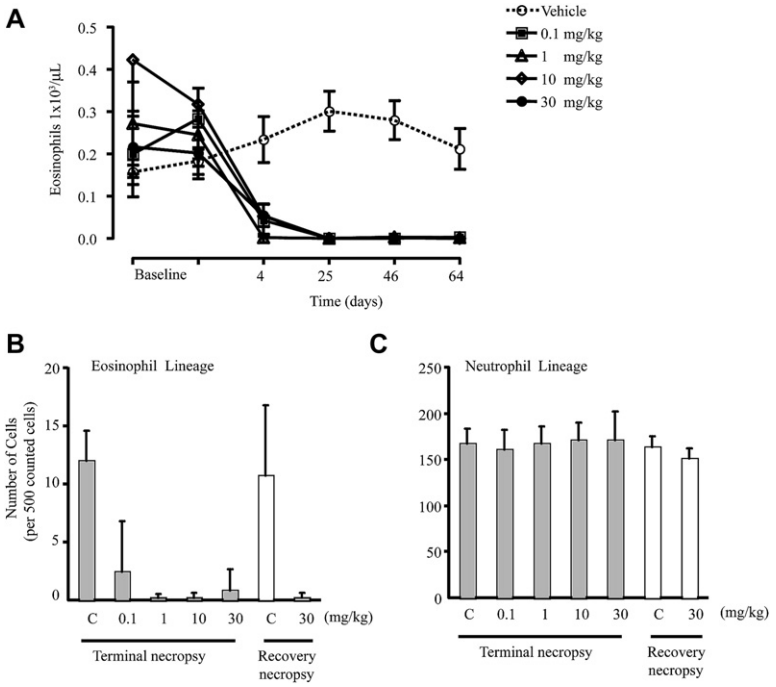

FIG 4. Eosinophil depletion in cynomolgus monkeys by MEDI-563. **A**, Peripheral blood eosinophil depletion in cynomolgus monkeys. **B** and **C**, Depletion of BM eosinophil precursors (Fig 4, *B*) and neutrophil precursors (Fig 4, *C*). Data indicate the average of 3 to 10 monkeys per group, and *error bars* represent the SD.

provide a more complete depletion of eosinophils through enhanced ADCC. MEDI-563 inhibits IL-5R signaling; however, this neutralizing effect is not central to its biological function. Afucosylation of MEDI-563 significantly increases its binding affinity to FcγRIIIa and consequently enhances its ability to engage with FcγRIIIa on effector cells, such as NK cells and macrophages. Indeed, only in the presence of NK effector cells did MEDI-563 mediate eosinophil and basophil apoptosis, with an EC_{50} of 0.9 and 0.5 pmol/L, respectively, and deplete IL-5Rα–expressing BMMNCs *in vitro*. However, even when used at concentrations 4 orders of magnitude greater than the MEDI-563 EC_{50}, the parental fucosylated αIL-5Rα mAb did not induce significant eosinophil killing, thus illustrating the importance of enhanced Fc engagement. Interestingly, although peripheral blood basophils expressed about 3-fold less IL-5Rα compared with eosinophils, the ADCC potency of MEDI-563 was indistinguishable. Therefore very low levels of cell-surface IL-5Rα expression seemed to be sufficient for the engagement of MEDI-563 in mediating potent ADCC of IL-5Rα–expressing target cells.

We have mapped the MEDI-563 binding site to an epitope that has previously been identified as a portion of the IL-5 binding site,[20] providing an explanation for its neutralizing activity. Furthermore, we have identified I61 on the human IL-5Rα D1 to be essential for MEDI-563 binding. I61 is conserved in human and cynomolgus monkey IL-5Rα, but not in murine IL-5Rα, explaining the cross-reactivity of MEDI-563 to cynomolgus monkey IL-5Rα but not to the murine homolog.

MEDI-563 bound to immobilized recombinant IL-5Rα and IL-5Rα expressed on human and cynomolgus monkey peripheral blood eosinophils with high affinity (26 and 40 pmol/L, respectively). However, the binding affinity of its F(ab) fragment to immobilized recombinant human and cynomolgus monkey IL-5Rα extracellular domains was about 100-fold lower, suggesting that avidity might enhance the binding of MEDI-563 to IL-5Rα. MEDI-563 also inhibited IL-5–induced proliferation of human IL-5Rαβ transfected CTLL-2 cells *in vitro*, although with a potency of 300-fold lower than its ADCC activity on eosinophils and basophils. It might be possible that neutralization of IL-5–mediated proliferation of transfected CTLL-2 cells requires a high degree of receptor occupancy by MEDI-563, whereas engagement of only a few receptors is sufficient to mediate potent ADCC. Eosinophils are the source of cationic granule proteins, such as major basic protein, ECP, EDN, and eosinophil peroxidase, which are stored in crystalloid granules. When released into the extracellular space, these basic proteins have been shown to be toxic to parasites and bacteria.[28,29] However, the toxicity is not only limited to foreign pathogens but also affects diverse cell types in the host. For example, major basic protein has been shown to induce airway hyperresponsiveness[5] and cytotoxicity against the airway epithelium,[30,31] whereas both ECP and EDN cause neurotoxicity.[32] Therefore ADCC-mediated killing of eosinophils might pose substantial safety risks associated with the potential release of cationic eosinophil proteins during cytolysis or accidental necrosis.[33] We measured the release of EDN and ECP in ADCC assays *in vitro* and found no indication that eosinophil apoptosis mediated by MEDI-563 was associated with increased levels of these proteins.

In nonhuman primates repeat administrations of MEDI-563 at all dose levels rapidly depleted peripheral blood eosinophils to less than the limit of detection, demonstrating the desired pharmacologic effect. Of particular note, MEDI-563 also depleted eosinophil precursors from the cynomolgus monkey BM without affecting granulocytic stem cell counts (myeloblast and promyelocyte stages). Because FcγRIIIa expression is conserved

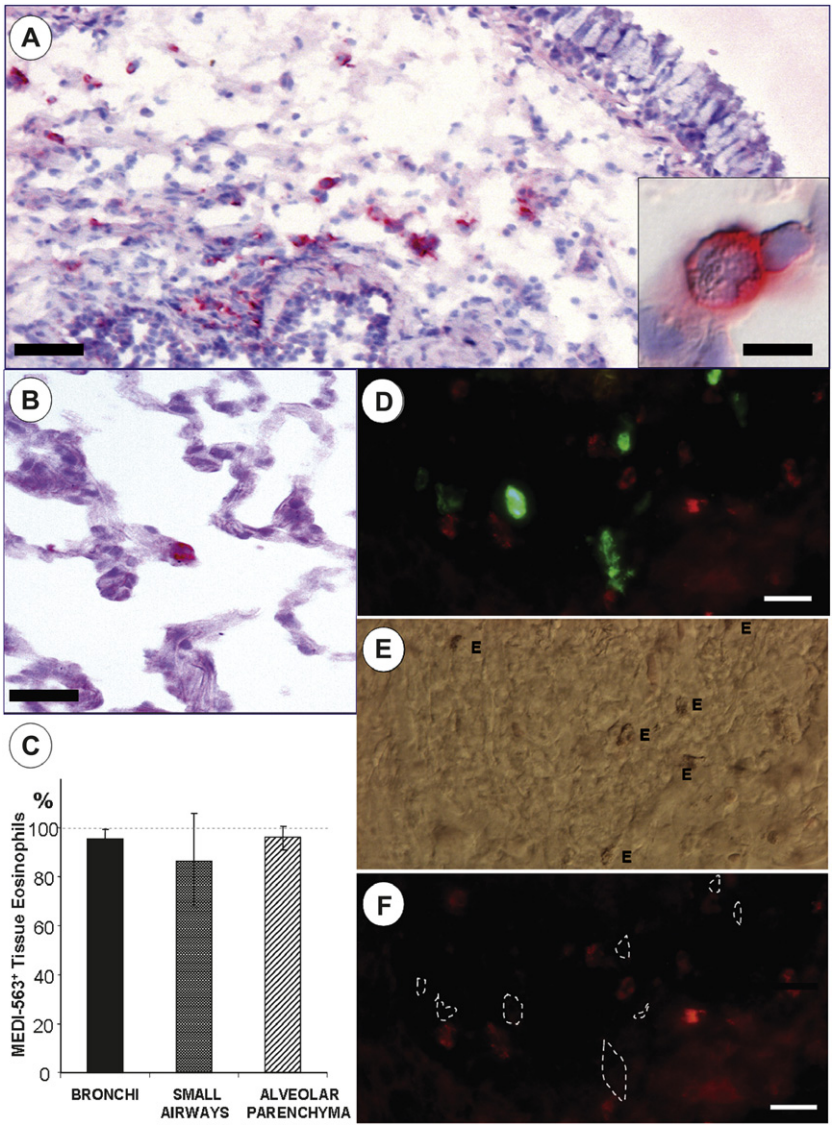

FIG 5. Immunohistochemical localization of IL-5Rα–expressing cells in lung tissue. **A** and **B**, Bright field micrographs depicting MEDI-563 immunoreactive cells *(red)* in the bronchial mucosa (Fig 5, *A*) and the alveolar parenchyma (Fig 5, *B*). Fig 5, *A*, *inset*, High-power magnification. **C**, Quantification of eosinophils positive for MEDI-563 staining (± SD). **D**, Mast cell tryptase *(green)* and IL-5Rα *(red)*. **E**, Red-stained eosinophils *(E)* in Fig 5, *D*, are identified through their distinct granule morphology. **F**, Lack of IL-5Rα staining in mast cells.

between human subjects and cynomolgus monkeys,[23,34,35] the data suggest that similar mechanisms might operate in both species for the depletion of peripheral blood eosinophils and possibly that of eosinophil precursors in the BM. Indeed, administration of single intravenous MEDI-563 doses in an initial phase I study of subjects with mild asthma has demonstrated rapid, long-lasting, and reversible depletion of peripheral blood eosinophils in a dose-dependent manner.[25] Whether the depletion of eosinophil precursors in the BM of human subjects might contribute to the sustained peripheral eosinopenia induced by MEDI-563 remains to be demonstrated. In 2 case studies eosinophil and basophil deficiency have been reported to be associated with reduced immunity and recurrent infections,[36,37] raising the possibility that sustained eosinophil and basophil depletion in human subjects by MEDI-563 might result in similar safety signals. Whether this will indeed be the case is currently being addressed in clinical trials with multiple administrations of MEDI-563.

Using immunohistochemical methods, we have demonstrated that MEDI-563 prominently stained all the eosinophils from lung biopsy specimens of subjects with mild asthma, a prerequisite for the successful and swift removal of eosinophils from the lung through mechanisms involving ADCC. Active and rapid depletion of lung eosinophils will also depend on the appropriate number and localization of effector cells and sufficient levels of MEDI-563 in lung tissue. Furthermore, MEDI-563–mediated depletion of eosinophils in subjects with asthma might be influenced by the downregulation of IL-5Rα on eosinophils in the lung after an allergen challenge,[38] IL-5 binding,[39,40] and the use of corticosteroids.[41] These factors alone or in combination might affect the degree and kinetics of MEDI-563–mediated

eosinophil depletion in subjects with asthma. Currently, clinical studies are in progress to address the effectiveness of MEDI-563 in depleting lung eosinophils in subjects with eosinophilic asthma.

Basophils are associated with allergic inflammation and, similar to eosinophils, are linked to asthma severity and exacerbations. Recent *in vitro* studies have demonstrated that basophils constitutively express IL-5Rα[8] but do not require the cytokine IL-5 for survival.[42] *In vivo* basophils have been shown to play a critical role in the development of IgE-mediated chronic allergic inflammation in the skin[43] and to increase in numbers in the airways of asthmatic subjects on allergen provocation.[44] Here we present data showing that MEDI-563 potently induces basophil apoptosis *in vitro*. However, the ability of MEDI-563 to deplete basophils *in vivo* and its downstream consequences remain to be demonstrated. Also, mast cells and mast cell–derived mediators, such as biogenic amines, leukotrienes, cytokines, and chemokines, have been implicated in asthma pathogenesis. In lung biopsy specimens from subjects with mild asthma, we have not been able to detect IL-5Rα expression in mast cells, although a recent study has demonstrated IL-5Rα expression in human mast cells *in vitro*.[45] One might speculate that mast cells could express IL-5Rα *in vivo* and become transiently sensitive to MEDI-563–mediated depletion under certain circumstances. Currently, no experimental evidence exists to support such a notion.

In summary, we report the characterization of a novel anti–IL-5Rα mAb, MEDI-563, that mediates the killing of eosinophils and basophils *in vitro* and in nonhuman primates through an enhanced ADCC function. Continued investigation of MEDI-563 activity in asthmatic subjects might provide new insights into the relative contributions of eosinophils and basophils, and active depletion of these cells might provide a novel approach for the treatment of bronchial asthma.

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Key message

- **MEDI-563 is a humanized anti–IL-5Rα mAb with enhanced ADCC function that potently induces eosinophil apoptosis *in vitro* and efficiently depletes peripheral blood eosinophils and eosinophil precursors in nonhuman primates.**

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METHODS

Reagents, proteins, and antibodies

Parent αIL-5Rα mAb and MEDI-563 were produced in wild-type Chinese Hamster Ovary (CHO) cells and *FUT8*-deficient CHO cells, respectively. Anti–IL-5Rα mAb KM1257 binds to an epitope on human IL-5Rα without interfering with MEDI-563 binding and *vice versa*. Antibodies were purified by means of Protein G affinity chromatography. Extracellular FcγR domains and extracellular IL-5Rα domains were constructed with C-terminal 6xHis tags, expressed in HEK293F cells, and purified by means of nickel affinity chromatography. Cytokines were purchased from R&D Systems (Minneapolis, Minn). RPMI 1640 medium containing high-glucose heat-inactivated FBS and horse serum was obtained from Invitrogen (Carlsbad, Calif).

Cell lines and primary cells

A CTLL-2 human IL-5Rαβ stably transfected cell line was received from Dr K. Takatsu (University of Tokyo). Cells were cultured in RPMI 1640 supplemented with 10% FBS, 50 μmol/L 2-mercaptoethanol, 1 ng/mL human IL-5, and 5 ng/mL murine IL-2 and cultured at 37°C and 5% CO_2 in saturating humidity. Eosinophils and basophils were isolated from heparinized peripheral blood of healthy donors. Red blood cells were removed by means of sedimentation in Hetasep (StemCell Technologies, Vancouver, British Columbia, Canada). Eosinophils or basophils were further enriched from the leukocyte fraction by means of negative selection with EasySep eosinophil or basophil enrichment kits, respectively (StemCell Technologies). The purity and viability of isolated cells were verified by means of flow cytometry. PBMCs were isolated by means of density gradient centrifugation with Histopaque-1077 (Sigma, St Louis, Mo). NK cells were isolated from PBMCs by means of negative selection with the EasySep NK enrichment kit (StemCell Technologies).

Measurements of kinetic rates and binding constants

All measurements were performed on a BIAcore 3000 instrument (BIAcore, Inc, Uppsala, Sweden). Human and cynomolgus monkey IL-5Rα was immobilized at low density onto separate flow cells on the same CM5 sensor chip by using a standard amino coupling chemistry, as outlined by the instrument's manufacturer. The final receptor domain densities were virtually identical at 585 RUs (human IL-5Rα) and 586 RUs (cynomolgus monkey IL-5Rα). A reference flow cell surface was also prepared on this sensor chip by using the identical immobilization protocol minus the protein. Serial dilutions of MEDI-563 and MEDI-563 F(ab) were prepared in HBS-EP buffer (pH 7.4) containing 0.01 mol/L HEPES, 0.15 mol/L NaCl, 3 mmol/L EDTA, and 0.005% P-20 surfactant. Samples were injected at a flow rate of 75 μL/min. Dissociation data were collected for 10 to 15 minutes, followed by a 60-second pulse of 4 mol/L $MgCl_2$ between injections to regenerate the receptor surfaces on the chip. MEDI-563 and parent αIL-5Rα were immobilized at high density onto separate flow cells on CM5 sensor chips by using a standard amino coupling chemistry, as outlined by the instrument's manufacturer, to determine the affinity (dissociation constant) for the binding of all available human and cynomolgus monkey FcγRs. The final IgG surface densities were very similar, ranging from 7,378 to 7,958 RUs. Stock solutions of FcγRs, starting at 4, 16, or 32 μmol/L were serially diluted to the desired concentrations with HBS-EP buffer. Different concentrations of FcγRs were then injected over both the IgG and reference cell surfaces at a flow rate of 5 μL/min. Binding data were collected for 50 minutes, followed by a 60-second pulse of 5 mmol/L HCl between injections to regenerate (remove bound FcγRs) from the IgG surfaces. Several buffer injections were also interspersed throughout the injection series. Select buffer injections were used along with the reference cell data to correct the raw datasets for injection artifacts, nonspecific binding interactions commonly referred to as double-referencing, or both.[E1] Fully corrected binding data were then globally fit to a 1:1 binding model (BIAevaluation 4.1 software; BIAcore, Inc) that included a term to correct for mass transport–limited binding, should it be detected. These analyses determined the kinetic rate (on or off) constants from which the apparent dissociation constant was then calculated as K_{off}/K_{on}.

Affymetrix gene array analysis

Total RNA was isolated from cells by using the RNeasy Total RNA Isolation Kit (Qiagen, Chatsworth, Calif) or TRIzol (Invitrogen Life Technologies), as per the manufacturer's instructions. cRNA was prepared and fluorescence intensities were measured on Affymetrix U133A and B arrays by using an Agilent GeneArray Laser Scanner, and absolute gene expressions were determined and scaled to 150 by using algorithms in MicroArray Analysis Suite 5.0 Software (Affymetrix). Spotfire (Somerville, Mass) was used to map gene expression patterns and produce heat maps. All of the datasets used in this study and the methods used for the purification of the relevant human leukocyte subsets have been previously described.[E2,E3]

CTLL-2 proliferation assay

CTLL-2 human IL-5Rαβ cells were washed twice with RPMI 1640 medium and cultured overnight in complete medium (no IL-2 and no IL-5). After overnight starvation, CTLL-2 human IL-5Rαβ cells were collected and adjusted to 0.2×10^6 cells/mL. Cells were plated at 50 μL per well in flat-bottom 96-well plates. IL-5 was adjusted to 1.2 ng/mL in complete medium, and 25 μL per well was added. Antibodies were adjusted to 40 μg/mL in complete medium, and serial 2-fold dilutions were prepared from these stocks. Antibody dilutions were added at 25 μL per well to the plate and incubated for 48 hours (at 5% CO_2 and 37°C). One hundred microliters of Titer Glo (Promega, Madison, Wis) assay solution was added to each well. After mixing, 150 μL per well was transferred into a new white 96-well plate (Corning, Lowell, Mass). Luminescence was read with the luminescence reader Victor 2 (PerkinElmer, Waltham, Mass).

Flow cytometry

Binding affinity of the parent αIL-5Rα mAb to eosinophils in whole blood from healthy human donors and cynomolgus monkeys was examined by means of flow cytometry. Human IgG1 was added to 100-μL whole blood serial dilutions of the parent αIL-5Rα mAb or control, and samples were vortexed briefly. After 1 hour of incubation at room temperature, samples were washed with PBS (2×2 mL). A 20 μL per tube 1:10 dilution of goat anti-human IgG Fcγ-specific $F(ab')_2$ allophycocyanin (APC) (Jackson ImmunoResearch Laboratories) was added and mixed well. After 1 hour at room temperature, 2 mL of FACS lysing buffer (BD Biosciences) was added. After 10 minutes, incubation samples were washed twice with PBS and analyzed on a LSRII flow cytometer. Eosinophils were identified in the granulocyte gate as cells with high autofluorescence in phycoerythrin and fluorescein isothiocyanate channels.[E4] Mean fluorescence intensity in the APC channel was recorded. Staining intensities were calculated by subtracting the isotype control mean fluorescence intensity from the parent αIL-5Rα mAb mean fluorescence intensity. Prism 5 software (Prism Software Corp, Irvine, Calif) was used to calculate binding curves and EC_{50} values.

ELISA

ECP and EDN levels in supernatants from ADCC experiments were quantified by means of ECP ELISA (MBL International, Woburn, Mass) and EDN ELISA (MBL International, Woburn, Mass), respectively.

Immunohistochemistry

A cohort consisting of 9 subjects (6 male and 3 female subjects) with mild asthma was selected. All subjects were atopic, and none were taking inhaled steroids at that time point in the study. Mean and range values for PD_{20} (methacholine) and FEV_1 percent predicted were 75.6 μg (16-145 μg) and 98.8% (88% to 124%), respectively. From each patient, multiple bronchial and transbronchial biopsy specimens were collected. Transbronchial biopsy specimens were performed under radiographic guidance. All subjects provided written informed consent to participate in the study, which was approved by the local ethics committee (Lund, Sweden). Immediately after, collected biopsy specimens were immersed in PLP fixative (2% paraformaldehyde, 0.075 mol/L

lysine, 0.037 mol/L sodium phosphate, and 0.01 mol/L periodate), subjected to fixation overnight, and thereafter rinsed in sucrose buffer before being mounted at −76°C in Tissue-Tek freeze mounting media. The usefulness of MEDI-563 for immunohistochemistry was tested through an initial screening of multiple immunohistochemical protocols. In the present study we used a protocol detecting MEDI-563 immunoreactivity in human tissues after direct labeling MEDI-563 or MEDI-563 $F(ab)_2$ with biotin or the fluorochrome Alexa-647 (Invitrogen). Briefly, 10 μmol/L cryosections were incubated with primary antibody for 2 hours at room temperature. As standard visualization, biotinylated MEDI-563 sections were incubated with AP-conjugated streptavidin, and the immunoreactivity was visualized with the New Fuchsin + Substrate-Chromogen System (K0625, Dako, Glostrup, Denmark), according to the manufacturer's directions. For visualization of the eosinophil IL-5Rα, eosinophils were identified by means of anti-ECP staining (EG-2, Pharmacia) or their characteristic granule morphology by using differential interference contrast microscopy. Mast cells were identified by antibodies directed at mast cell tryptase (clone G3; Chemicon, Temecula, Calif) and visualized by Alexa-488-labeled secondary antibodies (Molecular Probes, Eugene, Ore).

Euthanasia of cynomolgus monkeys

On the day of necropsy, animals were first sedated with ketamine and then weighed and anesthetized by using an intravenous injection of a commercial euthanasia solution containing pentobarbital and phenytoin. Euthanasia of the anesthetized animals was then performed by means of exsanguination.

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