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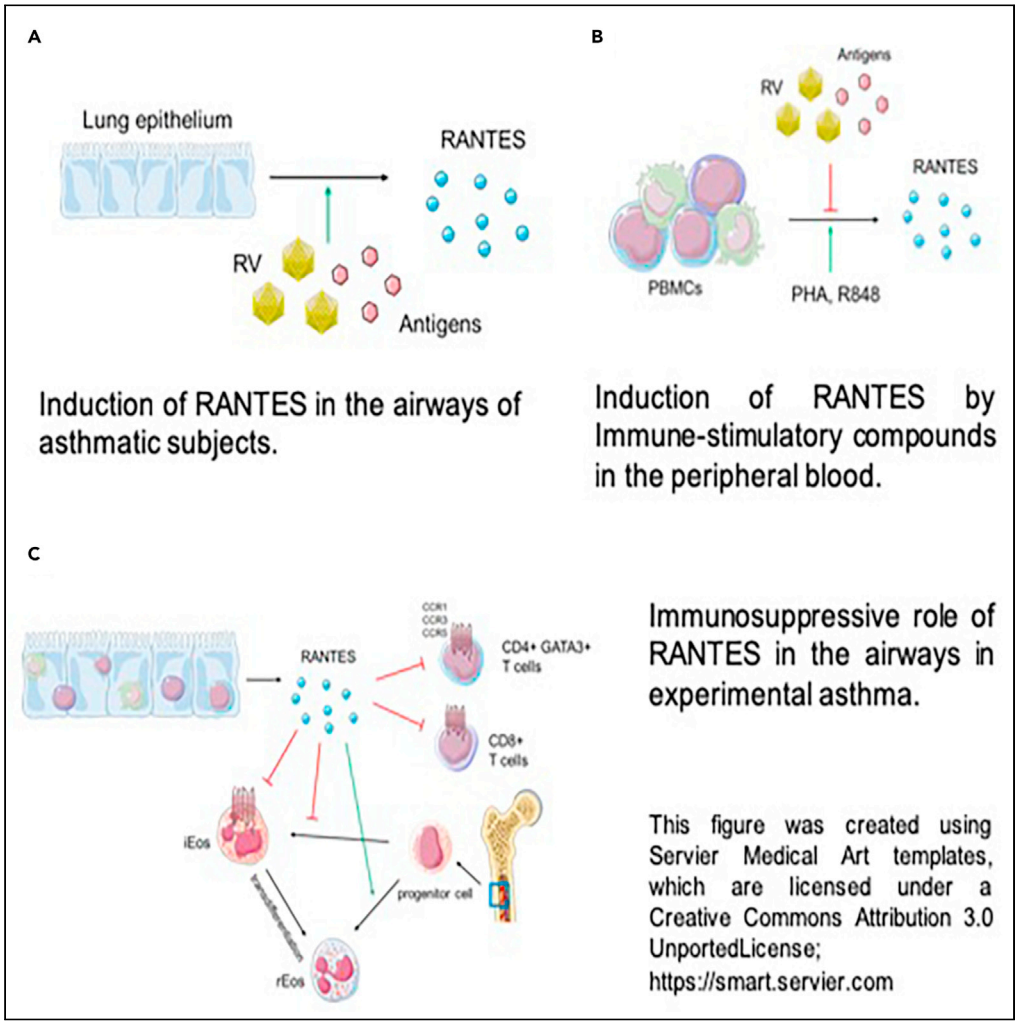

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Highlights

RANTES is associated with allergic asthma and T cell-dependent clearance of infections

RANTES is upregulated in asthmatic airways reflecting ongoing airway cell activation

Rhinovirus inhibited and antiviral agonist induced RANTES in PBMCs from asthmatics

Experimental treatment with RANTES (rRANTES) in the airways reduced local eosinophils

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Regulated on Activation, Normal T cell Expressed and Secreted (RANTES) drives the resolution of allergic asthma

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SUMMARY

RANTES is implicated in allergic asthma and in T cell-dependent clearance of infection. RANTES receptor family comprises CCR1, CCR3, and CCR5, which are G-protein-coupled receptors consisting of seven transmembrane helices. Infections with respiratory viruses like Rhinovirus cause induction of RANTES production by epithelial cells. Here, we studied the role of RANTES in the peripheral blood mononuclear cells in cohorts of children with and without asthma and validated and extended this study to the airways of adults with and without asthma. We further translated these studies to a murine model of asthma induced by house dust mite allergen in wild-type RANTES and CCR5-deficient mice. Here we show an unpredicted therapeutic role of RANTES in the resolution of allergen-induced asthma by orchestrating the transition of effector GATA-3+CD4+ T cells into immune-regulatory-type T cells and inflammatory eosinophils into resident eosinophils as well as increased IL-10 production in the lung.

INTRODUCTION

Asthma is a chronic inflammatory disease of the airways that affects approximately 300 million people in the world, especially children. Exacerbations requiring hospitalization are commonly triggered by respiratory viral infection alone or in combination with seasonal allergens. During asthma exacerbation there is an accumulation of eosinophils and activated type 2 CD4+ T helper lymphocytes producing IL-5, an eosinophil-regulating cytokine (Travers and Rothenberg, 2015). Clinical trials have been initiated to target interleukin (IL)-5, but anti-IL-5 treatment did not abolish pulmonary eosinophils accumulation in response to allergen. The recruitment of eosinophils into the different tissues is mediated in addition to IL-5 by chemokines like Eotaxin through the receptor for C-C type chemokines 3, CCR3, on eosinophils. CCR3 belongs to family 1 of the G-protein-coupled receptors.

Regulated on Activation, Normal T cell Expressed and Secreted (RANTES) can also activate CCR3 (Penido et al., 2001; Chvatchko et al., 2003), and it is chemotactic for T cells, eosinophils, and basophils into inflammatory sites. Infections with respiratory viruses like respiratory syncytial virus or interferon (IFN)γ treatment causes induction of RANTES by epithelial cells (Olszewska-Pazdrak et al., 1998; Konno et al., 2002). RANTES is a key chemokine involved also in the recruitment of CD4+ and CD8+ T cells into the lung and has been implicated in Th1 responses and eosinophilic disease driven by Th2 cells like allergic asthma (Culley et al., 2006; Luster, 1998; Homey and Zlotnik, 1999; Zhang and Gao, 2020). Thus, RANTES is responsible for T cell-dependent clearance of the infection (Holt and Sly, 2012). T cells may produce RANTES to recruit eosinophils and other inflammatory cells into the airways (Culley et al., 2006). Eosinophils in turn also regulate homeostatic processes at steady state (Rosenberg et al., 2013). Under baseline conditions, eosinophils rapidly leave the bloodstream to enter tissue, like gut and lung, where they regulate important functions. These resident eosinophils (rEos) have recently been demonstrated to be morphologically and phenotypically distinct from the inflammatory eosinophils (iEos) that are recruited to the lung during house dust mite (HDM)-induced allergic inflammation (Mesnil et al., 2016). In this study, we asked whether RANTES is

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involved in the resolution of experimental allergic asthma. Consistently, we set up to investigate the immunological mechanisms by which RANTES regulates the resolution of allergic asthma.

RESULTS

Increased RANTES in the airways of asthmatic subjects

To start to understand the role of RANTES in asthma, we first looked in the airways for RANTES expression after gene expression profiling in the bronchoalveolar lavage (BAL) of a subset of asthmatic subjects and healthy controls. Here we found a significantly increased expression of transcript variant 1 of RANTES (NCBI Reference Sequence [RefSeq]: NM_002985.2) in the BAL cells of subjects with asthma as compared with those of healthy adult controls, in the Asthma BRIDGE cohort (Raby et al., 2011) (Figure 1A). We thus thought that RANTES upregulation in asthmatic airways would reflect ongoing airway epithelial cells activation (Figure 1B).

Rhinovirus infection inhibited RANTES production by peripheral blood mononuclear cells from asthmatic children in the PreDicta study cohorts both *in vivo* and *in vitro*

We next asked if the RANTES induction seen in the airways in asthmatic subjects would also be present in PBMCs and if this induction would be dependent on the presence of Rhinovirus (RV) in the airways of the child. In two cohorts of preschool children with and without asthma recruited at the children hospital in Erlangen for the European Study PreDicta, we found that the supernatants of untreated PBMCs isolated from asthmatic children with RV infection in their upper airways *in vivo* had, by trend, reduced RANTES levels as compared with the control children with RV infection *in vivo* in our study cohort (Figures 2A–2C). To mimic the RV infection under *in vitro* conditions, we exposed PBMCs from control and asthmatic preschool children to RV and analyzed RANTES production in cell culture supernatants. We also considered the absence or presence of RV in the donor airways *in vivo* in the five European pediatric PreDicta cohorts (Xepapadaki et al., 2018; Bergauer et al., 2017). We observed that, by trend, RANTES was reduced in the supernatants of PBMCs isolated from asthmatic children with RV in their airways *in vivo* compared with control children with RV *in vivo* (Figure 2C, left-hand-side bars). Looking at their PBMCs after *in vitro* infection with RV, we found significantly lower levels of RANTES in all cohort groups when cultured with RV except for the control group without RV in the airways *in vivo* (Figure 2C, right-hand-side bars). Moreover, we cultured these PBMCs with phytohemagglutinin (PHA), a condition that activates T cells (Figure 2D). Here we found that PHA significantly induced RANTES levels in the asthmatics with RV in their airways *in vivo* (Figure 2D, right-hand-side bars). Additionally, we found that RV *in vitro* induced relatively low levels of IFNγ in PBMCs, in all groups (Figure 2E), as compared with IFNγ levels induced by PHA (Figure 2F).

Antiviral agonists of Toll-like receptors 7/8 induced RANTES

We next asked if RANTES would be associated with antiviral immune responses. We stimulated PBMCs from preschool children with a Toll-like receptors 7/8 (TLR7/8) agonist to mimic the activation of intracellular receptor upon single-stranded RNA virus infection (Krug et al., 2021). We found that the stimulation with the antiviral TLR7/8 agonist Resiquimod (R848) also induced RANTES significantly in asthmatic children (Figure 2G) and IFNγ in control children with an RV infection in the airways (Figure 2H). By contrast, TLR3 challenged via polyinosinic:polycytidylic acid (poly I:C), a synthetic analog of double-stranded RNA (Starkhammar et al., 2012), did not significantly induce RANTES production in these PBMCs (Figure S1A). Thus, these data indicate a key pathway inducing RANTES downstream of TLR7/8.

Increased RANTES levels are associated with lower C-reactive protein levels during symptomatic episodes of asthma in children

C-reactive protein (CRP) is an acute phase protein upregulated during ongoing inflammation and infections (Wu et al., 2015). Recent studies proposed that CRP could be used as marker of disease in asthma (Takemura et al., 2006). We then asked if CRP would correlate with RANTES. Here we found that, during exacerbations of disease at the symptomatic visits, not all CRP values (Figure S1B), but serum CRP levels under 20 mg/L, correlated indirectly with RANTES serum levels (Figure 2I). The cutoff <20 mg/L was chosen to exclude possible bacterial infections. In summary, RANTES was induced by RV in the airways and inhibited by RV in PBMCs, while being induced by TLR7/8 agonist R848 in PBMCs (Figure 2J).

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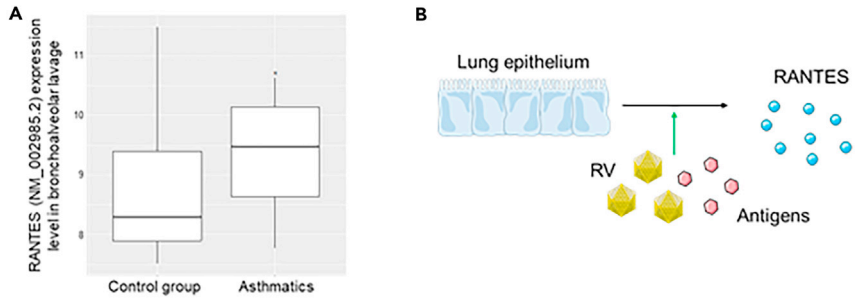

Figure 1. RANTES mRNA is induced in the bronchoalveolar lavage cells of asthmatic subjects compared with healthy controls

(A) A total of 47,009 tag probes to target individual exons, including 1 exon sequence linked to specific RANTES isoforms, were analyzed using a microarray. Gene expression profiling demonstrated a higher level of RANTES (CCL5 transcript variant 1) expression in the bronchoalveolar lavage cells of asthmatic than those of non-asthmatic healthy controls (fold change = 1.62, p = 0.027).

(B) Possible induction of RANTES in the airways of asthmatic subjects. This figure was created using Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 Unported License; https://smart.servier.com.

CCR5 is induced on Th1 cells but not on Th2 cells

We next investigated whether RANTES and its receptor components would associate with Th1 or Th2 development. We polarized murine naive spleen CD4+CD62L+ T cells to Th1 and Th2 cells (Figure 3A), as shown by the expression of T-bet (Figures 3B and 3C) and production of IFNγ (Figure 3D) in Th1 cells and the expression of GATA-3 (Figures 3E and 3F) and the production of IL-5 (Figure 3G) in Th2 cells. We found that, although Th0, Th1, and Th2 cells all produced high RANTES levels, RANTES was induced in activated T cells (Th0 cells) (Figure 3H), indicating that antiCD3/antiCD28 antibodies along with IL-2 induced RANTES while activating T cells. The receptor components of the chemokine RANTES comprise CCR1, CCR3, and CCR5 (Homey and Zlotnik, 1999), thereby RANTES has a greater affinity for CCR5 and CCR1 and lower affinity for CCR3 (Blanpain et al., 2001). We found that CCR1 is downregulated in activated Th0, Th1, and Th2 cells (Figure 3I), whereas the expression of CCR5 was significantly increased on Th1 cells compared with Th2 cells (Figure 3J). Since CCR5 is a common receptor for other chemokines besides RANTES, we then analyzed both wild-type (WT), RANTES$^{-/-}$, and CCR5$^{-/-}$ mice in a murine model of asthma.

RANTES$^{-/-}$ and CCR5$^{-/-}$ mice showed increased percentages of CCR3+ eosinophils in their airways

We next standardized a murine model of asthma after intraperitoneal sensitization and intranasal challenge with HDM (Figure 4A). We found that the CCR5$^{-/-}$ mice treated with HDM exhibited a significant increased airway hyperresponsiveness compared with WT mice treated with HDM and CCR5$^{-/-}$ control naive mice when challenged with 50 mg/mL methacholine (Figure 4B). This effect was not observed in RANTES$^{-/-}$ mice (Figure 4C). Total serum IgE was significantly reduced in the absence of RANTES but not in the absence of CCR5 (Figure S2A). RANTES levels in bronchoalveolar lavage fluid (BALF) were significantly higher in asthmatic CCR5$^{-/-}$ mice compared with the control group, where RANTES was barely detectable (Figure 4D). By contrast, in the supernatants of HDM-restimulated lung cells from WT and CCR5$^{-/-}$ asthmatic mice and in the serum of CCR5$^{-/-}$ asthmatic mice, we detected significantly reduced RANTES levels compared with the control naive mice (Figures 4E and 4F), indicating a local accumulation of RANTES-producing cells in experimental asthma. In serum, both the control and asthmatic CCR5$^{-/-}$ mice had significantly higher RANTES levels compared with the WT mice (Figure 4F). In this model of asthma, there was no apparent difference in lung inflammation between the HDM-treated groups after histological analysis (Figures 4G and 4H). Consistently, we observed that both asthmatic RANTES$^{-/-}$ and CCR5$^{-/-}$ mice had a significantly induced percentage of CCR3+ Gr-1 ^{low} eosinophils in the BALF (Figure 4H) as well as in total lung cells (Figure S2B) comparable with the WT asthmatic group (Figure 4I). Interestingly, we noticed fewer eosinophils in the BALF of naive CCR5-deficient mice. Together, these data show that the deficiency in CCR5 or RANTES in this asthma model did not affect the eosinophils in the BALF and in the lung.

Using flow cytometry analysis, we next examined the CD4+ and CD8+ T cell populations in the lung cells of the mice and found that asthmatic CCR5$^{-/-}$ but not RANTES$^{-/-}$ mice had a significantly higher percentage

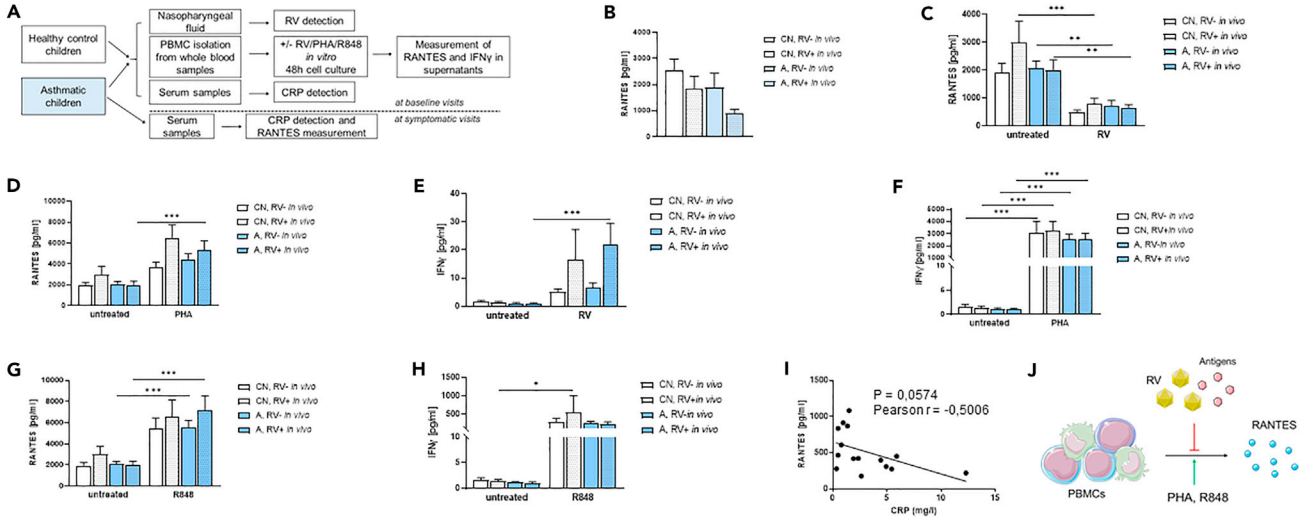

Figure 2. Rhinovirus infection *in vivo* and *in vitro* inhibited RANTES in PBMCs from asthmatic children

TLR7/8 agonist, R848 induced RANTES and IFN-gamma in PBMCs from asthmatic children with RV *in vivo* in the airways.

(A) Experimental design.

(B) RANTES was measured in untreated PBMCs supernatants by ELISA in the Predicta WP1 Erlangen cohort (Control group (CN), RV- *in vivo* n = 5; CN, RV+ *in vivo* n = 8; Asthmatic children (A), RV- *in vivo* n = 10; A, RV+ *in vivo* n = 10).

(C, D, and G) RANTES was measured in the supernatants of untreated or RV-infected (C, from left to right: n = 25, 31, 82, 57, 24, 36, 63, 55), PHA (D, from left to right: n = 25, 31, 82, 57, 23, 34, 78, 57), or R848-treated PBMCs (G, from left to right: n = 25, 31, 82, 57, 24, 35, 80, 57) by ELISA in all five European (Athens, Erlangen, Lodz, Ghent, and Turku) WP1 Predicta cohorts.

(E, F, and H) IFNγ was measured in supernatants of untreated or RV-infected (E, from left to right: n = 28, 34, 82, 59, 26, 33, 65, 55), PHA (F, from left to right: n = 28, 34, 82, 59, 28, 34, 80, 58), or R848-treated PBMCs (H, from left to right: n = 28, 34, 82, 59, 28, 34, 80, 58) by ELISA in all European WP1 Predicta cohorts.

(I) Linear regression analysis of serum levels of CRP (C-reactive protein) and RANTES in serum of asthmatic children with CRP <20 mg/L (n = 15) during symptomatic visits. Statistical analysis by Pearson correlation.

(J) Possible induction of RANTES by immune-stimulatory compounds in the peripheral blood. This figure was created using Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 Unported License; https://smart.servier.com. See also Figure S1. Data are shown as mean values ± SEM using two-way analysis of variance (B-H). *p, 0.05; **p, 0.01, ***p, 0.001.

of CD4+ T cells and both asthmatic RANTES$^{-/-}$ and CCR5$^{-/-}$ mice exhibited a higher percentage of CD8+ T cells as compared with their WT controls (Figure 4J). Interestingly, even the non-asthmatic RANTES$^{-/-}$ mice showed an induced CD8+ T cell percentage (Figure 4J). These data indicated an immunosuppressive function of RANTES mediated through CCR5.

Next, we looked at the Th1 and Th2 cell populations. Although the Th1 cell population was generally low in all groups (Figure S2E), GATA3+ CD4+ Th2 cells were significantly upregulated in the airways of both asthmatic RANTES$^{-/-}$ and CCR5−/− mice compared with their WT controls (Figure 4K). Together, these data demonstrate that a deficiency in RANTES or its main receptor CCR5 exacerbates the allergic response in asthmatic mice.

Intranasal delivery of recombinant RANTES in the airways reduced local inflammatory eosinophils and induced resident eosinophils

Based on our findings that mice with HDM-induced asthma have reduced RANTES levels in serum and the lung (Figures 4C and 4D), we next wanted to explore the therapeutic potential of RANTES in experimental asthma. In these therapeutic experiments, we chose an intranasal model of HDM challenge to induce asthma (Koch et al., 2019). These mice were in a BALB/c genetic background, and they were exposed to a mild protocol of asthma after intranasal allergen challenge. By contrast, the CCR5 and RANTES-deficient mice received a systemic allergen sensitization because they were in C57BL/6, a genetic background that is less predisposed to develop asthma. In addition, this intranasal allergen model is used to activate local cells of the innate immunity as we recently demonstrated (Koch et al., 2019), and thus we thought that would help to better understand the effect of RANTES in cells of the innate immunity like eosinophils as compared with the cells of adaptive immunity. In this intranasal allergen model, we performed intranasal application of recombinant RANTES (rRANTES) in BALB/c WT mice *in vivo* (Figure 5A). As a result, rRANTES administration decrease neither airway hyperresponsiveness

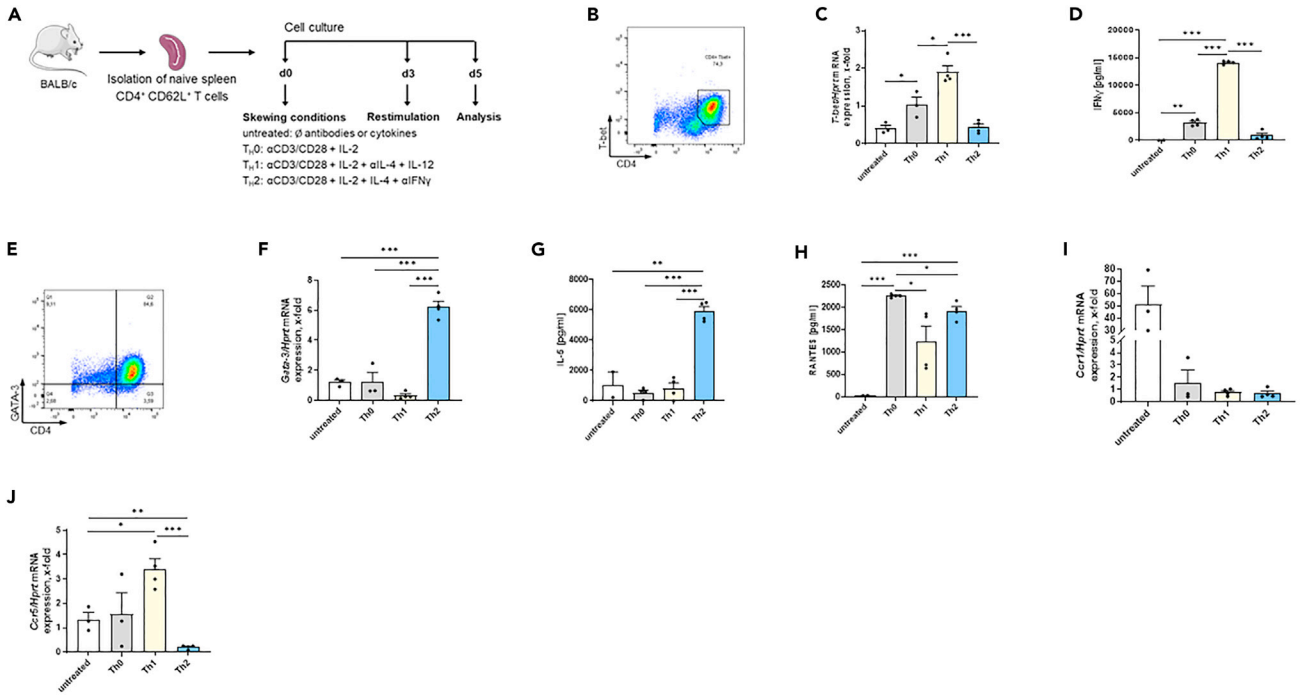

Figure 3. CCR5 is mainly expressed on Th1 cells but not in Th2 cells

(A) Experimental design of *in vitro* polarization of T cells.

(B and E) Representative dot plots of T-bet + CD4+ (B) and GATA-3 CD4+ (E) T cells gated on lymphocytes using flow cytometry.

(C, F, I, and J) T-bet (C), GATA-3 (F), CCR1 (I), and CCR5 (J) mRNA expression levels were measured by quantitative real-time PCR (from left to right: n = 3, 3, 4, 4).

(D, G, and H) IFNγ (D), IL-5 (G), and RANTES (H) were measured in the cell culture supernatants by ELISA (from left to right: n = 2, 4, 4, 4). Data are shown as mean values ± SEM. p Values were calculated using Student's two-tailed t test. *p,0.05; **p,0.01, ***p,0.001.

(AHR) (Figure 5B) nor peribronchial inflammation (Figures 5C and 5D) but led to decreased serum IgE in experimental asthma compared with the control group (Figure 5E). Furthermore, the immunosuppressive cytokine IL-10 was found to be upregulated in the lung of rRANTES-treated mice (Figure 5F). Furthermore, the numbers of CD4+ and CD8+ T cells were significantly decreased in the lung of rRANTES-treated asthmatic mice (Figure 5G). Finally, we analyzed the resident eosinophils (Siglec F^{int} $CD101^{low}$ CD45.2+ granulocytes, rEos) and inflammatory eosinophils (Siglec F^{hi} $CD101^{hi}$ CD45.2+ granulocytes, iEos) in the lung. Here we found that iEos were induced in this model of asthma, but rRANTES treatment *in vivo* reduced iEos and induced rEos (Figure 5H) in the asthmatic mice. In summary, these data indicate that RANTES is involved in the resolution of asthma by mediating the transition between lung iEos and rEos. In addition, we found that the expression of CCR5 increased on iEos as compared with rEos (Figure 5I). We next investigated eosinophils and neutrophils in the BAL of untreated and RANTES-treated mice. Here we found an induction of CCR3+ eosinophils and neutrophils after both allergen (HDM) and especially after allergen and RANTES treatment (Figures S3A and S3B). Moreover, the Th1 cytokine IFNγ was found to decrease in this asthma model and was restored after RANTES pretreatment (Figure S3C). Consistent with IL-10 upregulation after HDM and RANTES treatment, T-bet was found to be decreased (Figure S3D). By contrast, consistent with persistent lung airway hyperresponsiveness the Th2 cytokine IL-5 and the associated transcription factor GATA3 were not inhibited by RANTES treatment (Figures S3E and S3 F). By contrast, the T regulatory cells Foxp3+ were not changed by allergen challenge or by RANTES treatment (Figure S3G). In summary, RANTES treatment *in vivo* resulted in an induction of the immunosuppressing cytokine IL-10 and the suppression of effector T cells and iEos while inducing rEos locally. These data suggest a regulatory role of RANTES in the resolution of inflammation in asthma.

DISCUSSION

In our study we explored the role of RANTES in the resolution of asthma. To this end we examined the effect of RV on RANTES levels, since RV is implicated in the pathogenesis of asthma as well as its exacerbations (Kennedy et al., 2019; Anderson et al., 2017). RV infection *in vitro* is known to compromise the type 1 IFN and IFNγ response in PBMCs (Papadopoulos et al., 2002). Analogous to the effect of RV on IFNγ production, RV

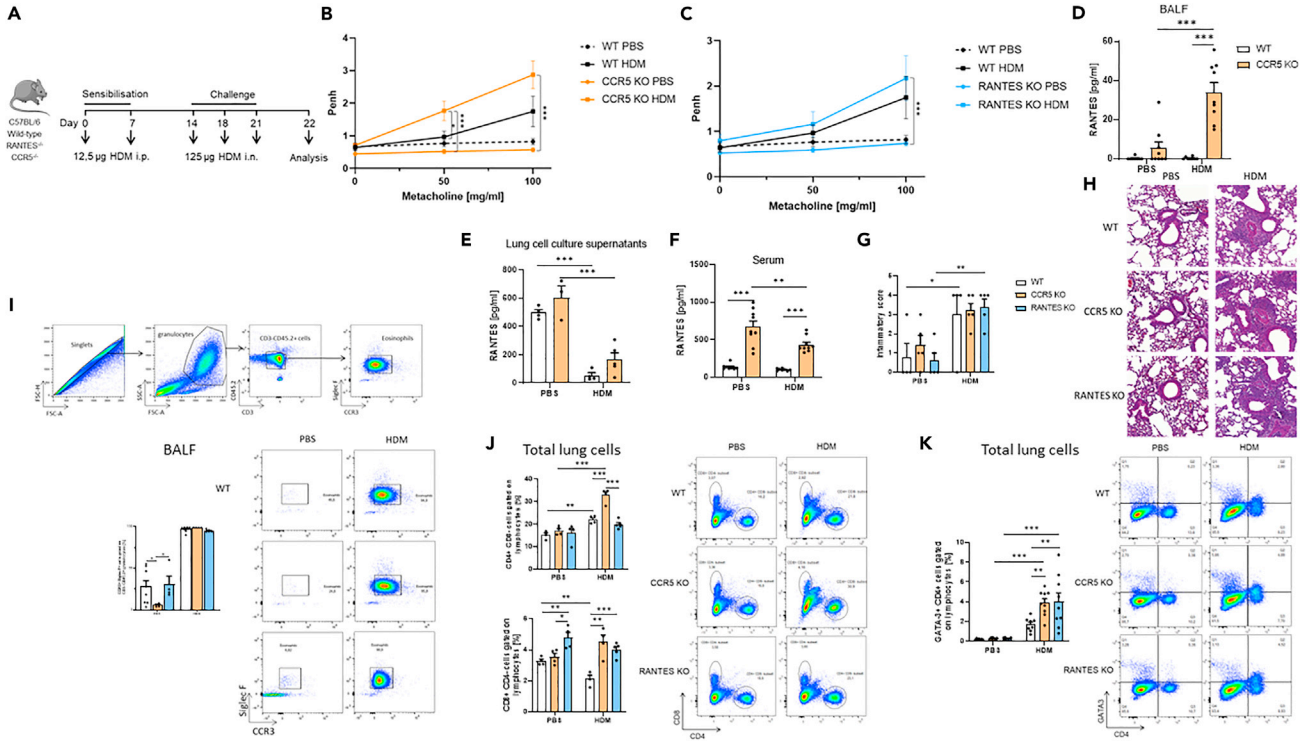

Figure 4. RANTES$^{-/-}$ and CCR5$^{-/-}$ mice have more eosinophils in the airways and a higher percentage of Th2 cells in the lung

(A) Experimental design of the murine asthma model.

(B and C) Lung function measured by Buxco system in response to increasing doses of methacholine.

(D, E, and F) RANTES was measured in BALF (D), re-stimulated lung cell supernatants (E), and serum (F) by ELISA.

(G and H) Representative hematoxylin and eosin-stained sections of the lung and respective inflammatory score. Scale bar, 150 µm.

(I) Flow cytometry analysis and representative dot plots of the CCR3+ Siglec F+ eosinophils gated on CD3- CD45.2+ granulocytes in the bronchoalveolar lavage.

(J) Flow cytometry analysis and representative dot plots of the CD4+ CD8- and CD8+ CD4- cells gated on lymphocytes in the lung.

(K) Flow cytometry analysis and representative dot plots of the GATA3+ CD4+ cells gated on lymphocytes in the lung. Data are shown as mean values ± SEM with n = 3–5 per group (B, C, E, I, and J) or n = 8–9 with data pooled from two independent experiments (n = 3–5 per group; (D, F, and K), each symbol representing individual mice. p Values were calculated using two-way analysis of variance. *p, 0.05; **p, 0.01, ***p, 0.001. See also Figures S2 and S3.

in vivo and *in vitro* inhibited RANTES in PBMCs from asthmatic children, indicating a defect in RANTES and IFNγ production in PBMCs after RV infection in the airways.

TLR7/8 are sensors of the single-stranded RNA viruses, and its agonists imiquimod (R837) and resiquimod (R848) have shown great therapeutic potential in treatment of allergic airway diseases (Drake et al., 2012). Treatment with TLR7/8 agonists in experimental asthma has been shown to induce T regulatory cells (Van et al., 2011) and inhibit the Th2 response (Quarcoo et al., 2004), both hallmarks of the resolution of asthma. The mechanism of the protective effect of TLR7/8 agonists is not fully understood: although there is evidence that the alleviation of inflammation is mediated by the production of IFNγ (Grela et al., 2011), IFNγ seems to be not the only reason as shown by experiments in which IFNγ-deficient mice could also be treated effectively with TLR7/8 agonists (Xirakia et al., 2010). We found that the challenge with TLR7/8 agonists, but not the TLR3 activator poly I:C, induced RANTES production in PBMCs. This demonstrates a key pathway downstream of TLR7/8 activating RANTES. Since TLR7/8 agonists induce the antiviral response that was impaired when RV suppressed RANTES production, it is possible that RANTES is involved in the increased IFN-type I and IFN-type III loop (Krug et al., 2021).Therefore, RANTES may play a notable role in the effectiveness of TLR7/8 agonists by supporting the IFN response.

CRP is known to correlate with the severity of asthma (Monadi et al., 2016). In fact, it has been reported that higher serum high-sensitivity CRP levels associated with increased sputum eosinophil numbers and decreased pulmonary function in steroid-naive patients. Further studies are needed to better understand the clinical association of CRP and asthma (Takemura et al., 2006). Here we found a negative correlation

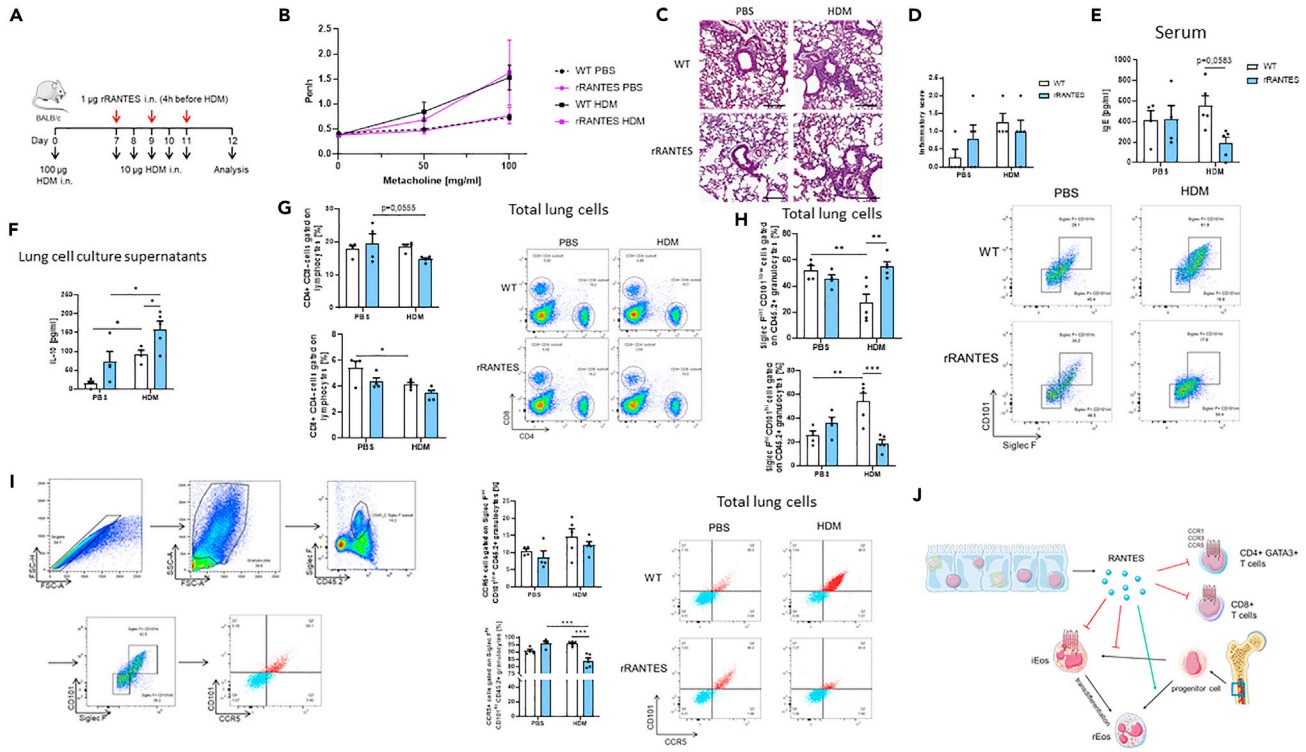

Figure 5. Intranasal treatment with recombinant RANTES *in vivo* is associated with immunosuppression and induced a shift from inflammatory eosinophils (iEos) to resident eosinophils (rEos) in the lung experimental asthma

(A) Experimental design.

(B) Airway hyperresponsiveness, measured by Buxco system, to increasing doses of methacholine.

(C and D) Representative hematoxylin and eosin-stained sections of the lung and respective inflammatory score. Scale bar, 150 µm.

(E) Total serum IgE was measured by ELISA.

(F) IL-10 was measured in the re-stimulated lung cells supernatants by ELISA.

(G) Flow cytometry analysis and representative dot plots of the CD4+ CD8- and CD8+ CD4- cells gated on lymphocytes in the lung.

(H) Flow cytometry analysis and representative dot plots of Siglec F^{int} $CD101^{low}$ cells gated on CD45.2+ granulocytes (rEos) and Siglec F^{hi} $CD101^{hi}$ cells gated on CD45.2+ granulocytes (iEos) in the lung.

(I) Flow cytometry analysis, gating strategy, and representative dot plots of CCR5+ cells gated on Siglec F^{int} $CD101^{low}$ CD45.2+ granulocytes (rEos) and CCR5+ cells gated on Siglec F^{hi} $CD101^{hi}$ CD45.2+ granulocytes (iEos) in the lung.

(J) Possible immunosuppressive role of RANTES in the airways. This figure was created using Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 Unported License; https://smart.servier.com. See also Figure S3. Data are shown as mean values ± SEM with n = 4–5 per group, each symbol representing individual mice. p Values were calculated using two-way analysis of variance. *p, 0.05; **p, 0.01, ***p, 0.001.

between RANTES and serum CRP levels below 20 mg/L (suggestive of non-bacterial illness), suggesting a protective function of RANTES during exacerbations of the disease.

Both human asthmatic subjects and $CCR5^{-/-}$ mice with HDM-induced asthma show elevated levels of RANTES in the BALF in the form of gene expression or cytokine levels, respectively. However, looking at PBMC supernatants of children with asthma or lung cell supernatants from asthmatic mice we found that these cells produce less RANTES compared with their healthy control groups. This apparent difference between cellular RANTES production and local levels of RANTES might reflect the migration of RANTES-producing cells into the airways during inflammatory processes. Another possible interpretation of this effect is that, in an organism with asthmatic disease, RANTES production might be depleted in the effector cells because the cytokine is excessively secreted into the airways. Further studies to investigate this are needed.

Chemokine receptors are highly shared among the family members to allow innate immediate recruitment of innate type cells to the site of infection, inflammation, and tumor development. In this study, we show that $RANTES^{-/-}$ mice with and without HDM-induced asthma exhibit an increased population of CD8+ T cells, whereas asthmatic $CCR5^{-/-}$ mice showed an increase in both CD4+ and CD8+ T cells compared

with their WT controls. This indicates that other ligands from the chemokine family that bind to CCR5, e.g., MIP-1alpha (CCL3) and MIP-1beta (CCL4), may play a role in regulating the development and local recruitment of T cells (Homey and Zlotnik, 1999; Luster, 1998). In line with a prior report about RANTES causing chemotaxis in Th1 but not Th2 cells (Siveke and Hamann, 1998), in translational studies we found that spleen naive CD4+ T cells polarized to Th1 cells have a higher CCR5 mRNA expression than Th2 cells, indicating a strong affinity of RANTES regarding the Th1 recruitment. Consistently, our results suggest that RANTES and CCR5 deficiency caused a shift from a Th1 to Th2 immune response, demonstrated by the upregulated Th2 population in the asthmatic RANTES$^{-/-}$ and CCR5$^{-/-}$ mice compared with their WT controls. Accordingly, the Th1 cells would be expected to be downregulated, but owing to an already low Th1 population in the WT mice with allergen-induced asthma, this effect was not visible. Eosinophils and effector T cells producing type 2 cytokines have been shown to play a pivotal role in the pathogenesis of asthma. The clinical relevance of this observation is highlighted by clinical trials showing that targeting of IL-4/IL-13, IL-5, or IL-5 signaling with antibodies such as dupilumab, mepolizumab, reslizumab, and benralizumab may treat asthma in humans (Busse et al., 2010; O'Byrne, 2007).

Although RANTES has been reported to bind to CCR3, which is associated with eosinophil chemotaxis (Sallusto et al., 1998), and we confirmed this in the BALF of RANTES-treated mice, our studies on RANTES-deficient mice demonstrate that the lack of RANTES resulted in similar CCR3+ eosinophil numbers in the BALF of these asthmatic mice. Therefore, other ligands to CCR3, like eotaxin-1, eotaxin-2, eotaxin-3, or CCL2, may play a dominating role in recruiting eosinophils during inflammation in the lung (Grozdanovic et al., 2019). The increased airway eosinophilia of RANTES$^{-/-}$ and CCR5$^{-/-}$ mice demonstrates the interchangeable role of chemokines, which is fundamental to maintain the homeostasis at the mucosal site in case of sudden changes like in respiratory infections and lung cancer. Additionally, the effect of the elevated percentage of CCR3+ eosinophils in the BAL of the RANTES$^{-/-}$ and CCR5$^{-/-}$ mice can be attributed to the elevated numbers of CD4+ and CD8+ T cells. In murine models of asthma, treatment with anti-CD4 antibody has been shown to reduce airway eosinophilia and AHR (Gavett et al., 1994) and CD8+ T cells have been shown to contribute to inflammation and AHR in the lung via type 2 cytokines (Cho et al., 2005; Schaller et al., 2005).

Our results are in apparent contradiction with previous studies that demonstrate a detrimental role of RANTES in allergic or autoimmune diseases, such as arthritis and atopic dermatitis (Chensue et al., 1999). We challenge the paradigm of RANTES as a merely destructive chemokine in asthma, demonstrating that the lack of RANTES or CCR5 aggravates the allergic disease by increasing the number of CD4+ and CD8+ T cells and an enhanced Th2 immune response, followed by an influx of eosinophils in the airways.

Previous studies have shown that treatment with recombinant RANTES can alleviate airway hyperresponsiveness in the long term, but not in short-term challenge (Koya et al., 2006). In Figure 5 we applied an asthma model of an early stage of disease. In fact, in this model, the innate immune response is targeted by RANTES. This model is different from models in which asthma is induced after systemic allergen sensitization or weekly repeated airway allergen challenges. Here we demonstrate that asthmatic mice that received three intranasal applications of rRANTES have decreased IgE serum levels, a well-established marker for allergic asthma that has been targeted in biologic therapies with monoclonal antibodies (Castillo et al., 2017). The asthmatic mice with intranasal rRANTES application also had elevated IL-10 levels in their lung compared with the group without rRANTES treatment. The immunosuppressive properties of IL-10 could be beneficial in allergen-induced asthma by downregulating airway inflammation (Zuany-Amorim et al., 1995). In the next study it would be interesting to analyze which cells are activated via RANTES to produce IL-10. IL-10 can be produced by several types of cells in the respiratory tract (Branchett et al., 2020) enclosing T cells. Moreover, in our setting we used HDM as restimulation in lung cell culture. Thus, it is possible that other cells of the innate immunity like ILC2 would be activated by RANTES treatment to produce IL-10 instead of type 2 cytokines (Seehus et al., 2017). In line with our finding that RANTES$^{-/-}$ and CCR5$^{-/-}$ mice have increased CD4+ and CD8+ T cells, the *in vivo* treatment with rRANTES caused decreased CD4+ and CD8+ T cell populations. This indicates an immunosuppressive effect mediated by RANTES either via IL-10 production by innate cells or T regulatory cells having an immunosuppressive effect on other T cells via IL-10R in allergic asthma. Further experiments in this direction are needed.

Moreover, during allergen-induced asthma, rEos are reported to remain in the parenchyma in order to preserve lung homeostasis, whereas iEos are recruited in the peribronchial areas contributing to airway inflammation (Mesnil et al., 2016; Weller and Spencer, 2017). In our studies, we demonstrate that intranasal

application of rRANTES *in vivo* reduced iEos and induced rEos in the lung of asthmatic mice, whereas CCR5 is expressed in a higher percentage of iEos than on rEos. Moreover, RANTES decreased iEos expressing CCR5, indicating that RANTES might have a direct suppressive effect on iEos via CCR5. Another possible explanation for the transition from iEos to rEos is that RANTES affects the maturation from a new progenitor cell present either in the bone marrow or in the lung parenchyma.

Our studies show a yet unrevealed role of RANTES. With the focus on effector cells like PBMCs, the secretion of RANTES is down-regulated and its lack may even be part of the pathogenesis of the asthmatic disease. The down-regulation of RANTES during exposure to RV illustrates a possible immunological escape to avoid viral clearance. Focusing on the airways, when overly secreted into the bronchial air spaces during asthma exacerbations it may aggravate inflammation, while in the early stages of the disease it may preserve the anti-viral function of eosinophils in asthma while resolving inflammation in the lung. Transferring this finding of our experiments to the human studies, this is also a possible explanation for the negative correlation between RANTES and CRP levels we observed in asthmatic children.

Further studies are needed to explore the role of RANTES as a regulator of the resolution of airway inflammation via pleiotropic effects on immune mediators such as effector T cells or eosinophils.

Limitations of study

In this study we describe a new role of RANTES as a regulator of the resolution of airway inflammation via pleiotropic effects on immune mediators such as effector T cells or eosinophils. Further studies are needed to extend this observation to different cohorts of asthmatic subjects. Furthermore, in murine model of disease the therapy with rRANTES needs to be further investigated at molecular and cellular levels, to better understand this new immunosuppressive effect mediated by RANTES via IL-10 production.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.103163.

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AUTHOR CONTRIBUTIONS

S.F. and N.L. designed the experiments related to the murine models. H.M., B.A.R., and S.T.W. generated Figure 1 obtained from the Asthma BRIDGE cohorts data. N.L. analyzed the results for Figures 2, 3, 4, and 5 and performed the experiments in Figures 4 and 5. J.K. performed the experiments for Figure 3 and helped N.L. perform the experiments for Figure 5. Z.Y. and S.K. helped N.L. perform the experiments for Figure 4. Moreover, Z.Y. and S.K. helped S.F. with the revision of the manuscript. A.K. and T.Z. are the pediatricians who recruited and provided all the clinical data of the PreDicta children at the Children Hospital Uniklinikum Erlangen. T.V. analyzed all the respiratory rhinoviruses in the pediatric cohorts of PreDicta. M.R. measured the CRP levels in the serum of the children. N.G.P. is the coordinator of PreDicta and designed this study. P.X., M.L.K., H.L., T.J., C.B., A.L.-P., and N.Z. contributed to the recruitment and analysis of the different European Centers of the PreDicta cohorts. B.S. and M.A. provided us with the PreDicta data performed with Multiplex on the peripheral blood mononuclear cells supernatants. S.F. supervised all the work and wrote the manuscript together with N.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Purified NA/LE Hamster Anti-Mouse CD3e	BD Biosciences	Cat# 553057, RRID:AB_394590
Purified NA/LE Hamster Anti-Mouse CD28	BD Biosciences	Cat# 553294, RRID:AB_394763
LEAF™ Purified anti-mouse IL-4 Antibody	Biolegend	Cat# 504115, RRID:AB_2295885
Ultra-LEAF™ Purified anti-mouse IFN-γ Antibody	Biolegend	Cat# 505834, RRID:AB_11150776
PerCP-Cy™5.5 Rat Anti-Mouse CD4	BD Biosciences	Cat# 550954, RRID:AB_393977
BV421 Mouse Anti-T-bet	BD Biosciences	Cat# 563318, RRID:AB_2687543
Alexa Fluor® 488 Mouse anti-GATA3	BD Biosciences	Cat# 560163, RRID:AB_1645302
V450 Hamster anti-Mouse CD3e	BD Biosciences	Cat# 560804, RRID:AB_2034004
FITC Mouse Anti-Mouse CD45.2	BD Biosciences	Cat# 553772, RRID:AB_395041
BV510 Rat Anti-Mouse Siglec-F	BD Biosciences	Cat# 740158, RRID:AB_2739911
V500 Rat anti-Mouse CD8a	BD Biosciences	Cat# 560776, RRID:AB_1937317
Alexa Fluor® 647 Rat Anti-Mouse CD101 (Igsf2)	BD Biosciences	Cat# 564473, RRID:AB_2738821
PE Rat Anti-Mouse CD195	BD Biosciences	Cat# 559923, RRID:AB_397377
PE Rat Anti-Mouse Ly-6G and Ly-6C	BD Biosciences	Cat# 553128, RRID:AB_394644
BV421 Rat Anti-Mouse CD25	BD Biosciences	Cat# 564370, RRID:AB_2738772
Alexa Fluor® 488 anti-mouse/human CD45R/B220 Antibody	Biolegend	Cat# 103225, RRID:AB_389308
PerCP-Vio770 anti-mouse CD193 (CCR3) Antibody REAfinity™	Miltenyi Biotec	Cat# 130-102-144, RRID:AB_2655883
APC anti-mouse FOXP3 Antibody	Miltenyi Biotec	Cat# 130-113-470, RRID:AB_2733420
CD62L (L-Selectin) Monoclonal Antibody (MEL-14), PE-Cyanine5, eBioscience™	Invitrogen	Cat# 15-0621-81, RRID:AB_468766
Bacterial and virus strains		
RV1b	Athens Prof. Nikolaos Papadopoulos	
Biological samples		
Chemicals, peptides, and recombinant proteins		
rmRantes	Peprotech	Cat Nr. 250-07-50µg
House dust mite protein from *Dermatophagoides pteronyssinus*	Greer Laboratories	
PHA-M	Sigma	REF L8902
R848	InvivoGen	REF tlrl-R848
rmIL2	Immunotools	12340024
Recombinant Mouse IL-12 (p70) (carrier-free)	Biolegend	577002
Recombinant Mouse IL-4 (carrier-free)	Biolegend	574302
Methacholin		
H&E		
Critical commercial assays		
Human Rantes Bioplex Multiplex Immunoassay	Biorad	Custom
Human IFNy Bioplex Multiplex Immunoassay	Biorad	Custom

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
CD4+CD62L+ T Cell Isolation Kit, mouse	Miltenyi Biotec	130-106-643
mIFNy elisa	BD Biosciences	Cat# 555138, RRID:AB_2869028
mIL5 ELISA	BD Biosciences	Cat# 555236, RRID:AB_2869048
mRantes ELISA	R&D Systems	DY478
mIgE Elisa	BD Biosciences	Cat# 555248, RRID:AB_2869051
mIL10 ELISA	BD Biosciences	Cat# 555252, RRID:AB_2869052
Deposited data		
Human BRIDGE cohort		
Experimental models: Organisms/strains		
Balb/c mice	Janvier	BALB/CJRJ(W 6)
C57Bl/6 CCR5 KO	Jackson Laboratory	002782
C57Bl7& RANTES KO	Jackson Laboratory	005090
C57Bl/6 mice	Janvier	C57BL/6"J"RJ(W 6)
Oligonucleotides		
CCR1 (5′-ACTCTGGAAACACAGACTCACTG-3′, 5′-AGTTGTGGGGTAGGCTTCTG-3′)	Eurofins	Custom
CCR5 (5′-GATTTGTACAGCTCTCCTAGCCA-3′, 5′AATCCATCCTGCAAGAGCCAGA-3′)	Eurofins	Custom
GATA3 (5′-GTCATCCCTGAGCCACATCT-3′, 5′-TAGAAGGGGTCGGAGGAACT-3′)	Eurofins	Custom
HPRT (5′-GCCCCAAAATGGTTAAGGTT-3′, 5′-TTGCGCTCATCTTAGGCTTT-3′)	Eurofins	Custom
Tbet (5′-CCTGGACCCAACTGTCAACT-3′, 5′-AACTGTGTTCCCGAGGTGTC-3′)	Eurofins	Custom
Software and algorithms		
BD DIVA	BD Biosciences	https://www.bdbiosciences.com/en-us/products/software/instrument-software/bd-facsdiva-software#Overview
FlowJo V10	BD Biosciences	https://www.flowjo.com/
Revelation Quicklink 4.2	Dynex Tech	
Biorad CFX Maestro	Biorad	https://www.bio-rad.com/de-de/product/cfx-maestro-software-for-cfx-real-time-pcr-instruments?ID=OKZP7E15
Fine point 2.3	DSI	https://www.datasci.com/products/buxco-respiratory-products/finepointe-whole-body-plethysmography
Microsoft Office	Microsoft	

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources, methods and reagents should be directed to and will be fulfilled by the lead contact, Prof. Dr. Dr. Susetta Finotto (susetta.finotto@uk-erlangen.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Data reported in this paper will be shared by the lead contact if required upon request.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cohort of pre-school children

In the European Study PreDicta (Post-infectious reprogramming and its association with persistence and chronicity of respiratory allergic diseases), we examined healthy and asthmatic pre-school children at the age of 4-6 years in collaboration with the Department of Allergy and Pneumology at the children hospital in Erlangen (Graser et al., 2016; Koch et al., 2016). Boys are consistently reported to have more prevalent wheeze and asthma than girls. In the adolescence this pattern changes. In this study the cohorts of children recruited in Erlangen was composed of 41% females and 59% males for the control group and 37,5% females and 62,5% for the male. All experiments with human samples were approved by the Ethics Committee of the Friedrich-Alexander University Erlangen-Nürnberg, Germany under the approval number 'Re.-No. 4435.

Mice

Wild-type BALB/c females mice used for T cell isolation and *in vitro* polarization, wild-type mice BALB/c mice that received intranasal rRANTES treatment and their corresponding controls and wild-type C57BL/6 were purchased from JANVIER LABS (Saint-Berthevin, France). RANTES$^{-/-}$ and CCR5$^{-/-}$ mice with C57BL/6 genetic background were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). The experiments were performed with female mice aged 6-8 weeks. The animals were bred in individually ventilated cages at the animal facility adjacent to our institute and had free access to food and water. The government of Mittelfranken, Bavaria approved the experiments (55.2.2,-2532-2-633).

Replication of RANTES in bronchoalveolar lavage

Asthma BRIDGE is an open-access biorepository for subjects participating in genetic studies of asthma in the EVE Consortium (Raby et al., 2011). In this study, sample collection and processing were carried out at each participating institution according to standardized and validated protocols. A subset of asthmatic subjects and healthy controls also provided bronchoalveolar lavage (BAL) samples for gene expression profiling. In this cohort, asthma was defined based on having a physician diagnosis of the disease with evidence of reversible airflow obstruction with a bronchodilator. The collected samples were centralized at the Data Coordinating Center at the Channing Division of Network Medicine at Brigham and Women's Hospital at the Harvard Medical School (Boston, MA, USA).

METHODS DETAILS

RANTES microarray analysis

For our differential expression analyses and replication of differential expression of RANTES in bronchoalveolar lavage, forty-three of these subjects (25 with asthma) of the asthma BRIDGE cohort also had with log2-transformed and quantile-normalized gene expression (n = 47,009 probes) from cells in the bronchoalveolar lavage measured by Illumina HumanHT-12 v4 BeadChips. The R Bioconductor limma package (version 3.6.2) (Ritchie et al., 2015) was used to perform differential expression analysis. A linear model was fitted along with the implementation of empirical Bayes statistics to assess whether expression levels of the candidate genes of RANTES were significantly altered between asthmatics and healthy non-asthmatics in BAL after adjustment for race, age, gender and batch effect. Significance was defined using a *p*-value cutoff of less than 5%.

In vitro cell culture of human peripheral blood mononuclear cells (PBMCs) and cell culture

Heparinized blood was collected from children with and without asthma at the Baseline Visit of the PreDicta study and subsequently PBMCs were isolated with Ficoll using density centrifugation. A part of the cells were cultured in a concentration of 1 x 106 cells/ml for 48 hours in RPMI 1640 medium supplemented with 25 mmol/L HEPES, 100 IU/mL penicillin, 100 μg/mL streptomycin, 1% L-glutamine (200 mmol/L) (all anprotec, Bruckberg, Germany), 50 μmol/L β-mercaptoethanol, 1% MEM Vitamin (all Sigma-Aldrich, Steinheim, Germany), 1% non-essential amino acids, 1% sodium pyruvate (all Gibco®, Thermo Fisher Scientific,

Waltham, USA), and 10% FBS (Biochrom GmbH, Berlin, Germany) at 37°C and 5% CO2 and stimulated with 10 μg/ml Phytoheamagluttinin (PHA) (Sigma-Aldrich, Steinheim, Germany) or 20 μg/ml Poly I:C (Sigma-Aldrich, Steinheim, Germany). Additionally, PBMCs were cultured for 48 hours in complete culture medium at 37°C and 5% CO_2, whereby parts of them were challenged *in vitro* with RV1b or 200μl/ml R848 (0.2mg/ml) (InvivoGen, Toulouse, France). This R848 is very specific for: TLR7 and TLR8, CAS number: 144875-48-9 (free base). The company: 'R848 (Resiquimod) is an imidazoquinoline compound with potent anti-viral activity. This low molecular weight synthetic molecule activates immune cells via the TLR7/TLR8 MyD88-dependent signaling pathway (Hemmi et al., 2002; Jurk et al., 2002). InvivoGen's R848 is water soluble, validated for TLR7/8 potency and tested to ensure the absence of TLR2 or TLR4 contamination. It has a vaccine grade. The growth of RV1b and the description of the RV1b infection itself have been published elsewhere (Bergauer et al., 2017). Afterwards, supernatants were carefully removed and stored at −80°C.

Detection of C-reactive protein (CRP)

CRP values in serum samples of the children were analyzed as previously described (Eda et al., 1998). Briefly, CRP was measured on a routine clinical chemistry analyser (cobas c501, Roche, Mannheim, Germany). The method is based on particle-enhanced immunoturbidimetry (Tina-quant C-Reactive Protein, Roche, Mannheim, Germany, interday CV 1.9% (9.72 mg/L), limit of detection 0.3 mg/L). The methods is calibrated to ERM-DA474/IFCC.

Nasopharyngeal fluid collection with swab and RV detection

For the detection of the Rhinovirus in the upper airways of the children, a nasopharyngeal specimen was collected using a per-nasal applicator swab with a flocked soft nylon fiber tip (E-Swab 482CE; Copan, Brescia, Italy) as described in detail elsewhere (Bergauer et al., 2017). Specifically, the swab was inserted into one nostril, its tip with flocked soft nylon fiber was rotated for 5 seconds over the surface of posterior nasopharynx to allow for mucus absorption. Moreover, before exiting the nose, the swab was rotated against the mucosa of the anterior nares. The nylon tip was eluted by turning into the E-Swab's medium. The nasopharyngeal fluid was aliquoted under sterile conditions and stored at −80°C until further analysis. RV and other respiratory viruses detection was performed at the Department of Virology, University of Turku (Finland).

There, nucleic acids were extracted using a NucliSENS® easyMag® extractor (bioMérieux, Marcy l'Etoile, France). An in house PCR and commercial Seeplex RV 12 or Anyplex RV 16 kits were used to detect respiratory viruses (Seegene, Soul, Korea).

For evaluation, only children with no detectable virus were included in the "-RV" group, while children in the "+RV" group had at least a positive RV detection in NPF.

The detection of the Rhinovirus was performed at the Department of Virology, University of Turku (Finland). The description of this analysis is already published in detail elsewhere (Bergauer et al., 2017). In short, nucleic acids were extracted from 200 μl of the swab medium using easyMag extractor (BioMeriex, Marcy l'Etoile, France) according to the manufacturer's instruction. Afterwards different rhinovirus strains were detected using an earlier described in house PCR method (Peltola et al., 2008) as well as the Anyplex™ II RV16 Detection Kit (Seegene, Soul, Korea) according to the manufacturer's protocol.

Mouse naïve $CD4^+$ $CD62L^+$ T cell isolation and *in vitro* differentiation into T cell subsets

Spleens were removed from naïve Balb/c WT mice at the age of 10 weeks. Single cell suspension was prepared by pushing the spleen through a 40 μm cell strainer, lysing with 10 ml of an Ammonium-Chloride-Potassium (ACK)-Lysis buffer (0.15 M NH_4Cl, 0.1 mM $KHCO_3$, 0.1 mM Na_2-EDTA dissolved in ddH_2O) and washing with PBS. Spleen $CD4^+$ $CD62L^+$ T cells were positively sorted by magnetic bead isolation using the mouse $CD4^+$ $CD62L^+$ T cell isolation kit (MACS, Miltenyi Biotec, Bergisch-Gladbach, Germany) in accordance to the manufacturer's protocol. Cells were plated at a concentration of 1 x 10^6 cells/ml and cultured in RPMI 1640 medium containing 100 IU/mL penicillin, 100 μg/mL streptomycin (all anprotec, Bruckberg, Germany), and 10% FCS (Biochrom GmbH, Berlin, Germany) at 37°C and 5% CO_2. For the unstimulated condition naïve $CD4^+$ $CD62L^+$ T cells were cultured without any further stimulant, whereas for all other conditions the naïve T cells were activated by plate bound anti-CD3 (0.5 μg/ml; BD Biosciences, Heidelberg, Germany) and soluble anti-CD28 (1 μg/ml; Biolegend, San Diego, USA). Furthermore different cytokines (rmIL-2, rmIL-4, and rmIL-12 from Immunotools, Friesoythe, Germany; rhTGFβ from Miltenyi Biotec,

Bergisch-Gladbach, Germany) and antibodies (anti-IL-4 from Biolegend, San Diego, USA; anti-IFNγ from Miltenyi Biotec, Bergisch-Gladbach, Germany) were added to the cell culture medium to generate T_H0 cells (20 ng/ml rmIL-2), T_H1 cells (20 ng/ml rmIL-2, 20 ng/ml rmIL-12, 10 μg/ml anti-IL-4), T_H2 cells (20 ng/ml rmIL-2, 20 ng/ml rmIL-4, 10 μg/ml anti-IFNγ) and T_{reg} cells (100 ng/ml rmIL-2, 30 ng/ml rhTGFβ). After three days the cells were restimulated with the same conditions. On day 5 cells and cell culture supernatants were harvested for analysis.

HDM and recombinant RANTES (rRANTES) treatment

Wild-type, $RANTES^{-/-}$ and $CCR5^{-/-}$ mice on a C57BL/6 genetic background received an intraperitoneal injection of saline or 12,5 μg house dust mite (HDM) protein obtained from *Dermatophagoides pteronyssinus* whole body extract (Greer Laboratories, Lenoir, USA) in a volume of 200 μl saline on day 0 and 7. Additionally, the mice received intranasally 125 μg HDM extract or PBS on day 14, 18 and 21. Airway hyperresponsiveness was measured 24h after the last intranasal HDM application; on day 22, lung cells were isolated. Balb/c wild-type mice received intranasally 100 μg HDM on day 0 and 10 μg HDM on five consecutive days (day 7-11). On day 7, 9 and 11 they were treated with 1 μg rRANTES intranasally 4 hours before they received HDM. On day 12, lung cells were isolated. All murine experiments were approved by the government of Mittelfranken, Bavaria (55.2.2,-2532-2-633).

Airway hyperresponsiveness

The airway hyperresponsiveness was measured on day 22 or day 12 performing a non-invasive whole-body plethysmography 24 hours after the last challenge with HDM by using a Buxco Electronics apparatus (Buxco Research Systems, Wilmington, NC). Thereby mice were challenged with increasing doses of nebulized methacholine (MCh) in an exposure chamber, and Penh responses were measured as previously described (Menachery et al., 2015). The mice were undergoing 6 minutes of acclimatisation period before a nebulisation time of 1:30 minutes. The response time to the methacholin challenge was 6 minutes and 1 minute of recovery to go back to baseline after each dose (0mg/ml, 50mg/ml, 100mg/ml MCH in PBS). The data are expressed as mean values of enhanced pause ± SEMs.

ELISA

Mouse IgE, IFNγ, IL-5 and IL-10 were detected by using OptEIA™ sandwich ELISA kits from BD Bioscience (Heidelberg, Germany). Mouse RANTES was detected by using a Duoset™ sandwich ELISA kit from R&D Systems (Wiesbaden, Germany).

RNA isolation and quantitative real time–PCR

Total lung tissue was homogenized and total RNA from the tissue, total lung cells, or spleen was then extracted by using PeqGold RNA Pure according to the manufacturer's protocol (PeqLab, Erlangen, Germany). RNA (1μg) was reverse transcribed using the first strand cDNA synthesis kit for RT-PCR (MBI Fermentas, Sat. Leon-Rot, Germany). The resulting template-cDNA was amplified by quantitative real-time PCR using SsoFast EvaGreen Supermix (Bio-Rad Laboratories, München, Germany). The qPCR was performed with a cycle of 2 min 98°C, 50 cycles at 5s 95°C, 10s 60°C, followed by 5s 65°C and 5s 95°C and a cycle of 2 min 95°C, 42 cycles at 15s 95°C, 15s 56°C, 15s 72°C, followed by 30s 95°C, 5s 60°C and 5s 95°C in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, München, Germany). The primers used for mouse were as follows:

Ccr1 (5′-ACTCTGGAAACACAGACTCACTG-3′, 5′-AGTTGTGGGGTAGGCTTCTG-3′)

Ccr5 (5′-GATTTGTACAGCTCTCCTAGCCA-3′, 5′AATCCATCCTGCAAGAGCCAGA-3′)

Gata-3 (5′-GTCATCCCTGAGCCACATCT-3′, 5′-TAGAAGGGGTCGGAGGAACT-3′)

T-bet (5′-CCTGGACCCAACTGTCAACT-3′, 5′-AACTGTGTTCCCGAGGTGTC-3′)

The mRNA was normalized using the mRNA levels of the housekeeping gene *Hprt* (5′-GCCCCAAAATGGTTAAGGTT-3′, 5′-TTGCGCTCATCTTAGGCTTT-3′).

Collection and analysis of the BAL

Bronchoalveolar lavage was performed 24h after the last allergen challenge, by intratracheally injecting and aspirating 0.8 ml saline twice. After its collection the BALF was centrifuged for 5 min at 1500 rpm. The cell pellets were resuspended in 1 ml PBS and an aliquot was stained with trypan blue solution and cells were counted using a Neubauer chamber. Eosinophils and neutrophils were detected by fluorescence-activated cell sorting (FACS) analysis. The cell surface staining was performed with antibodies against CD3, GR-1 (BD Bioscience, Heidelberg, Germany), anti-CCR3 (Miltenyi Biotech, Bergisch Gladbach, Germany) and CD45R (eBioscience, Frankfurt, Germany) for 30 min at 4°C. The samples were analyzed by using a FACS-Canto and FlowJo (Treestar Inc).

Histological analysis

Lung tissues were analyzed by using paraffin-embedded tissue slices for histology. After staining with Hematoxylin/Eosin, the pathologist performed a blind analysis of the peribronchial and perivascular inflammation, by using a semi-quantitative scoring system with a range pending between 1 (mild) and 4 (severe) as described before (Doganci et al., 2008).

Total lung cell isolation and *in vitro* cell culture

Lungs were removed at the end of the asthma protocol, and total cells were isolated as previously described (Sauer et al., 2006). Briefly, lung tissue was cut into small pieces by a scalpel and digested with 300 U/ml Collagenase Typ Ia and 0.015% DNase (10 mg/ml) in PBS at 37°C for 1h. Digested lung was then pushed through a 40 μm cell strainer and subsequently lysed with an Ammonium-Chloride-Potassium (ACK)-Lysis buffer (0.15 M NH_4Cl, 0.1 mM $KHCO_3$, 0.1 mM Na_2-EDTA dissolved in ddH_2O). Finally the lung cells were washed with PBS and counted using a hemocytometer. Cells were platted at a concentration of 1 x 10^6 cells/ml and cultured in RPMI 1640 medium containing 100 IU/mL penicillin, 100 μg/mL streptomycin (all anprotec, Bruckberg, Germany), and 10% FCS (Biochrom GmbH, Berlin, Germany) at 37°C and 5% CO_2 and restimulated with 50 μg/ml HDM on a 48 well plate where indicated.

Flow cytometry analysis and intracellular staining

Total lung cells were stained with anti-CD4, anti-CD8, anti-CD25, anti-CD45.2, anti-CD101, anti-CCR5, anti-Siglec F (BD Bioscience, Heidelberg, Germany) and anti-CD62L (Invitrogen) antibodies for 30 min at 4°C and washed once before measuring. For intracellular staining the cells were fixed with fixation/permeabilization solution (eBioscience, Frankfurt, Germany) for 35 min at 4°C and then stained with an antibody against GATA3, T-bet (BD Bioscience, Heidelberg, Germany) and Foxp3 (Miltenyi Biotec)for 30 min at 4°C in permeabilization buffer. Afterwards cells were washed once with permeabilization buffer (eBioscience, Frankfurt, Germany) and finally with PBS. The samples were analyzed by using a FACS-Canto (BD Bioscience, Heidelberg, Germany) and FlowJo (Treestar Inc).

QUANTIFICATION AND STATISTICAL ANALYSIS

For statistical analysis, we used Prism version 8 for Windows (GraphPad, La Jolla, CA, USA). Data from are presented as mean values ± s.e.m. and differences were evaluated for significance by using two-way analysis of variance (Figures 2, 4, and 5) or two-tailed Student's *t* test (Figure 3) to generate p -value data (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$). To correct for multiple comparisons in Figures 2, 4, and 5 Sidak's test was used.

ADDITIONAL RESOURCES

The human study PreDicta is registered in the German Clinical Trials Register (www.germanctr.de: DRKS00004914).