

Expression of histamine receptors H2R and H4R are predominantly regulated via the IL-4/IL-13 receptor type II on human M2 macrophages

To The Editor,

The pathophysiology of allergic skin diseases such as atopic dermatitis (AD) is characterized by complex interactions between susceptibility genes of the disease, a defect skin barrier and aberrant immune responses. Among other immune cells, monocytes differentiating to macrophages are attracted into inflamed skin and are exposed to Th2 cytokines and to histamine detectable in high concentrations in AD patients' skin contributing to immunomodulation and pruritus.^{1,2} Increasing expression levels of functional histamine receptors, in particular of the histamine H4 receptor (H4R), on immune cells within the inflammatory infiltrate of eczematous skin lesions may exacerbate inflammation by mediating cytokine and chemokine release in contact to local histamine.³ Knowledge of these mechanisms may result in therapeutic strategies to alleviate inflammation.

Two specific antagonists of the H4R were studied in phase II studies with AD patients.^{2,51} A number of other substances, particularly dupilumab⁵², currently approved for treatment of type II inflammatory diseases, have shown meaningful reduction of pruritic symptoms and clinical AD severity scores.

Since little is known about the cross talk of type II cytokines and histamine receptors on macrophages known to be increased in the dermis of inflamed skin in AD⁵³, we investigated in this study if the Th2 cytokines IL-4 and IL-13 influence the histamine receptor mRNA expression levels in human monocyte-derived M2 macrophages differentiated in the presence of M-CSF for 10 days (see additional information in the Material and Methods section and Figure S1 in this article's Online Repository).

As shown previously, the H1R-, H2R- and H4R- but not H3R mRNA expressions are detectable in fully differentiated human M2 macrophages.⁴

The stimulation of M2 macrophages with IL-4 led to an up-regulation of H2R and H4R, whereas IL-13 selectively up-regulated the H4R at mRNA level in M2 macrophages (Figure 1B,C,E,F). The H1R mRNA expression was neither up-regulated by IL-4 nor by IL-13 (Figure 1A,D).

Macrophage M2 marker CD206, also known as mannose receptor C-type I, expressing cells were detected in the dermis of AD patients pointing to a possible role for macrophages in disease

pathology⁵³. Interestingly, M2 macrophages generated from monocytes from patients with moderate-to-severe AD showed higher constitutive expression levels of the H4R mRNA when compared to healthy controls, which was not seen for the H2R (Figure 1G,H). Since it is suggested that serum IgE levels correlate with disease severity of AD⁵⁴, we also measured total IgE levels in the serum of respective patients by ImmunoCap, Thermo Fisher Scientific. AD patients with elevated H4R mRNA expression levels showed also high total IgE levels in their serum as shown in Table S1 in this article's Online Repository. These data obtained in human macrophages strongly fit observations in human eosinophils in a recently published work.⁵ In human eosinophils, H4R mRNA expression was also up-regulated by IL-4 and IL-13 whereas up-regulation of H2R mRNA expression was, just as in macrophages, mediated via IL-4 only.⁵ Similar to M2 macrophages, eosinophils from AD patients constitutively express high levels of H4R when compared to cells from healthy controls.⁵

The up-regulation of H4R mRNA expression also in response to IL-13 as well as the high levels of constitutive mRNA expression of the receptor shown in eosinophils⁵ and in this work in M2 macrophages from AD patients appoint to a relevant role of the H4R in the pathophysiology of AD. Under these conditions, the H4R is also referred to as a pathological H4R. The observation of a significant up-regulation of IL-13 mRNA expression in subacute and chronic AD skin lesions, which was not detected for IL-4⁵⁵, fosters this hypothesis.

Both cytokines IL-4 and IL-13 determine potent type 2 inflammatory processes by signaling through their cell surface receptor heterodimers.¹ Heterodimerisation of type I or II receptor subunits by IL-4 or IL-13 leads to catalytic activation of associated Janus/Tyrosine kinases (JAK/TYK comprising of JAK1, JAK2, JAK3, and TYK2). Subsequent phosphorylation of tyrosine residues in the receptors takes place thereby signal transducer and activator of transcription 6 (STAT6) was activated and translocated to the nucleus to induce transcription of cytokine-induced genes⁵⁶ (Figure 2A).

We investigated the signaling pathways of IL-4 and IL-13 in order to find the checkpoints for H2R and H4R up-regulation in human M2 macrophages.

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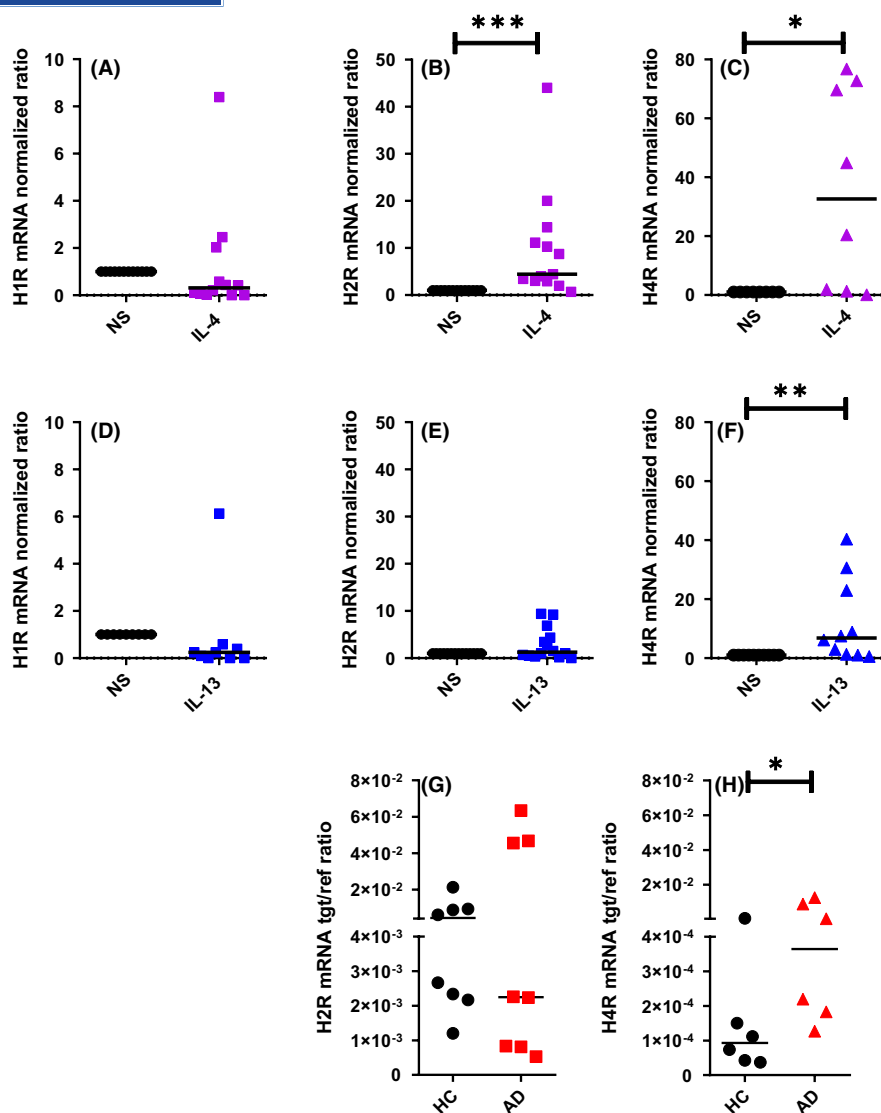


FIGURE 1 Regulation of the H1R, H2R, and H4R mRNA expression in response to Th2 cytokines in human M2 macrophages. In the presence of M-CSF differentiated macrophages from residual blood samples from platelet apheresis A-F or from peripheral blood from healthy controls or from atopic dermatitis patients G and H were stimulated with IL-4 (20 ng/ml) or IL-13 (15 ng/ml) for 48 h. The mRNA expression of A, H1R, B, H2R, and C, H4R was measured in fully differentiated and in IL-4- or D, H1R, E, H2R, F, H4R in IL-13-activated M2 macrophages. G, H2R and H, H4R constitutive mRNA expression was measured in fully differentiated M2 macrophages (10 days) from healthy controls compared with cells from patients with atopic dermatitis. Significant differences, as determined by the Wilcoxon matched-pairs signed rank test A-F and the Mann Whitney test G and H, are indicated as follows: * $p < .05$; ** $p < .01$; *** $p < .001$; medians are shown in the graphs. A, ($n = 12$ independent donors and experiments), B, ($n = 13$ independent donors and experiments), C, ($n = 8$ independent donors and experiments), D, ($n = 9$ independent donors and experiments), E, ($n = 14$ independent donors and experiments), F, ($n = 10$ independent donors and experiments), G, ($n = 8$ independent donors and experiments), H, ($n = 6$ independent donors and experiments), NS = non stimulated, HC = healthy controls, AD = atopic dermatitis, tgt/ref ratio = target/reference ratio

Therefore, IL-4 or IL-13 stimulated (48 h) M2 macrophages were pre-incubated with antibodies targeting the IL-4 and IL-13 receptor complex subunits: Type I (IL-4) receptor composed of the IL-4 receptor alpha (IL-4R α) (dupilumab) and the common γ chain/IL-2R γ , and type II (IL-4/IL-13) receptor composed of the IL-4R α and IL-13R α 2 subunit.⁶ The binding of IL-13 to the IL-13R α 2 was also blocked by a specific antibody. Inhibitors of the downstream regulators JAK3, TYK2 and of the activator protein-1 (AP-1) were also added to the cells before Th2 cytokine stimulation. We did

not block JAK1 signaling because JAK1 is associated with both the type I and type II receptor and thus does not differentiate between both^{S6} (dose-response experiments for antibodies and inhibitors see Figure S2 in this article's Online Repository).

Dupilumab, a humanized IgG4 monoclonal antibody targeting the IL-4R α subunit, significantly inhibited the IL-4-mediated H2R (Figure 2B) as well as the IL-4- and IL-13-mediated H4R mRNA up-regulation. Targeting the other IL-4 type I or IL-4/IL-13 type II receptor subunits by specific antibodies was less effective (Figure 2D,F). A

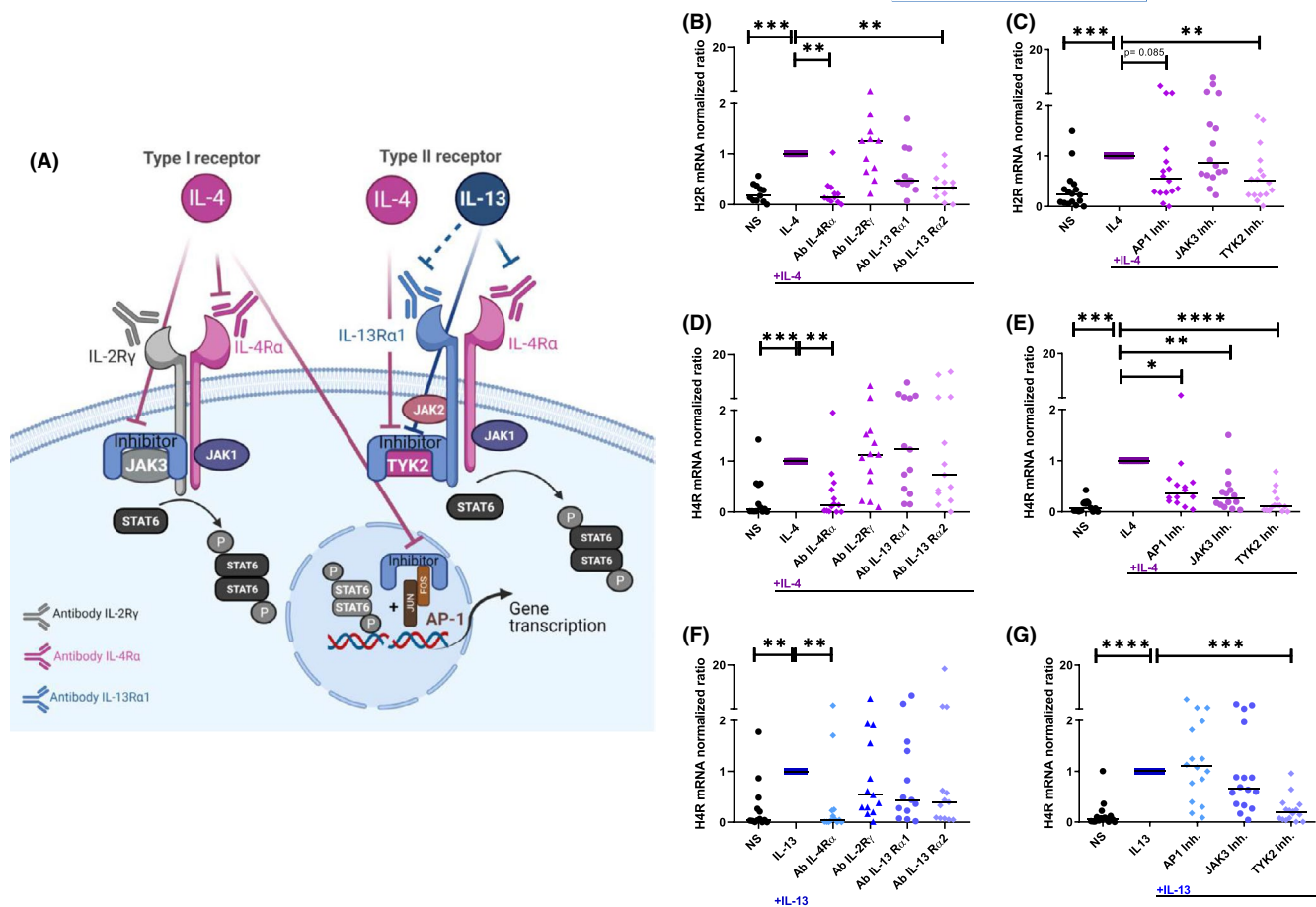


FIGURE 2 The IL-4- or IL-13-induced up-regulation of H2R and H4R mRNA expression in human M2 macrophages is mainly mediated via the IL-4/IL-13 receptor type II. A, The binding of IL-4 and IL-13 to their cognate receptors (type I and type II) triggers the activation of receptor subunit associated signaling adaptor molecules which induce the recruitment and phosphorylation of signal transducer and activator of transcription 6 (STAT6) that can cooperate with activator protein-1 (AP-1) to activate specific gene transcription programs (simplified diagram, created with BioRender.com.). B–G, In the presence of M-CSF differentiated macrophages were stimulated with IL-4 (20 ng/ml) or IL-13 (15 ng/ml) for 48 h. 20 min before stimulation, the subunits of IL-4R type I and IL-4/IL-13R type II cell surface receptors were either selectively blocked by specific antibodies (Ab): Dupilumab (10 μ M), a monoclonal IgG4 antibody which blocks the IL-4R α subunit, a polyclonal goat IgG antibody was used to block the common γ chain/IL-2R γ (20 μ M), the IL-13R α 1, and IL-13R α 2 subunits were blocked by monoclonal antibodies (10 μ M) each, or their downstream signaling adaptor molecules were blocked by selective inhibitors (Inh.): T-5224 for AP-1 (10 μ M), PF 06651600 malonate (10 μ M) for JAK3 and TC JL 37 (10 μ M) for TYK2. B and C, H2R mRNA expression in IL-4 stimulated M2 macrophages, D and E, H4R mRNA expression in IL-4 stimulated M2 macrophages, F and G, H4R mRNA expression in IL-13 stimulated M2 macrophages. Significant differences, as determined by Friedman Dunn's Multiple Comparison test selected pairs and as determined by the Wilcoxon matched-pairs signed rank test (NS versus IL-4 or IL-13) are indicated as follows: * p < .05; ** p < .01; *** p < .001; **** p < .0001; medians are shown in the graphs. NS = non-stimulated; B, (n = 11 independent donors and experiments), C, (n = 16 independent donors and experiments), D, (n = 13 independent donors and experiments), E, (n = 14 independent donors and experiments), F, (n = 13 independent donors and experiments), G, (n = 16 independent donors and experiments). NS = non-stimulated

possible explanation for this is offered by the following hypothesis: If the common γ chain/IL-2R γ is exclusively blocked, the IL-13R α 1 subunit can be recruited to form heterodimers with IL-4R α leading to signal transduction. A corresponding result would be seen vice versa when IL-13R α 1 is blocked alone. In our study, an anti-IL-13R α 1 antibody was able to inhibit the IL-13-induced H4R up-regulation by trend (Figure 2F).

Interestingly, the IL-4-induced up-regulation of H2R mRNA expression was also reversed by an antibody directed against the IL-13R α 2 subunit (Figure 2B). The mechanisms of the IL-13R α 2 subunit are not clearly understood until yet. Some reports describe that the

IL-13R α 2 acts as a decoy receptor and does not transduce for IL-4/IL-13 signaling into the cell^{57,58}. However, some data support the hypothesis that the cytoplasmic tail of IL-13R α 2 may function as a regulator of IL-4 signaling by physical interactions with the IL-4 R α ^{7,58} which may fit our observation.

Regarding the selectively associated JAK3 or TYK2 signaling molecules for type I and type II receptors, respectively, we observed significant inhibition of IL-4-induced H2R mRNA up-regulation using the selective TYK2 inhibitor (Figure 2C).

The IL-4-induced H4R mRNA expression was blocked by a JAK3 inhibitor which is selective for type I receptor (IL-4R), and

even stronger by the inhibitor of TYK2 which is selective for type II (IL-4/IL-13R). Inhibition of TYK2 also prevents the IL-13-induced up-regulation of H4R mRNA expression (Figure 2E,G). These results set the type II receptor in the focus of H2R and H4R regulation.

Blocking the transcription factor AP-1 by small molecules inhibited the IL-4-mediated up-regulation of H4R mRNA expression only (Figure 2E). The AP-1 family proteins play a critical part in several severe as well as in inflammatory disorders by regulating transcription of specific genes involved in production of cytokines, chemokines, proliferation, and differentiation of immune cells⁵⁹. Remarkably, it has been demonstrated that the IL-4 dependent induction of germ-line ϵ transcripts, an essential step preceding Ig isotype switching to IgE in mice requires the interaction of AP-1 proteins Jun/Fos and STAT6⁵¹⁰.

For human M2 macrophages, we show here for the first time a novel function of AP-1 regulating the IL-4-dependent up-regulation of the H4R mRNA expression.

In parallel, we investigated the phosphorylation of STAT6 at tyrosine 641, which is considered to be a major pathway involved in IL-4 or IL-13 responsiveness, by flow cytometry.⁶ After respective blocking of the IL-4/IL-13 pathways by antibodies and inhibitors of the signaling molecules, inhibition of the phosphorylation of STAT6 mostly corresponded to the picture of the inhibition of IL-4- and IL-13-induced H4R mRNA up-regulation. The phosphorylation of STAT6 at tyrosine 641 was blocked by dupilumab, by an antibody against IL-13R α 1, and by JAK3 and TYK2 inhibitors (see Figure S3 in this article's Online Repository) pointing to a crucial role of STAT6 in the regulation of H4R gene transcription.

The H4R was shown *in vitro* and *in vivo* to be involved in mechanisms which are characteristic for the pathogenesis of AD.³ In two clinical proof of concept trials in patients with AD selective H4R antagonists showed reduction of pruritus and/or improvement of eczema.^{2,51} A novel role of the H2R as a possible amplifier of inflammatory type II responses in AD resulting in up-regulating the chemokine CCL17 in monocytes and macrophages was recently described by us.⁸

Dupilumab, the first monoclonal antibody approved for the treatment of AD⁵² and also small molecules targeting the JAK/TYK pathways showed positive outcomes in the treatment of inflammatory diseases of patients or in clinical trials.⁹

Our experiments with specific substances blocking the IL-4R type I and IL-4/IL-13R type II receptor subunits as well as the downstream signaling molecules, and in particular the transcription factor AP-1, showed novel mechanisms of action leading in the prevention of the up-regulation of H2R and most pronounced of H4R in human M2 macrophages.

Our findings should contribute to an improved understanding of the regulation of histamine receptors with possible relevance for AD and the related mode of action of substances interfering with type II inflammatory responses in allergic skin diseases such as AD.

KEYWORDS

atopic dermatitis, histamine, macrophages

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CONFLICT OF INTEREST

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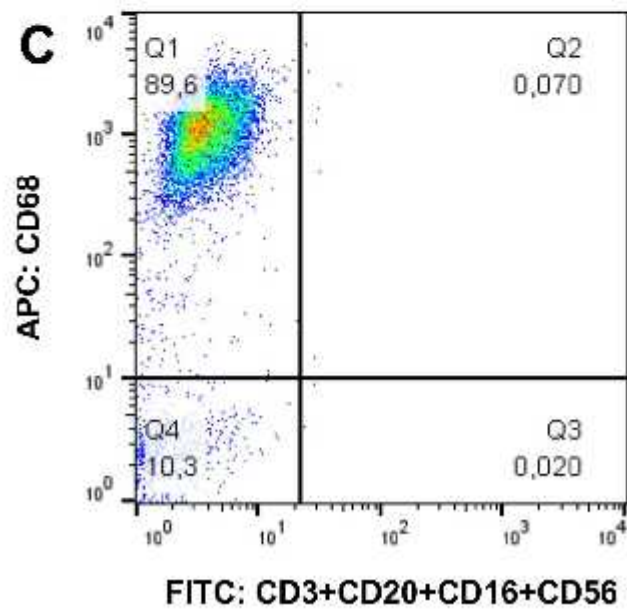
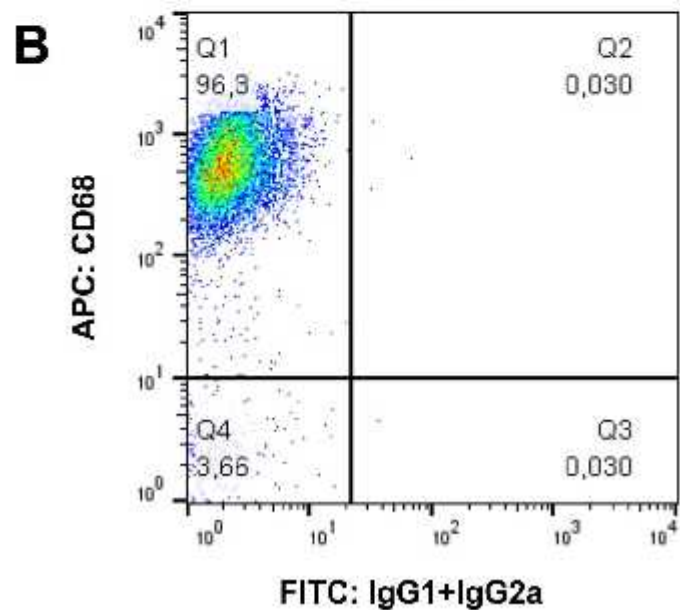
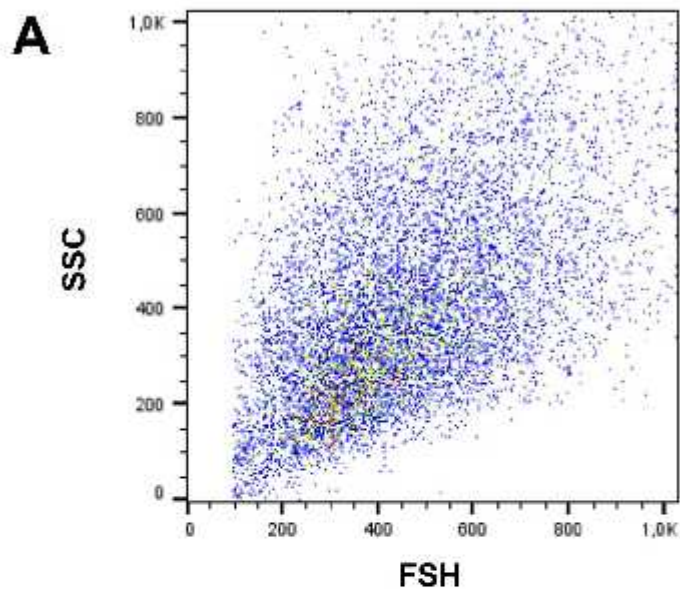
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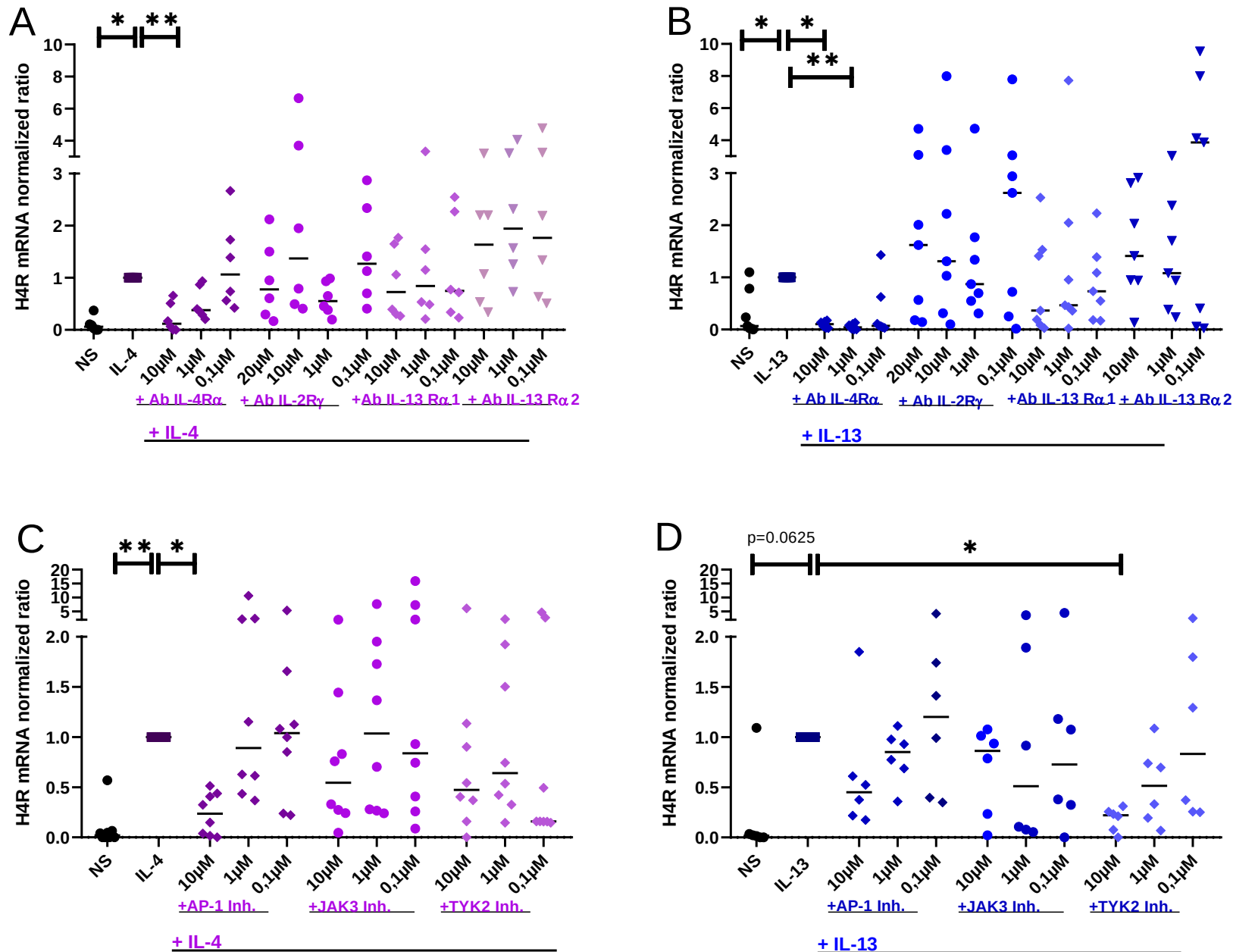
SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

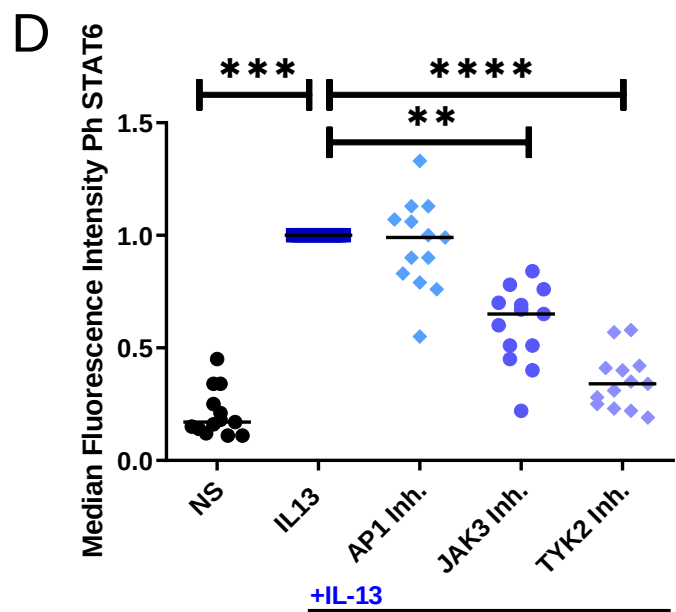
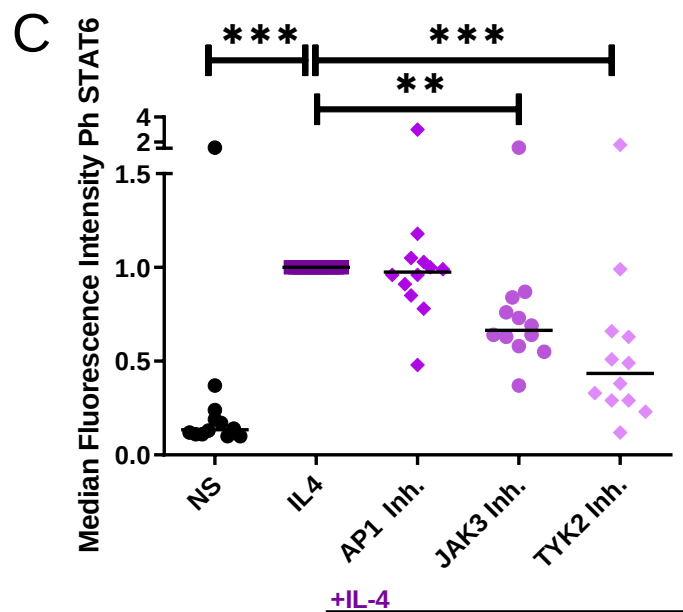
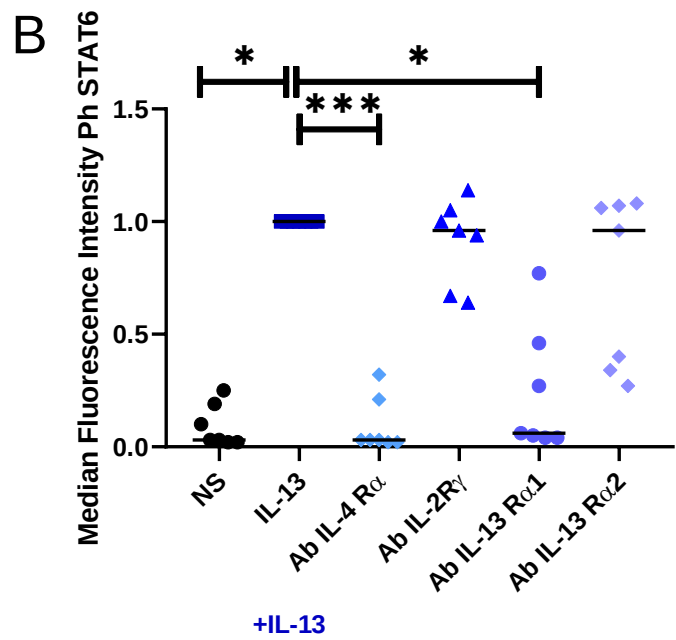
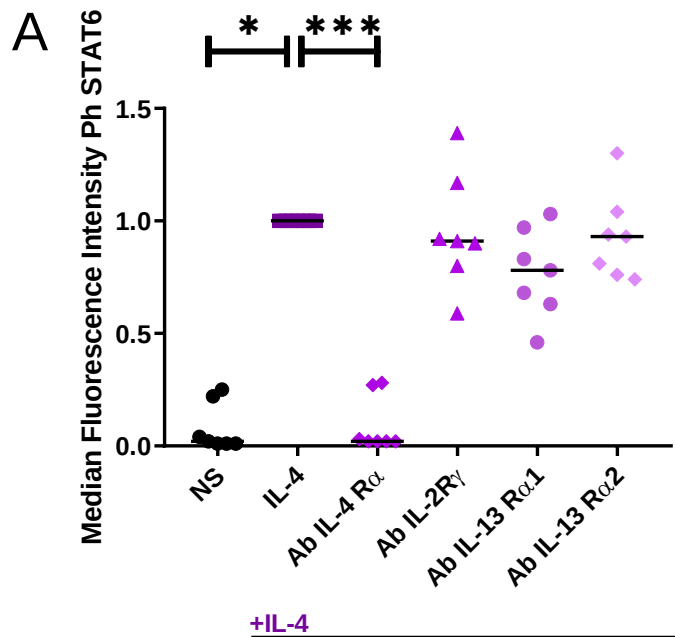
Supplementary Figure O1



Supplementary Figure O2



Supplementary Figure O3



Supplementary Table 01

ID	Total IgE (kU/l)	Tgt/Ref ratio H4R mRNA expression
01	11	1,11E-04
02	136	1,83E-04
03	6267	1,26E-02
04	13465	5,09E-04
05	4825	8,85E-03

Online Repository

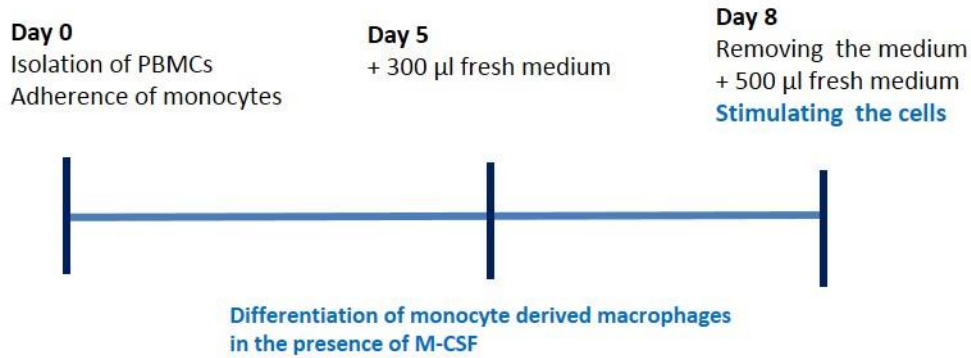
Materials and Methods

Differentiation of M2 macrophages

Residual blood samples from platelet (PLT) apheresis disposables used for routine PLT collection and of regular anonymous healthy donors served as source material for the isolation of human peripheral blood mononuclear cells (PBMCs). Peripheral blood samples were taken from patients with extrinsic AD and from healthy controls comparing the constitutive H4R mRNA expression. PBMCs were separated by density gradient centrifugation on lymphoprep (Fresenius Kabi Norge AS, Oslo, Norway). With a seeding density of 1×10^6 cells pro well, PBMCs were plated in a 24 well plate in Iscoves Medium supplemented with AB serum (2,5 % v/v).

We differentiated M-CSF stimulated macrophages from PBMCs/monocytes comparable to our previous publications according to the protocol of promocell ^{S11, S12, S13}. To attach the monocytes, cells were incubated for 1.5 h at 5% CO₂ and 37°C. Non-adherent cells were removed by vigorously washing of adherent cells three times with PBS. An appropriate amount of RPMI 1640, supplemented with 2 mM l-glutamine, 100 mg/ml penicillin/streptomycin, 12 mM Hepes, and 5% v/v human AB serum heat inactivated (PAN-Biotech, Aidenbach, Germany; all other media components from Biochrom, Berlin, Germany) and 10 ng/ml macrophage colony-stimulating factor (M-CSF) (R&D, San Diego, CA, USA) was added. Cells were incubated at 37°C and 5% CO₂.

At day 5: 300 µl fresh medium was added, and at day 8: the medium was completely removed and fresh medium was added. From this point on the macrophages were ready for stimulation. According to our protocols we stimulated the cells at day 8 for 48 h with IL-4 or IL-13.



Differentiation of monocyte derived macrophages in the presence of M-CSF.

At day 0 monocytes were attached to the culture dishes. Non-adherent cells were removed by vigorously washing of the adherent cells. At day 5: 300 µl new medium was added and at day 8: the medium was completely removed and fresh medium was added. From this point on the macrophages were ready for stimulation.

After 10 days M2 macrophages showed expression and up-regulation of the co-stimulatory molecule CD86 by IL-4 and IL-13. Also M2 markers CD206 and CD163 were expressed on M2 macrophages regulated by IL-4 or IL-13.

We also checked expression of CD80 on M-CSF differentiated M2 macrophages. CD80 was not expressed and neither regulated by IL-4 nor by IL-13. The expression of co-stimulatory molecules and M2 markers on M2 macrophages in this study are in accordance to the data described in our previous publications^{S11, S12}.

To exclude the possibility that non-adherent cells influence the purity of the M-CSF generated macrophages, we stained the cells at day 10 before stimulation with FITC conjugated surface lineage markers for T cells: CD3, for B cells: CD20 (Beckman, Coulter, Krefeld, Germany), for granulocytes: CD16 and for NK cells: CD56 (BioLegend, San Diego, CA, USA). The respective FITC conjugated isotype controls IgG2a and IgG1 (Beckman, Coulter, BioLegend), were tested in parallel. Cells were fixed, permeabilized using the BD Cytofix/Cytoperm fixation/permeabilization kit (BD Bioscience) and the macrophage differentiation marker CD68 belonging to the family of lamp proteins, which are intracellularly expressed in cytoplasmic granules was stained with anti-human CD68-Allophycocyanin (APC) (BioLegend). The

respective APC conjugated isotype control IgG2b (BioLegend) was tested in parallel. Sample acquisition was performed by flow cytometry (FACS Calibur, Becton Dickinson, Heidelberg, Germany) and % gated cells was calculated in the quadrants by FlowJow software (Becton Dickinson).

As depicted in Supplementary Figure S1 up to 96% of the cells show expression of CD68 as described in our previous publications where a significant up-regulation of CD68 during the period of differentiation of monocytes to macrophages was observed^{S12}. We found no contamination of the cell culture with immune cells expressing one of the lineage markers CD3, CD16, CD20 or CD56.

Reagents to block the IL-4R type I and IL-4/IL-13R type II receptor complex subunits, the Janus/Tyrosine kinases (JAK/TYK) and the transcription factor activator protein-1(AP-1)
Monoclonal mouse IgG4 antibody dupilumab (Dupixent, Sanofi-Aventis GmbH, Frankfurt am Main, Germany) inhibiting the IL-4R α chain; Polyclonal goat IgG antibody (common γ chain/IL-2R γ R&D Systems) inhibiting the IL-4R common γ chain/IL-2R γ ; Monoclonal mouse antibody (Aldevron, Freiburg, Germany) inhibiting the IL-13R subunit α 1; Monoclonal mouse antibody (BioLegend, Germany) inhibiting the IL-13R subunit α 2.

T-5224 for activator-protein 1 (AP-1) (10 μ M) (Cayman, Chemical, USA), PF 06651600 malonate, as a potent and selective Janus kinase 3 (JAK3) inhibitor, TC JL 37, as a potent inhibitor of Tyrosine kinase 2 (TYK2) (both from Tocris Bioscience, Bristol, UK) were used to inhibit the IL-4R type I and IL-4/IL-13R type II receptor down-stream signalling adaptor molecules.

Stimulation of fully differentiated M2 macrophages

For assessment of H2R and H4R mRNA expression, fully differentiated M2 macrophages were stimulated at day 8 with IL-4 (20 ng/ml) (R&D Systems), IL-13 (15 ng/ml) (R&D Systems) for

48 h. For blocking the IL-4R type I, IL-4/IL-13R type II and the down-stream signalling molecules, antibodies and inhibitors were added in concentrations ranging from 0.1 μ M to 20 μ M 20 min before adding the Th2 cytokines. To assess the levels of STAT6 phosphorylation on Tyrosine 641, we added the antibodies and inhibitors 10 min before stimulation with Th2 cytokines for further 20 min.

Quantitative Real Time-PCR

Total RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The cDNA was synthesized by reverse transcription (QuantiTect reverse transcription kit, Qiagen, Germany). Quantitative Real Time-PCR (qRT-PCR) was performed with Quantitect[®] primer assays for H1R (QT00199857), H2R (QT00210378), H4R (QT00032326) and H4R 390 (1912485, 1912486) (TIB Molbiol Syntheselabor, Berlin, Germany) and RPS 20 (ribosomal protein S20) (QT00079247) using SYBR[®] Green according to the manufacturer's instructions (Qiagen, Hilden, Germany) using the LightCycler 1.5 and 480. The amount of the target mRNA relative to the amount of the reference gene, ribosomal protein S20 (rps 20) mRNA in the same sample was calculated using the comparative Ct method also known as the Δ Ct method provided by the Software LC 480 (Roche Molecular Biochemicals) and target/reference ratios are depicted. Otherwise Ct values of the targets are normalized to the Ct values of the housekeeping gene ribosomal protein S20 (rps 20) and expressed as normalized mRNA ratio to the IL-4- or IL-13-stimulated samples. This was calculated using the comparative Ct method also known as the $\Delta\Delta$ Ct method provided by the Software LC 480 (Roche Molecular Biochemicals).

Flow cytometric analysis of STAT6 phosphorylation at Tyrosine 641 residue

In response to IL-4 or IL-13 the tyrosine residue 641 (Tyr641) of STAT6 is phosphorylated. STAT6 forms heterodimers, translocates to the nucleolus and regulates specific gene transcription. Stimulated and non-stimulated monocyte derived M2 macrophages were

carefully scraped from the culture plates and seeded (5×10^5 cells per well) into 96 well plates. Cells were fixed, permeabilized using the BD Cytofix/Cytoperm fixation/permeabilization kit (BD Bioscience). The intracellular phosphorylation of Tyr641 of STAT6 was stained with a monoclonal anti-human Phycoerythrin-labelled antibody which recognizes the phosphorylated STAT6 form (mouse monoclonal IgG1, 0,125 μ g/100 μ l) (BioLegend). The matched isotype control IgG1 (BioLegend) was tested in parallel in the same concentration. Sample acquisition was performed by flow cytometry (FACS Calibur, Becton Dickinson, Heidelberg, Germany) and median fluorescence intensities (MFI) were calculated by FACS CellQuest Pro (Becton Dickinson).

The Median fluorescence (MFI) values of the isotype controls were in the same range in all samples. The MFI values were normalized to MFI values of the IL-4 and IL-13-stimulated M2 macrophages.

Statistics

For statistical analyses, the software GraphPad Prism Version 8.0 was used (GraphPad software, San Diego, CA, USA). First we performed methods to test the normal Gaussian distribution of the data. In all our experiments due to the individual variations of the data the normality tests were failed. The non-parametric tests Wilcoxon matched-pairs signed rank test, Mann Whitney test or Friedman Dunn's Multiple Comparison test selected pairs were performed and the median is shown in the graph. A p-value < 0.05 was regarded as statistically significant (p < 0.05 was labelled with *, p < 0.01 was labelled with **, p < 0.001 was labelled with ***, p < 0.0001 was labelled with ****).

Figure legends

Supplementary Figure S1

M-CSF differentiated macrophages express up to 90% the macrophage differentiation marker CD68.

Macrophages differentiated in the presence of M-CSF were stained with FITC conjugated surface lineage markers for T cells: CD3, for B cells: CD20, for granulocytes: CD16 and for NK cells: CD56. The respective FITC conjugated isotype controls IgG2a and IgG1 were tested in parallel. Cells were fixed, permeabilized using the BD Cytotfix/Cytoperm fixation/permeabilization kit (BD Bioscience).

The macrophage differentiation marker CD68 belonging to the family of lamp proteins, which are intracellularly expressed in cytoplasmic granules was stained with anti-human CD68-Allophycocyanin (APC) (BioLegend). The respective APC conjugated isotype control IgG2b was tested in parallel. Sample acquisition was performed by flow cytometry (FACS Calibur, Becton Dickinson, Heidelberg, Germany) and % gated cells was calculated in the quadrants by FlowJow software (Becton Dickinson). One representative example out of 5 is shown. A, Forward scatter versus Side scatter, B, APC-conjugated anti-CD68 versus FITC-conjugated isotypes, C, APC-conjugated anti-CD68 versus FITC-conjugated anti-CD3, anti-CD16, anti-CD20, anti CD56 (Lineage marker).

Supplementary Figure S2

The IL-4-induced up-regulation of H4R mRNA expression is dose dependently reversed by antibodies directed against the IL-4R type I and IL-4/IL-13R type II receptor subunits and by inhibitors of IL-4/IL-13 down-stream signaling pathways.

Macrophages differentiated in the presence of M-CSF were stimulated with IL-4 (20 ng/ml) or IL-13 (15 ng/ml) for 48 min. 20 min before stimulation, the subunits of IL-4 and IL-13 cell surface receptors were either selectively blocked by specific antibodies (Ab) (ranging from 20 to 0.1 μ M each): an IgG4 antibody which targets the IL-4R α subunit (dupilumab), a polyclonal goat IgG antibody was used targeting the common γ chain/IL-2R γ , and monoclonal antibodies were used targeting the IL-13R α 1 or IL-13R α 2 subunits, or the IL-4/IL-13 receptor down-stream signalling adaptor molecules were blocked by specific inhibitors (Inh.) (ranging from

229 10 to 0.1 μ M each): T-5224 targeting activator protein-1 (AP-1), PF 06651600 malonate
 230 targeting JAK3 and TC JL 37 targeting TYK2.

231 H4R mRNA expression A and C, in IL-4- and B and D, in IL-13-stimulated M2 macrophages.
 232 The mRNA expression of the H4R was measured by qRT-PCR. Significant differences, as
 233 determined by Friedman Dunn's Multiple Comparison test selected pairs and as determined by
 234 the Wilcoxon matched-pairs signed rank test (NS versus IL-4 or IL-13) are indicated as follows:
 235 * $p < 0.05$; ** $p < 0.01$; medians are shown in the graphs. NS = non-stimulated; A, (n = 6
 236 independent donors and experiments), B, (n = 7 independent donors and experiments), C, (n =
 237 8 independent donors and experiments), D, (n = 6 independent donors and experiments). NS =
 238 non-stimulated.

239 **Supplementary Figure S3**

240 **Phosphorylation of STAT6 on tyrosine 641 is mediated via the IL-4 type I and IL-4/IL-13** 241 **type II receptor.**

242 Macrophages differentiated in the presence of M-CSF were stimulated with IL-4 (20 ng/ml) or
 243 IL-13 (15 ng/ml) for 20 min. 10 minutes before stimulation, the subunits of IL-4 and IL-13 cell
 244 surface receptors were either selectively blocked by specific antibodies (Ab): an IgG4 antibody
 245 which blocks the IL-4R α subunit (dupilumab) (10 μ M), a polyclonal goat IgG antibody was
 246 used to block the common γ chain/IL-2R γ (20 μ M), the IL-13R α 1 or IL-13R α 2 subunits were
 247 blocked by monoclonal specific antibodies (10 μ M) each, or the IL-4/IL-13 receptor down-
 248 stream signalling adaptor molecules were blocked by specific inhibitors (Inh.): T-5224 for
 249 activator protein-1 (AP-1) (10 μ M), PF 06651600 malonate for JAK3 (10 μ M) and TC JL 37
 250 for TYK2 (10 μ M). A and C, IL-4- and B and D, IL-13-activated M2 macrophages.

251 The phosphorylation of STAT6 was measured using a monoclonal Ab which recognizes human
 252 STAT6 when phosphorylated on tyrosine 641 and analysed by flow cytometry. The median
 253 fluorescence intensities normalized to the IL-4- and IL-13-stimulated samples are depicted.

Significant differences, as determined by Friedman Dunn's Multiple Comparison test selected pairs and as determined by the Wilcoxon matched-pairs signed rank test (NS versus IL-4 or IL-13) are indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; medians are shown in the graphs. A and B ($n = 7$ independent donors and experiments C, ($n = 12$ independent donors and experiments), D, ($n = 13$ independent donors and experiments). NS = non-stimulated.

Supplementary Table S1

H4R mRNA expression levels in M2 macrophages from AD patients show a slight trend to be associated with total IgE levels in their serum.

The constitutive H4R mRNA expression levels were analysed in fully differentiated M2 macrophages (10 days) from patients with atopic dermatitis (AD). Target/reference (Tgt/Ref) ratio of H4R versus housekeeping gene ribosomal protein S20 (rps 20) is depicted. Total IgE levels were measured by an in vitro test system for the quantitative measurement of circulating total IgE in the serum of respective AD patients (ImmunoCap, Thermo Fisher Scientific).

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