ORIGINAL ARTICLE

# Early and Long-Term Effects of Dupilumab Treatment on Circulating T-Cell Functions in Patients with Moderate-to-Severe Atopic Dermatitis

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Dupilumab, a mAb targeting IL-4 receptor alpha (IL-4Rα), markedly improves disease severity in patients with atopic dermatitis. However, the effect of IL-4Rα blockade on dynamics of circulating skin-homing T cells, which are crucial players in the pathologic mechanism of atopic dermatitis, has not been studied yet. In addition, it remains unknown whether dupilumab treatment induces long-lasting T- and B-cell polarization. Therefore, we studied the short- and long-term effects of dupilumab treatment on IL-4Rα expression and T-cell cytokine production within total and skin-homing (cutaneous lymphocyte antigen$^+$/CCR4$^+$) subpopulations in patients with moderate-to-severe atopic dermatitis. Dupilumab treatment completely blocked IL-4Rα expression and signal transducer and activator of transcription 6 phosphorylation in CD19$^+$ B cells and CD4$^+$ T cells within 2 hours of administration and through week 52. Although no change in the proportion of skin-homing T-cell subsets was found, dupilumab treatment significantly decreased the percentage of proliferating (Ki67$^+$) and T helper type 2 and T helper type 22 cytokine–producing skin-homing CD4$^+$ T cells at week 4. No evidence of general T helper type cell skewing following a year of dupilumab treatment was found. In summary, dupilumab treatment rapidly and stably inhibited IL-4Rα, which was accompanied by a strong early functional immunological effect specifically on skin-homing T cells without affecting overall T helper type cell skewing in the long term.

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## INTRODUCTION

The pathogenesis of atopic dermatitis (AD), a common chronic inflammatory skin disease, is mainly driven by CD4$^+$ T helper type (Th) 2 cell–mediated responses. The type 2 inflammation in AD is characterized by profound overexpression of type 2–related cytokines, such as IL-4, IL-5, IL-13, IL-31, and TARC or CCL17, in both skin and blood (Czarnowicki et al., 2014). Recently, activation of other T-cell axes, such as Th1, Th17/IL-23, and Th22, has also been reported in patients with AD (Werfel et al., 2016).

During the past decades, more insight into the pathogenesis of AD has led to the development of novel targeted therapies. Dupilumab (Dupixent) is the first targeted antibody-based treatment for moderate-to-severe AD that has been approved in the European Union, United States, Japan, and other countries (Paller et al., 2017). It is a fully human monoclonal IgG4 antibody, targeting IL-4 receptor alpha (IL-4Rα), best known for the regulation of IgE production by B cells and, in a lesser amount, also expressed on T cells, where it promotes differentiation of Th2 cells (Gandhi et al., 2016; Tanaka et al., 2007). By dually inhibiting the signaling of IL-4 and IL-13, dupilumab has demonstrated significantly improved clinical and patient-reported outcomes in patients with moderate-to-severe AD (Ariëns et al., 2020; Blauvelt et al., 2017; de Bruin-Weller et al., 2018; de Wijs et al., 2020; Faiz et al., 2019; Olesen et al., 2019; Tauber et al., 2019). Previous studies have shown that dupilumab significantly reduces circulating serum levels of type 2 biomarkers and suppresses Th2, Th17, and Th22 inflammatory pathways in lesional skin as early as 4 weeks after treatment initiation (Ariëns et al., 2020; Hamilton et al., 2014). However, the effect of IL-4Rα blockade on circulating, skin-homing T cells, which are crucial players in the pathologic mechanism of AD, has not been studied yet. Peripheral tissue-homing receptors enable T-cell subsets to traffic through distinct domains of nonlymphoid peripheral tissues (Brinkman et al., 2013). Cutaneous lymphocyte antigen (CLA) and chemokine receptors CCR4 and CCR10 have been proposed as critical mediators of skin-specific Th-cell homing by binding

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*Abbreviations: AD, atopic dermatitis; CLA, cutaneous lymphocyte antigen; HC, healthy control; IL-4Rα, IL-4 receptor alpha; Th, T helper type*

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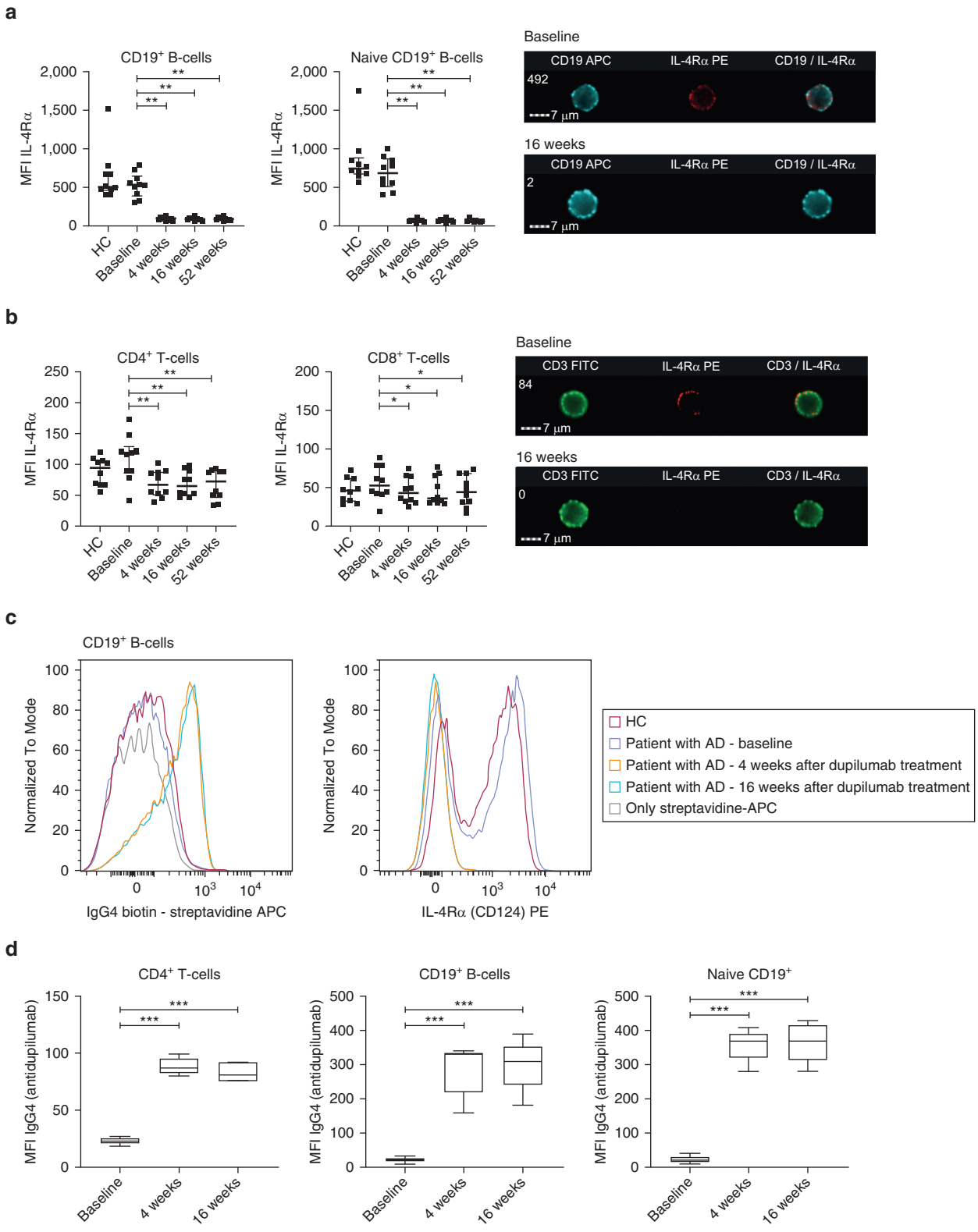


**Figure 1. Effect of dupilumab binding on measurable IL-4Rα levels.** (**a**) MFI of IL-4Rα on total and naive (CD27$^-$ IgD$^+$) CD19$^+$ B cells after 4, 16, and 52 weeks of treatment compared with HCs (left and middle panel). Medians with interquartile range are presented. Representative ImageStream visualization of measurable IL-4Rα levels (red) on a CD19$^+$ (blue) B cell at baseline and after 16 weeks of treatment (right panel). (**b**) MFI of IL-4Rα on CD4$^+$ and CD8$^+$ T cells before initiation of dupilumab treatment (baseline) and after 4, 16, and 52 weeks of treatment compared with HCs (left and middle panel). Medians with interquartile range are presented. Representative ImageStream visualization of measurable IL-4Rα levels (red) on a CD3$^+$ (green) T cell at baseline and after 16 weeks of treatment (right panel). (**c**) Representative histogram of flow cytometry analysis of IgG4 (dupilumab) (left) and IL-4Rα (right) on CD19$^+$ B cells from a patient with AD before initiation of dupilumab (purple) and after 4 weeks (green) and 16 weeks of dupilumab treatment (blue), an HC (red), and the control staining including only the secondary antibody streptavidin-APC (yellow). (**d**) MFI of IgG4 (dupilumab) on CD4$^+$ T cells, CD19$^+$ B cells, and naive CD19$^+$ B

to E-selectin, TARC or CCL17, and CTACK/CCL27, respectively (Ferran and Santamaria-Babi, 2010; Soler et al., 2003). $CLA^+$ T cells recirculate between skin and peripheral blood, where they might reflect effector T cells in AD skin lesions (Czarnowicki et al., 2017). Therefore, circulating skin-homing T cells might serve as cellular peripheral biomarkers and as a source of translational knowledge in T-cell–mediated skin diseases such as AD (Ferran et al., 2013). In addition, an important unanswered question is whether long-term blockade of IL-4Rα may lead to skewed T-cell responses with increasing Th1 and Th17 polarization. Several recent case reports have reported Th1- and Th17-mediated adverse effects developing newly in patients with AD during dupilumab treatment, including psoriasis (Safa and Paumier, 2019; Tracey et al., 2018; Varma and Levitt, 2020), alopecia areata (Heibel et al., 2021), and rosacea (Barroso-García et al., 2018; Chung et al., 2019; Salgüero-Fernández et al., 2019).

In this study, we investigated the effects of dupilumab on the peripheral total and skin-homing T-cell functional dynamics and polarization in patients with moderate-to-severe AD after 4, 16, and 52 weeks of treatment. In addition, we measured IL-4Rα expression on T and B cells and responsiveness of these cells to recombinant human IL-4.

## RESULTS

### Dupilumab treatment rapidly blocks IL-4Rα on B and T cells and downstream signaling, reflecting clinical response

To longitudinally study short- and long-term effects of dupilumab treatment on IL-4Rα expression and T-cell cytokine production, we included 10 patients with moderate-to-severe AD (6 male; median age = 50.0, interquartile range = 46.0–54.5; median Eczema Area Severity Index score = 16.8, interquartile range = 13.8–21.1) treated with 300 mg dupilumab every other week for at least 52 weeks (experiment 1, Supplementary Table S1). Comparable with previous studies (Ariëns et al., 2020; Blauvelt et al., 2017; de Bruin-Weller et al., 2018; Simpson et al., 2017), dupilumab treatment significantly improved measures of disease severity from week 4 through week 52, including Eczema Area Severity Index scores and serum TARC or CCL17 levels, with the most robust decrease during the first 4 weeks of treatment ($P = 0.002$; Supplementary Figure S1a and b).

The rapid clinical response in our patients was reflected by a complete blockade of IL-4Rα on total and naive ($CD27^-IgD^+$) $CD19^+$ B cells at week 4, which remained stable through week 52 (Figure 1a and Supplementary Figure S2). Although T cells expressed much lower levels of IL-4Rα than naive B cells at baseline and in healthy control (HC) samples, T cells (especially $CD4^+$ T cells) also showed a significant reduction in measurable IL-4Rα levels from baseline through weeks 4–52 (Figure 1b). No differences in peripheral blood naive $CD19^+$ B-cell and $CD3^+$ T-cell numbers (data not shown) and $CD4^+/CD8^+$ T-cell ratio were observed over time (Supplementary Figure S3). Reduced measurable IL-4Rα levels were accompanied by clear binding of dupilumab antibodies to the surface of both T and B cells after 4 and 16 weeks of treatment, with no differences between these two time points (Figure 1c and d).

Next, we investigated the very early effects of dupilumab on T- and B-cell IL-4Rα occupancy and on their reactivity to IL-4. No appreciable differences were found between patients at baseline and HCs in respect to receptor expression and functional response. However, within 2 hours after the first dupilumab administration, IL-4Rα detection was abolished, and reciprocal dupilumab binding was detected on the surface of all cell types studied (Figure 2a and b). On a functional level, this finding indicated a weaker signaling of intracellular phosphorylated signal transducer and activator of transcription 6 in response to stimulation with recombinant human IL-4 in vitro (Figure 2c and Supplementary Figure S4).

Correspondingly, TARC or CCL17 levels in supernatants of lesional and nonlesional skin biopsies decreased (although not significantly) from baseline through week 4 in a clinically comparable cohort of eight patients with AD (experiment 5, Supplementary Table S1) treated with dupilumab (Supplementary Figure S1c). TARC or CCL17 levels in supernatants of lesional skin biopsies were significantly higher ($P = 0.028$) than in supernatants of nonlesional skin biopsies at baseline and at 4 weeks. Total serum IgE levels were significantly higher at baseline than HC samples and steadily decreased from baseline through week 52 in all patients (Supplementary Figure S1d). In summary, dupilumab showed a rapid and stable blockade of IL-4Rα accompanied by a decrease in clinical and severity-related molecular markers, both locally and systemically.

### Dupilumab treatment modulates proliferation of skin-homing T cells without affecting the proportion of total and skin-homing T-cell subsets

Next, we analyzed the effect of dupilumab treatment on the proportion and proliferation of different T-cell populations. Surface staining was used to examine changes in the total ($CD3^+$), helper ($CD4^+$), cytotoxic ($CD8^+$), and skin-homing T cells ($CLA^+$) and the skin-homing subpopulations ($CCR4^+/CLA^+$, $CCR10^+/CLA^+$, and $CRTH2^+/CLA^+$) during dupilumab treatment (Figure 3a). Absolute lymphocyte counts measured at the time PBMCs were isolated did not change during dupilumab treatment besides a slight increase from baseline through week 16 (data not shown). At baseline, the proportion of total skin-homing ($CLA^+$) $CD4^+$ T cells and specific $CCR10^+$ $CLA^+$ $CD4^+$ T cells in PBMCs from patients with AD was slightly lower than in HC samples (Figure 3a). The vast majority of $CLA^+$ $CD4^+$ T cells consisted of CD45RA memory T cells (Supplementary Figure S5), with no difference between HCs and patients. $CCR4^+$ and $CRTH2^+$ $CLA^+$ $CD4^+$ T cells in patients at baseline did not significantly differ from HCs. There were no differences in $CD8^+$ T-cell

cells from five patients with AD before dupilumab treatment (baseline) and after 4 and 16 weeks of treatment. MFI IgG4 was corrected for MFI only secondary antibody streptavidin-APC. Boxes represent median with interquartile range, whiskers indicate minimum and maximum. Significance levels correspond to the following *P*-values: $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.005$. AD, atopic dermatitis; APC, allophycocyanin; HC, healthy control; IL-4Rα, IL-4 receptor alpha; MFI, median fluorescence intensity; PE, phycoerythrin.

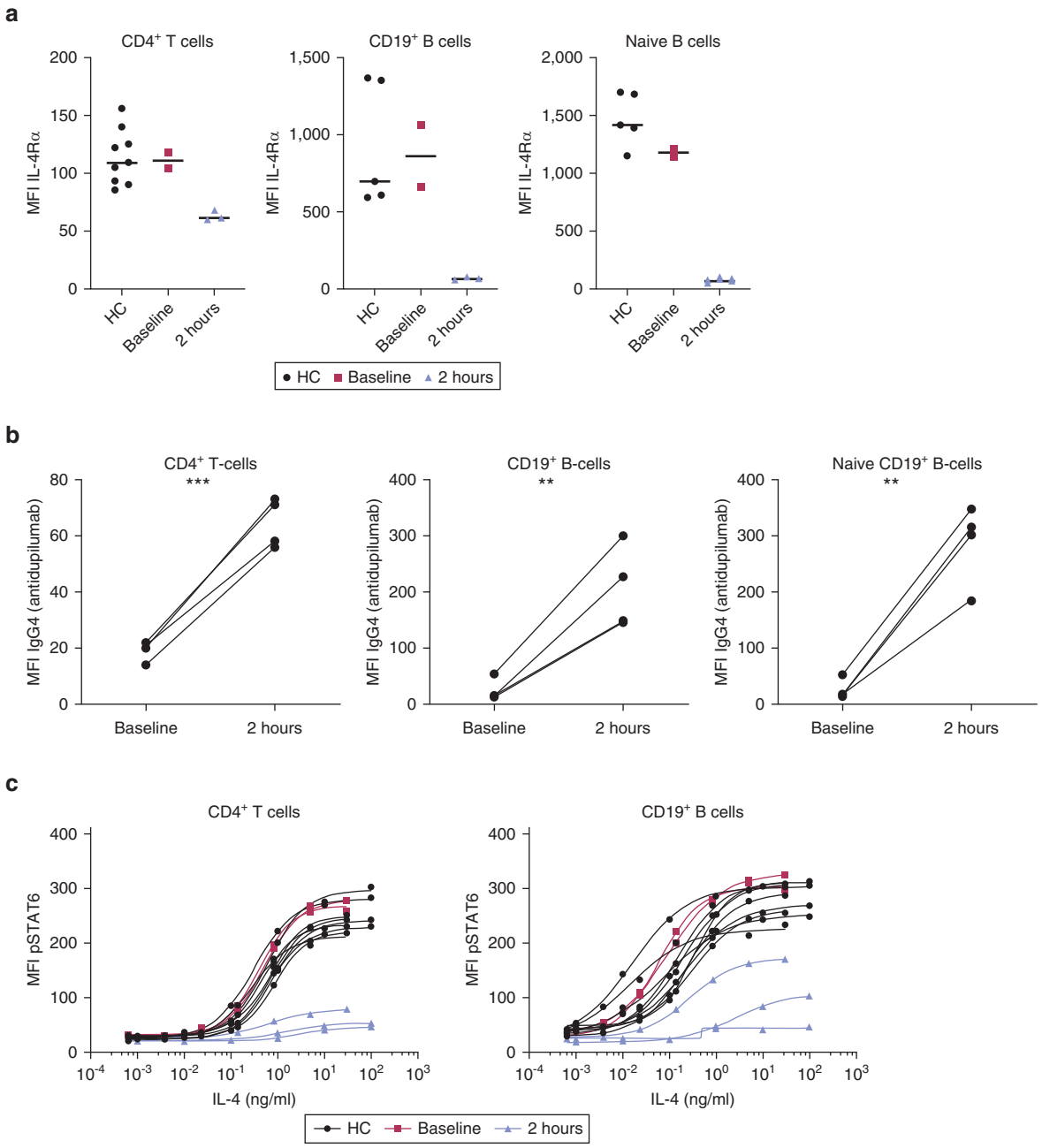


**Figure 2. Very early effects of dupilumab binding on measurable IL-4Rα levels and signaling.** (**a**) MFI of IL-4Rα on $CD4^+$ T cells and total and naive ($CD27^-$ $IgD^+$) $CD19^+$ B cells before (in two patients) and within 2 hours after first dupilumab administration (in three patients) compared with HCs. Horizontal lines represent median values. (**b**) MFI of IgG4 (anti-dupilumab) on $CD4^+$ T cells, $CD19^+$ B cells, and naive $CD19^+$ B cells from four patients with AD before and within 2 hours after the first dupilumab administration. Significance levels correspond to the following *P*-values: $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.005$. (**c**) Dose-response curves of pSTAT6 response to IL-4 of $CD4^+$ T cells and total $CD19^+$ B cells. AD, atopic dermatitis; HC, healthy control; IL-4Rα, IL-4 receptor alpha; MFI, median fluorescence intensity; pSTAT6, phosphorylated signal transducer and activator of transcription 6.

(skin-homing) subsets between patients and controls (data not shown).

Although the percentage of skin-homing $CD4^+$ and $CD8^+$ T cells in PBMCs from patients with AD remained relatively stable during dupilumab treatment (Figure 3a and b), a striking difference in the proliferation of skin-homing T cells before and during dupilumab treatment was noted. First of all, the frequency of proliferating ($Ki67^+$) T cells was highly increased in the $CLA^+$ population compared with the $CLA^-$ population, especially in $CD4^+$ T cells (Figure 3c), which might be explained by the (effector) memory phenotype of the skin-homing T cells. Furthermore, the proportion of proliferating ($Ki67^+$) $CLA^+$ $CD4^+$ and $CLA^+$ $CD8^+$ T cells was significantly higher in PBMCs of patients with AD at baseline than in HCs and significantly decreased from baseline through weeks 16 and 52 of dupilumab treatment (Figure 3c). Similar effects during treatment were observed in the $CLA^-$ $Ki67^+$ $CD8^+$ and $CD4^+$ T cells. Taken together, these results might indicate that dupilumab treatment suppresses proliferation of skin-homing T cells, although their relative proportion remains unaffected.

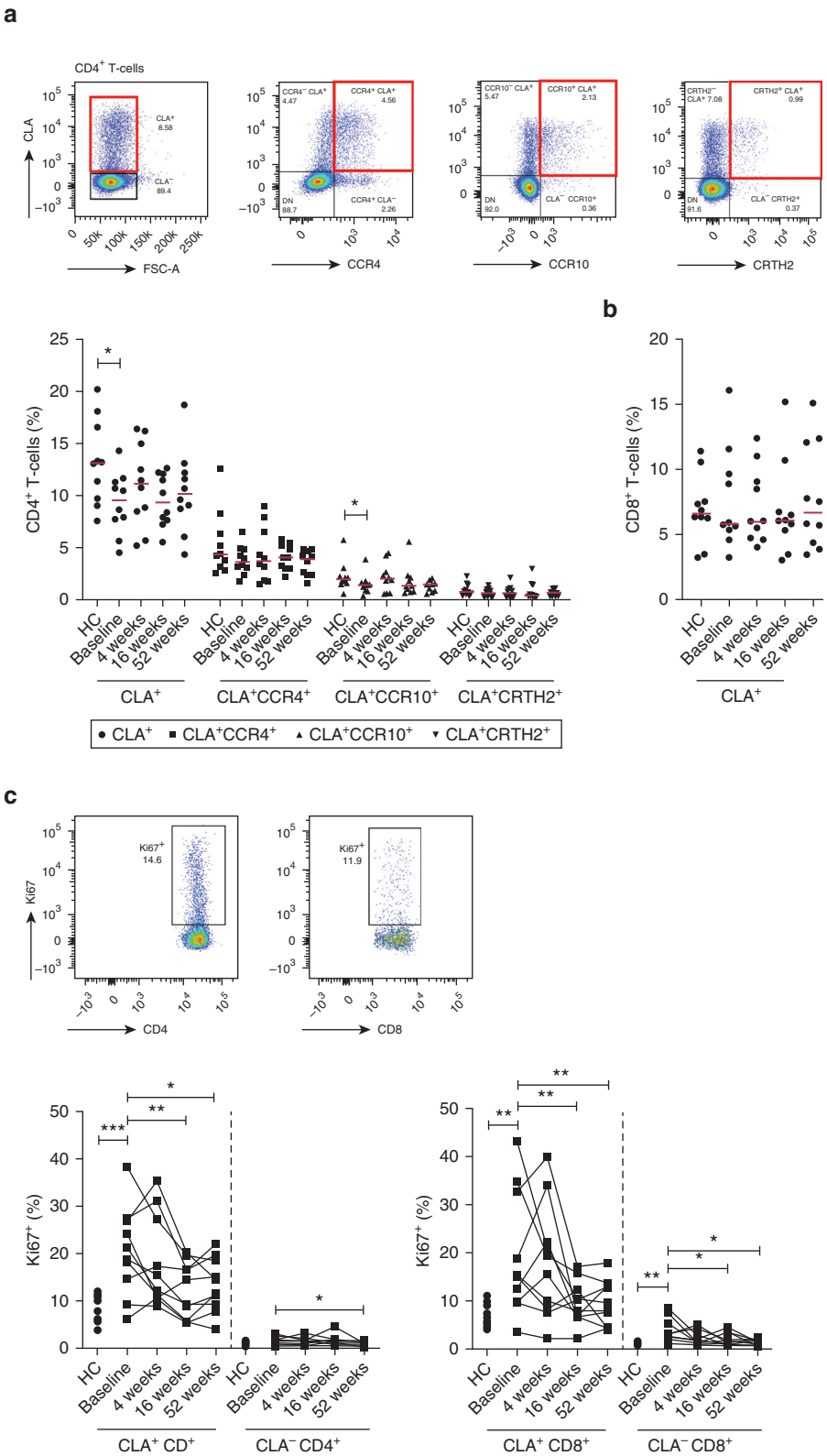


**Figure 3. Effect of dupilumab treatment on skin-homing CD4$^+$ and CD8$^+$ T-cell subtypes.** Representative flow cytometry gating strategy for and percentages of several skin-homing subpopulations within (**a**) total CD4$^+$ T cells and (**b**) total CD8$^+$ T cells. (**c**) Percentage of proliferating (Ki67$^+$) skin-homing (CLA$^+$) and non–skin-homing (CLA$^-$) CD4$^+$ and CD8$^+$ T cells in PBMCs from 10 patients with AD during dupilumab treatment compared with HCs. Significance levels correspond to the following *P*-values: $*P < 0.05$, $**P < 0.01$, and $***P < 0.005$. AD, atopic dermatitis; CLA, cutaneous lymphocyte antigen; FSC-A, forward scatter area; HC, healthy control.

**Dupilumab treatment downregulates Th2 and Th22 cytokine–producing skin-homing T cells**

To study the early functional effect of dupilumab treatment on T-cell cytokine production, PBMCs were stimulated with phorbol 12-myristate 13-acetate (Sigma-Aldrich, St. Louis, MO) and ionomycin (Sigma-Aldrich) for 4 hours and intracellularly stained for Th subset–related cytokines (Supplementary Figure S6). At baseline, a significantly higher production of Th2- and Th22-related cytokines by CLA$^+$ CD4$^+$ T cells was observed in PBMCs from patients with AD than in HCs (Figure 4a). After 4 weeks of dupilumab treatment, a significant reduction of IL-4 ($P = 0.013$), IL-5

($P = 0.007$), IL-13 ($P = 0.005$), and IL-22 ($P = 0.007$) production in $CLA^+$ $CD4^+$ T cells was found. Similar effects were observed in $CCR4^+$ (Supplementary Figure S7) and $CCR10^+$ $CLA^+$ $CD4^+$ T cells (data not shown). No differences were observed in cytokine production in $CLA^-$ $CD4^+$ T cells after 4 weeks of treatment, suggesting a selective effect on skin-homing T cells.

Production of Th1-related cytokines was significantly higher for IFN-γ and significantly lower for TNF-α in $CLA^+$ $CD4^+$ T cells from patients with AD at baseline compared with HCs (Figure 4b). This effect was not visible in the $CLA^-$ $CD4^+$ T-cell compartment, and this did not change during the first 4 weeks of treatment in $CD4^+$ $CLA^+$ and $CLA^-$ T-cell populations. A decline in IL-17 production by $CLA^+$ $CD4^+$ T cells was detected in 7 of 10 patients at week 4 (Figure 4c). In addition, cytokine analyses by Luminex immunoassays of the PBMC culture supernatant 72 hours after anti-CD3 stimulation at baseline and 4, 16, and 52 weeks after initiation of dupilumab treatment were performed to evaluate the effect on total cytokine-producing capability of all PBMCs. In line with the previous results, no changes in total IL-5, IL-13, IL-17, IL-22, TNF-α, or IFN-γ total cytokine production were observed over time, except for a transient decrease in total IL-4 production (Supplementary Figure S8). Overall, rapid effects of dupilumab treatment on Th2 and Th22 cytokine production were selectively observed in the skin-homing $CD4^+$ T-cell population.

**No evidence of long-term polarization toward Th1, Th17, and Th22 cytokines is observed during dupilumab treatment**

In the long term, the overall effect of dupilumab treatment on the Th2 cytokine–producing $CLA^+$ $CD4^+$ and $CLA^-$ $CD4^+$ T cells remained relatively stable (Figure 5a). However, after a decrease in the first 4 weeks of treatment, the production of Th2-related cytokines by $CCR4^+$ $CLA^+$ $CD4^+$ T cells significantly increased from week 4 through week 52, not exceeding baseline levels (Figure 5b). The percentage of $CD4^+$ regulatory T cells ($CD25^+$ $FOXP3^+$) significantly and stably increased in patients with AD during dupilumab treatment (Figure 6a). The increase could be attributed almost completely to the $CLA^+$ $CD4^+$ T-cell compartment, again pointing to a specific effect on skin-homing cells.

Concerning the long-term possible polarization of T cells toward the production of Th1-, Th17-, and Th22-related cytokines, a significant increase of IL-22–producing $CLA^+$ $CD4^+$ T cells from week 4 through week 52 was noted (Figure 6b). Although not significant, the mean percentages of IFN-γ-, TNF-α- and IL-17–producing $CLA^+$ $CD4^+$ T cells also increased from week 4 to week 52 during dupilumab treatment. In 4 of 10 individual patients, IL-17 production by $CCR4^+$ $CLA^+$ $CD4^+$ T cells exceeded baseline levels after 52 weeks of treatment (Supplementary Figure S9). One of these patients developed severe rosacea after 40 weeks.

In sum, after a rapid downregulation of the Th2 and Th22 cytokine–producing skin-homing $CD4^+$ T-cell population, a slight increase was observed after 52 weeks. Overall, dupilumab does not seem to have strong long-term polarizing effects on the peripheral Th composition. However, in some individuals, the balance may be tipped toward a more Th1 and Th17 phenotype, especially within the skin-homing T-cell population.

## DISCUSSION

The clinical efficacy of dupilumab treatment supports the hypothesis that type 2 cytokines IL-4 and IL-13 are critical mediators of AD pathogenesis. Elucidating the mechanisms of action of this targeted drug is of great importance for a better understanding of the pathogenesis of the disease. To our knowledge, previously unreported mechanistic effects of IL-4Rα blockade on the peripheral T- and B-cell compartment in patients with moderate-to-severe AD were evaluated in this study.

This study confirmed the mechanism of action of dupilumab by demonstrating a substantial functional blockade of IL-4Rα on B and T cells within 2 hours after the first dosage. The very rapid initial response was followed by a stable blockade of IL-4Rα on $CD19^+$ B cells and $CD4^+$ T cells from week 4 through week 52, which was concomitant with clinical efficacy. The significant decrease of IL-4Rα median fluorescence intensity observed is most likely reflecting full occupancy of IL-4Rα by dupilumab mAbs, as confirmed by IgG4 antibody detection on T and B cells during treatment. In addition, we confirmed that the IL-4Rα blockade by dupilumab is of functional relevance, by showing weaker phosphorylated signal transducer and activator of transcription 6 responsiveness to IL-4 within 2 hours after the first administration. Although no signs of receptor internalization were observed until 16 weeks of treatment, we cannot rule out a change in IL-4Rα expression levels owing to shedding or changes in gene expression in the long term. Pharmacokinetic studies have shown that, following the first subcutaneous dose of 600 mg, the maximum serum concentration of dupilumab is achieved after approximately 1 week (D'Ippolito and Pisano, 2018). However, actual functional blockade of IL-4Rα on effector cells has not been confirmed before.

Previous studies have shown that the number of circulating type 2 cytokine–producing skin-homing T cells is increased in the peripheral blood of patients with AD (Piletta et al., 1996; Teraki et al., 2000). Our study confirmed this by showing a higher production of type 2 cytokines by $CLA^+$ $CD4^+$ T cells in patients with AD at baseline than HCs. Data regarding mechanistic changes during dupilumab treatment are scarce. Recently, dupilumab treatment was observed to downregulate the expression of genes related to type 2 inflammation in lesional skin after 4 and 16 weeks of treatment and decrease Th2-related serum biomarkers (Guttman-Yassky et al., 2019; Hamilton et al., 2014). Our study showed that, during the first 4 weeks of treatment, dupilumab significantly suppressed type 2 cytokine production in $CLA^+$, but not in $CLA^-$ $CD4^+$, peripheral blood T cells. Similar effects were observed in specific subtypes ($CCR4^+$, $CCR10^+$, $CRTH2^+$) of skin-homing $CLA^+$ $CD4^+$ T cells. In accordance with our findings, a recent in vitro study including 12 patients with AD and 6 HCs showed that IL-4Rα blockade could reduce production of the type 2 cytokines IL-4, IL-5, IL-13, and IL-31 by proliferating $CD4^+$ T cells (Brøgger et al., 2020). However, this study found an increase of IFN-γ production in $CD4^+$ T cells, whereas in our study, no increase in the production of Th1- and Th17-related cytokines was observed. The skin-homing peripheral T-cell population in our study might have been affected by (re)migrating T cells

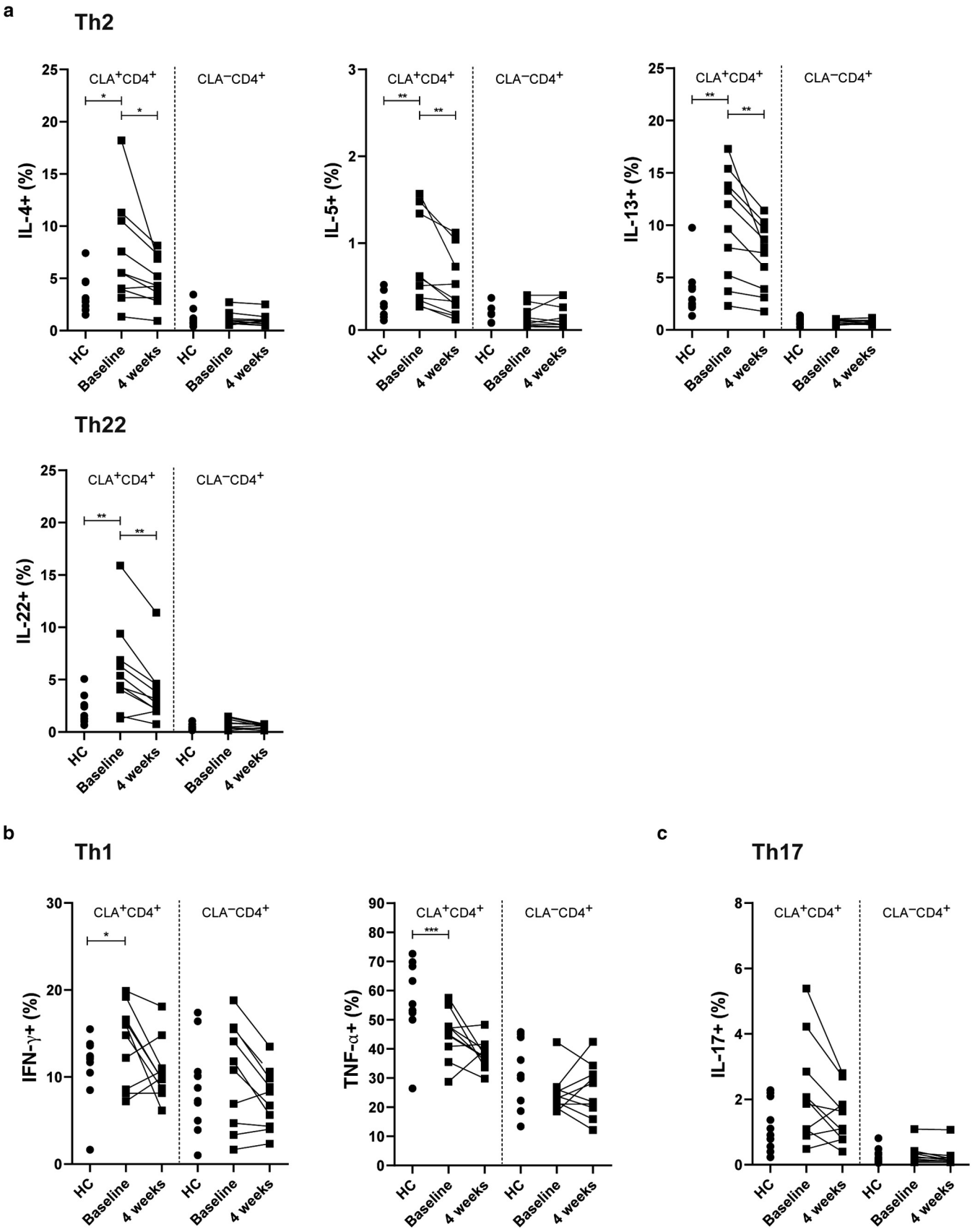


**Figure 4. Short-term effect of dupilumab treatment on cytokine-producing (skin-homing) CD4$^+$ T cells.** Percentages of (**a**) Th2- (IL-4, IL-5, IL-13) and Th22-related (IL-22), (**b**) Th1-related (IFN-γ, TNF-α), and (**c**) Th17-related (IL-17) cytokine-producing skin-homing (CLA$^+$) and non–skin-homing (CLA$^-$) CD4$^+$ T cells in PBMCs from 10 patients with AD at baseline and after 4 weeks of dupilumab treatment compared with HCs. Significance levels correspond to the following *P*-values: $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.005$. AD, atopic dermatitis; CLA, cutaneous lymphocyte antigen; HC, healthy control; Th, T helper type.

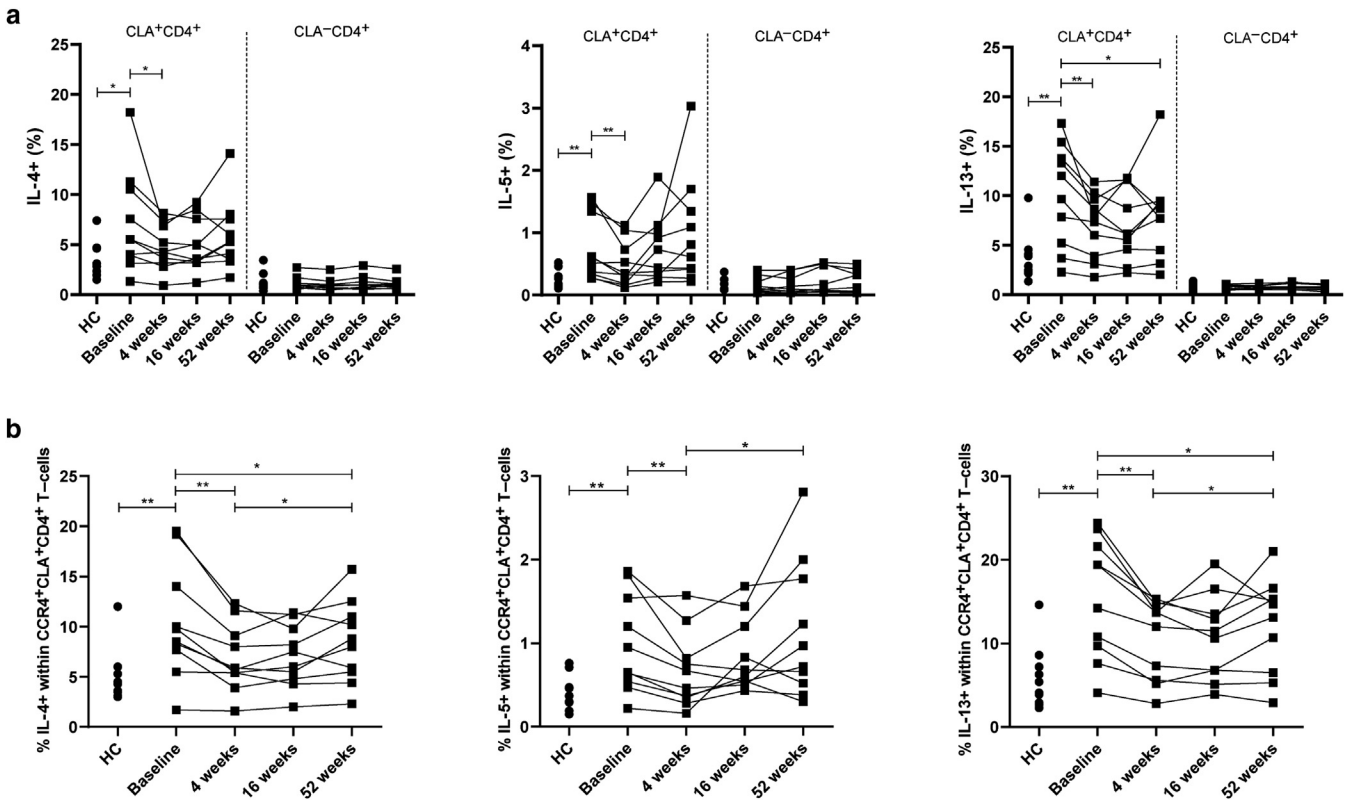


**Figure 5. Long-term effect of dupilumab treatment on Th2-related cytokine-producing (skin-homing) $CD4^+$ T cells.** Percentages of IL-4−, IL-5−, and IL-13−producing (**a**) total skin-homing ($CLA^+$) and non−skin-homing ($CLA^-$) and (**b**) specific $CLA^+$ $CCR4^+$ $CD4^+$ T cells in PBMCs from patients with AD during 52 weeks of dupilumab treatment compared with HCs. Significance levels correspond to the following *P*-values: $^*P < 0.05$ and $^{**}P < 0.01$. AD, atopic dermatitis; CLA, cutaneous lymphocyte antigen; HC, healthy control; Th, T helper type.

from the skin compartment because of reduced TARC or CCL17 expression in skin. In contrast to the study of Brøgger et al. (2020), in which PBMCs were stimulated in vitro with and without a neutralizing mAb against IL-4Rα, our study analyzed actual in vivo and ex vivo effects of dupilumab treatment in patients with AD, and our results suggest that dupilumab treatment selectively affects only the skin-homing T-cell population. No difference in expression of IL-4Rα on skin-homing T-cell subsets was observed between patients at baseline and HCs. Another reason for increased sensitivity to dupilumab treatment may be the relatively high turnover of the $CLA^+$ T-cell compartment we observed at baseline, which decreased on treatment. Finally, priming of naive T cells may also be affected by dupilumab, possibly preventing induction and/or differentiation of skin-homing T cells.

IL-4Rα plays an important role in inducing B-cell proliferation and isotype switching, resulting in high levels of circulating IgE (Gandhi et al., 2016). Our study showed that after blocking IL-4Rα, dupilumab treatment steadily decreased IgE levels from baseline through week 52 in all 10 patients, even including the patients who showed normal IgE (<100 IU/ml) levels at baseline. Our findings are in accordance with previous studies, showing significant reduction in total serum IgE levels from baseline through week 16 in patients with AD (Fargnoli et al., 2019; Guttman-Yassky et al., 2019; Olesen et al., 2019) and a gradual reduction throughout 52 weeks of dupilumab treatment in patients with asthma (Corren et al., 2020). The long-term IgE suppression found in our study suggests that dupilumab treatment adequately suppresses IL-4 and IL-13 activity and might indicate long-term effects on the atopic phenotype.

The functional immunological effect of dupilumab treatment was mainly observed in the first 4 weeks of treatment. After the significant decrease from baseline through week 4, the production of type 2 cytokines by $CCR4^+$ $CLA^+$ $CD4^+$ T cells gradually increased again. A similar trend in the production of IL-4, IL-5, and IL-13 in total skin-homing T-cell population was observed. This effect may be explained by a small decrease in biological efficacy after the initial high pulse dosing or compensatory mechanisms on a biological level, for example, upregulation of IL-4Rα. At the same time, our results suggest that IL-4Rα occupancy remains stable over time. Despite the gradual increase in type 2 cytokine−producing skin-homing T cells in the long term, lasting effects of dupilumab treatment were observed on clinical efficacy and total IgE levels, which both continued to decrease until 1 year of treatment. These results show the differential dynamics of immune-modulating effects on T and B cells by dupilumab treatment.

Recent case reports describing new onset or worsening of rosacea (Heibel et al., 2021), alopecia areata (Barroso-García et al., 2018; Chung et al., 2019; Flanagan et al., 2018; Salgüero-Fernández et al., 2019), and psoriasis (Safa and Paumier, 2019; Tracey et al., 2018; Varma and Levitt, 2020) during dupilumab treatment suggest possible skewing of the helper T-cell profile toward a Th1 and Th17 phenotype as a

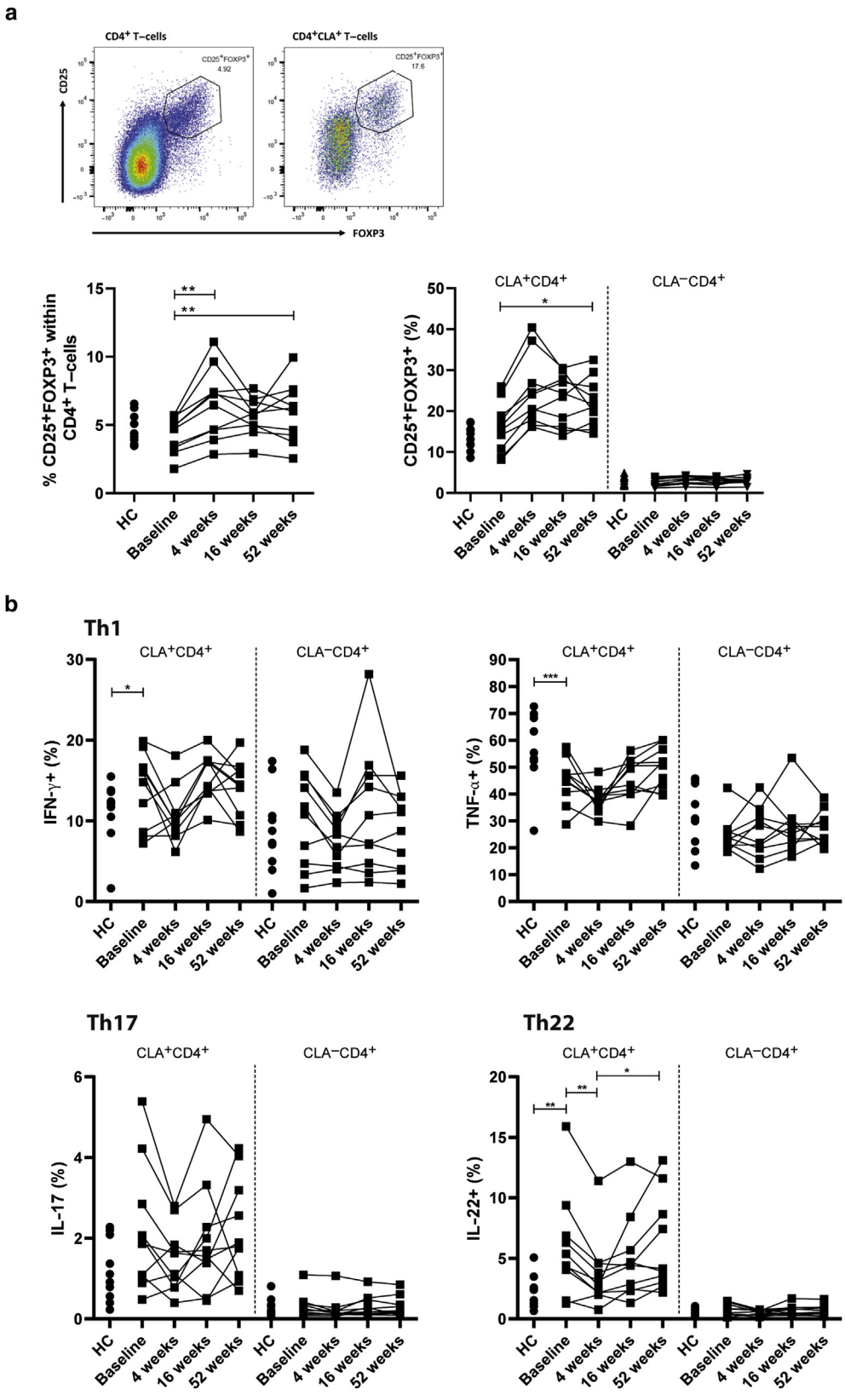


**Figure 6. No signs of long-term Th1, Th17, or Th22 T-cell skewing but significantly increasing Treg proportions.** (**a**) Representative flow cytometry gating strategy for and percentages of regulatory ($CD25^+$ $FOXP3^+$) within $CD4^+$ T cells (lower left panel) and within $CLA^+$ and $CLA^-$ $CD4^+$ T cells (lower right panel) in PBMCs from patients with AD during 52 weeks of dupilumab treatment compared with HCs. (**b**) Percentages of Th1- (IFN-γ, TNFα), Th17- (IL-17), and Th22-related (IL-22) cytokine-producing skin-homing ($CLA^+$) and non–skin-homing ($CLA^-$) $CD4^+$ T cells in PBMCs from patients with AD during 52 weeks of dupilumab treatment compared with HCs. Significance levels correspond to the following *P*-values: $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.005$. AD, atopic dermatitis; CLA, cutaneous lymphocyte antigen; HC, healthy control; Th, T helper type; Treg, regulatory T cell.

result of IL-4R signaling antagonism. Because it has been shown that IL-4 can act as a negative regulator of the Th1 and Th17 pathways, suppression of the IL-4/IL-13 signaling pathway could result in alteration of the Th1, Th2, and Th17 balance and may predispose patients to Th1- and Th17-mediated diseases (Guenova et al., 2015). However, our study showed no signs of general immune skewing toward Th1 or Th17 pathways in the total $CD4^+$ T-cell (data not shown) and

specific skin-homing T-cell levels during the first year of dupilumab treatment. This might be the effect of increased control and suppression of T-cell responses, as especially the proportion of skin-homing regulatory $CD4^+$ $CD25^+$ FOXP3 T cells (regulatory T cells) significantly increased in PBMCs from patients with AD during dupilumab treatment. Regulatory T cells are known to suppress immune responses by suppressing effector T cells and play a major role in controlling asthma and allergy (Umetsu et al., 2003). Accordingly, the expansion of regulatory T cells might also contribute to the improvement of clinical signs and symptoms of AD in our patients. The occurrence of Th1- and Th17-mediated diseases as adverse effects during dupilumab treatment might be explained by T-cell skewing on the level of individual patients, because our results show that in 4 of 10 patients, IL-17 production by $CCR4^+$ $CLA^+$ $CD4^+$ T cells exceeded baseline levels after 52 weeks of treatment. Remarkably, one of these patients developed severe rosacea after 40 weeks of dupilumab treatment. It remains to be shown whether dupilumab-induced IL-17 production is able to induce neutrophil activation in patients with AD, because it has been shown that IL-4Rα—mediated signaling can inhibit neutrophil migration and function (Egholm et al., 2019; Impellizzieri et al., 2019).

The strength of this is study is the inclusion of both patients with AD and HCs and the long-term follow-up until 52 weeks of dupilumab treatment. In addition, the evaluation of skin-homing $CLA^+$ T cells in peripheral blood, which might reflect cutaneous immune responses, creates an opportunity for less invasive, translational approaches and might eliminate the need for skin biopsies in future studies. Although results were very consistent between the different cohorts included, the small number of included patients for the assessment of very early treatment effects was a limitation of this study.

Overall, this study confirms the mechanism of action of dupilumab treatment by demonstrating a (very) rapid and stable blockade of IL-4Rα on B cells and T cells accompanied by a strong early functional immunological effect (after 4 weeks), specifically in skin-homing T cells of patients with AD treated with dupilumab. Although there were no signs of general immune skewing on the T-cell level following a year of dupilumab treatment, the continuous decrease in total IgE levels and increase in IL-17 production by skin-homing T cells in a selection of patients may indicate long-term effects on the atopic phenotype. For the future, monitoring peripheral (skin-homing) T-cell responses might be a useful tool to predict and/or monitor treatment efficacy and potential side effects and guide tapering strategies in patients with AD.

## MATERIALS AND METHODS

### Study design

This prospective study enrolled patients from a larger prospective, observational cohort (the Dutch BioDay registry, ClinicalTrials.gov identifier: NCT03549416, retrospectively registered 08 June 2018), including adult patients with moderate-to-severe AD (Supplementary Table S1) who were treated with dupilumab in daily practice under an institutional review board—approved protocol. At baseline, patients received a loading dose of 600 mg dupilumab subcutaneously, followed by 300 mg dupilumab subcutaneously every other week. We included 10 patients who were treated with dupilumab for at least 52 weeks for longitudinal analyses (experiment 1). For specific subquestions (experiments 2—5), we selected additional patients from the same cohort. Patient characteristics are summarized in Supplementary Table S1. Concomitant treatment with topical corticosteroids was allowed. Patients using oral immunosuppressive drugs within 2 (fast-acting drugs) or 4 (slow-acting drugs) weeks before screening were excluded. Clinical data were extracted from an online Good Clinical Practice database (BioDay registry). All patients signed institutional review board—approved written, informed consent, adhering to the Declaration of Helsinki Principles.

Blood samples were collected from patients in experiments 1—4 before initiation of dupilumab treatment (baseline) and after 2 hours and 4, 16, and 52 weeks of treatment. PBMCs were isolated and analyzed using flow cytometry (Supplementary Table S2). Serum IgE levels were measured using ELISA assays (BD Biosciences Pharmingen, San Diego, CA). Blood samples from 10 adult healthy volunteers were obtained from the University Medical Center Utrecht (Utrecht, The Netherlands). Skin biopsies (3 mm) of lesional and nonlesional skin were collected from eight patients with moderate-to-severe AD included in experiment 5 at baseline and week 4 of dupilumab treatment. Concentrations of 16 cytokines and chemokines were measured in supernatants of anti-CD3 stimulated PBMCs and 24-hour cultured biopsies by Luminex multiplex immunoassay (de Jager et al., 2005) (Supplementary Table S3). Detailed methods related to sample collection, assessments, and statistical analysis are described in Supplementary Materials and Methods.

### Data availability statement

No datasets were generated during this study.

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### CONFLICT OF INTEREST

MSDBW is a principal investigator and advisory board member for AbbVie and Regeneron Pharmaceuticals, Inc; a principal investigator, advisory board member, and consultant for Sanofi Genzyme; an advisory board member for Eli Lilly and UCB; and a principal investigator and advisory board member for Pfizer. MSDBW has been a consultant, advisory board member, and/or speaker for Almirall, Arena, Galderma, Janssen, Leo Pharma, and Pfizer. JLT and DSB are speakers for Sanofi Genzyme. All other authors declare no relevant conflicts of interest.

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### AUTHOR CONTRIBUTIONS

Conceptualization: DSB, MMVDW, MSDBW, JLT, FVW; Formal Analysis: DSB, MMVDW; Investigation: DSB, MMVDW, LEMH, BG, MA; Methodology: MMVDW, LEMH, BG, FVW; Resources: MMVDW, LEMH, BG; Supervision: MSDBW, JLT, FVW; Validation: FVW; Visualization: DSB, MMVDW; Writing - Original Draft Preparation: DSB, MMVDW; Writing - Review and Editing: LEMH, BG, MA, EMD, JD, SN, OB, MSDBW, JLT, FVW

## SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2021.01.022.

## REFERENCES

Ariëns LFM, van der Schaft J, Bakker DS, Balak D, Romeijn MLE, Kouwenhoven T, et al. Dupilumab is very effective in a large cohort of difficult-to-treat adult atopic dermatitis patients: first clinical and biomarker results from the BioDay registry. Allergy 2020;75:116–26.

Barroso-García B, Rial MJ, Molina A, Sastre J. Alopecia areata in severe atopic dermatitis treated with dupilumab. J Investig Allergol Clin Immunol 2018;28:420–1.

Blauvelt A, de Bruin-Weller M, Gooderham M, Cather JC, Weisman J, Pariser D, et al. Long-term management of moderate-to-severe atopic dermatitis with dupilumab and concomitant topical corticosteroids (LIBERTY AD CHRONOS): a 1-year, randomised, double-blinded, placebo-controlled, phase 3 trial. Lancet 2017;389:2287–303.

Brinkman CC, Rouhani SJ, Srinivasan N, Engelhard VH. Peripheral tissue homing receptors enable T cell entry into lymph nodes and affect the anatomical distribution of memory cells. J Immunol 2013;191:2412–25.

Brøgger P, Blom LH, Simonsen S, Thyssen JP, Skov L. Antagonism of the interleukin 4 receptor alpha promotes TH 1-signalling among T cells from patients with atopic dermatitis after stimulation. Scand J Immunol 2020;91:e12835.

Chung J, Slaught CL, Simpson EL. Alopecia areata in 2 patients treated with dupilumab: new onset and worsening. JAAD Case Rep 2019;5:643–5.

Corren J, Castro M, O'Riordan T, Hanania NA, Pavord ID, Quirce S, et al. Dupilumab efficacy in patients with uncontrolled, moderate-to-severe allergic asthma. J Allergy Clin Immunol Pract 2020;8:516–26.

Czarnowicki T, Krueger JG, Guttman-Yassky E. Skin barrier and immune dysregulation in atopic dermatitis: an evolving story with important clinical implications. J Allergy Clin Immunol Pract 2014;2:371–81.

Czarnowicki T, Santamaria-Babí LF, Guttman-Yassky E. Circulating $CLA^+$ T cells in atopic dermatitis and their possible role as peripheral biomarkers. Allergy 2017;72:366–72.

de Bruin-Weller M, Thaçi D, Smith CH, Reich K, Cork MJ, Radin A, et al. Dupilumab with concomitant topical corticosteroid treatment in adults with atopic dermatitis with an inadequate response or intolerance to ciclosporin A or when this treatment is medically inadvisable: a placebo-controlled, randomized phase III clinical trial (LIBERTY AD CAFÉ). Br J Dermatol 2018;178:1083–101.

de Jager W, Prakken BJ, Bijlsma JW, Kuis W, Rijkers GT. Improved multiplex immunoassay performance in human plasma and synovial fluid following removal of interfering heterophilic antibodies. J Immunol Methods 2005;300:124–35.

de Wijs LEM, Bosma AL, Erler NS, Hollestein LM, Gerbens LAA, Middelkamp-Hup MA, et al. Effectiveness of dupilumab treatment in 95 patients with atopic dermatitis: daily practice data. Br J Dermatol 2020;182:418–26.

D'Ippolito D, Pisano M. Dupilumab (Dupixent): an interleukin-4 receptor antagonist for atopic dermatitis. P T 2018;43:532–5.

Egholm C, Heeb LEM, Impellizzieri D, Boyman O. The regulatory effects of interleukin-4 receptor signaling on neutrophils in type 2 immune responses. Front Immunol 2019;10:2507.

Faiz S, Giovannelli J, Podevin C, Jachiet M, Bouaziz JD, Reguiai Z, et al. Effectiveness and safety of dupilumab for the treatment of atopic dermatitis in a real-life French multicenter adult cohort. J Am Acad Dermatol 2019;81:143–51.

Fargnoli MC, Esposito M, Ferrucci S, Girolomoni G, Offidani A, Patrizi A, et al. Real-life experience on effectiveness and safety of dupilumab in adult patients with moderate-to-severe atopic dermatitis [e-pub ahead of print]. J Dermatolog Treat 2019. https://doi.org/10.1080/09546634.2019.1682503 (accessed April 30 2020).

Ferran M, Romeu ER, Rincón C, Sagristà M, Giménez Arnau AM, Celada A, et al. Circulating CLA+ T lymphocytes as peripheral cell biomarkers in T-cell-mediated skin diseases. Exp Dermatol 2013;22:439–42.

Ferran M, Santamaria-Babi LF. Pathological mechanisms of skin homing T cells in atopic dermatitis. World Allergy Organ J 2010;3:44–7.

Flanagan K, Sperling L, Lin J. Drug-induced alopecia after dupilumab therapy. JAAD Case Rep 2018;5:54–6.

Gandhi NA, Bennett BL, Graham NM, Pirozzi G, Stahl N, Yancopoulos GD. Targeting key proximal drivers of type 2 inflammation in disease. Nat Rev Drug Discov 2016;15:35–50.

Guenova E, Skabytska Y, Hoetzenecker W, Weindl G, Sauer K, Tham M, et al. IL-4 abrogates T(H)17 cell-mediated inflammation by selective silencing of IL-23 in antigen-presenting cells. Proc Natl Acad Sci USA 2015;112:2163–8.

Guttman-Yassky E, Bissonnette R, Ungar B, Suárez-Fariñas M, Ardeleanu M, Esaki H, et al. Dupilumab progressively improves systemic and cutaneous abnormalities in patients with atopic dermatitis. J Allergy Clin Immunol 2019;143:155–72.

Hamilton JD, Suárez-Fariñas M, Dhingra N, Cardinale I, Li X, Kostic A, et al. Dupilumab improves the molecular signature in skin of patients with moderate-to-severe atopic dermatitis. J Allergy Clin Immunol 2014;134:1293–300.

Heibel HD, Hendricks AJ, Foshee JP, Shi VY. Rosacea associated with dupilumab therapy. J Dermatolog Treat 2021;32:114–6.

Impellizzieri D, Ridder F, Raeber ME, Egholm C, Woytschak J, Kolios AGA, et al. IL-4 receptor engagement in human neutrophils impairs their migration and extracellular trap formation. J Allergy Clin Immunol 2019;144:267–79.e4.

Olesen CM, Holm JG, Nørreslet LB, Serup JV, Thomsen SF, Agner T. Treatment of atopic dermatitis with dupilumab: experience from a tertiary referral centre. J Eur Acad Dermatol Venereol 2019;33:1562–8.

Paller AS, Kabashima K, Bieber T. Therapeutic pipeline for atopic dermatitis: end of the drought? J Allergy Clin Immunol 2017;140:633–43.

Piletta PA, Wirth S, Hommel L, Saurat JH, Hauser C. Circulating skin-homing T cells in atopic dermatitis. Selective up-regulation of HLA-DR, interleukin-2R, and CD30 and decrease after combined UV-A and UV-B phototherapy. Arch Dermatol 1996;132:1171–6.

Safa G, Paumier V. Psoriasis induced by dupilumab therapy. Clin Exp Dermatol 2019;44:e49–50.

Salgüero-Fernández I, Gonzalez de Domingo MA, Suarez D, Roustan-Gullón G. Dermatitis and alopecia in a patient treated with dupilumab: a new adverse effect? Clin Exp Dermatol 2019;44:e41–3.

Simpson EL, Akinlade B, Ardeleanu M. Two phase 3 trials of dupilumab versus placebo in atopic dermatitis. N Engl J Med 2017;376:1090–1.

Soler D, Humphreys TL, Spinola SM, Campbell JJ. CCR4 versus CCR10 in human cutaneous TH lymphocyte trafficking. Blood 2003;101:1677–82.

Tanaka Y, Hamano S, Gotoh K, Murata Y, Kunisaki Y, Nishikimi A, et al. T helper type 2 differentiation and intracellular trafficking of the interleukin 4 receptor-alpha subunit controlled by the Rac activator Dock2. Nat Immunol 2007;8:1067–75.

Tauber M, Apoil PA, Richet C, Laurent J, De Bonnecaze G, Mouchon E, et al. Effect of dupilumab on atopic manifestations in patients treated for atopic dermatitis in real-life practice. Br J Dermatol 2019;180:1551–2.

Teraki Y, Hotta T, Shiohara T. Increased circulating skin-homing cutaneous lymphocyte-associated antigen (CLA)+ type 2 cytokine-producing cells, and decreased CLA+ type 1 cytokine-producing cells in atopic dermatitis. Br J Dermatol 2000;143:373–8.

Tracey EH, Elston C, Feasel P, Piliang M, Michael M, Vij A. Erythrodermic presentation of psoriasis in a patient treated with dupilumab. JAAD Case Rep 2018;4:708–10.

Umetsu DT, Akbari O, Dekruyff RH. Regulatory T cells control the development of allergic disease and asthma. J Allergy Clin Immunol 2003;112:480–8.

Varma A, Levitt J. Dupilumab-induced phenotype switching from atopic dermatitis to psoriasis. JAAD Case Rep 2020;6:217–8.

Werfel T, Allam JP, Biedermann T, Eyerich K, Gilles S, Guttman-Yassky E, et al. Cellular and molecular immunologic mechanisms in patients with atopic dermatitis. J Allergy Clin Immunol 2016;138:336–49.

## SUPPLEMENTARY MATERIALS AND METHODS

### Patient characteristics and blood and biopsy collection

This study included adult patients with moderate-to-severe atopic dermatitis (AD) from a larger prospective, observational cohort study in which patients were treated with dupilumab in daily practice and were enrolled in the Dutch BioDay Registry at the National Expertise Center for Atopic Dermatitis from the University Medical Center Utrecht (Utrecht, The Netherlands) (ClinicalTrials.gov Identifier: NCT03549416, retrospectively registered 08 June 2018). At baseline, patients received a loading dose of 600 mg dupilumab subcutaneously, followed by 300 mg dupilumab subcutaneously every other week. Concomitant treatment with topical corticosteroids was allowed. Patients using oral immunosuppressive drugs within 2 (fast-acting drugs, including systemic steroids or cyclosporine A) or 4 (slow-acting drugs, including azathioprine, methotrexate, and mycophenolate mofetil) weeks before screening were excluded. Blood samples were collected before initiation of dupilumab treatment (baseline) and after 4, 16, and 52 weeks of treatment from 10 patients with moderate-to-severe AD (experiment 1). Blood samples from 10 adult healthy controls who have not experienced AD or any other atopic disease were obtained from the Mini Donor Service at University Medical Center Utrecht. To study actual binding of dupilumab antibodies to IL-4 receptor alpha after 4 and 16 weeks of dupilumab treatment (experiment 2), a clinically comparable subgroup of five adult patients with AD were included. For the study of very early effects, blood samples of four (experiment 3) and three (experiment 4) patients, respectively, were taken within 2 hours after first dupilumab administration. Skin biopsies (3 mm) of lesional and nonlesional skin were collected from a clinically comparable cohort of eight adult patients with moderate-to-severe AD at baseline and week 4 of dupilumab treatment (experiment 5). Posttreatment biopsies were taken from the same location as pretreatment biopsies, close to prior biopsy scars. Clinical data were extracted from an online Good Clinical Practice database (BioDay registry). All patients signed institutional review board–approved written, informed consent, adhering to the Declaration of Helsinki Principles.

The Eczema Area and Severity Index score was used to evaluate disease severity. In addition, TARC or CCL17 levels, currently the best performing and accepted biomarker for disease severity (Thijs et al., 2015), were measured in routine care using Quantikine ELISA immunoassays (R&D Systems, Minneapolis, MN).

### Assessments

***Cell isolation.*** PBMCs were isolated using Ficoll-Paque (GE Healthcare, Chicago, IL) density gradient centrifugation. PBMCs were frozen in RPMI 1640 medium supplemented with 2mM L-glutamine, 100 IU/ml penicillin-streptomycin, 20% fetal bovine serum (FBS), and 10% DMSO (Sigma-Aldrich, St. Louis, MO) and stored at −170 °C until use.

***Flow cytometry.*** PBMCs were thawed in a 37 °C water bath, washed, and resuspended in RPMI 1640 medium (Gibco, Grand Island, NY) containing 10% FBS with the addition of L-glutamine and penicillin-streptomycin.

***Experiment 1.*** A total of 250,000–500,000 PBMCs were plated in round bottom 96-well plates. To determine cell death, eBioscience Fixable Viability Dye eFluor 506 (Invitrogen, Carlsbad, CA) in PBS was used. Surface staining of multiple T- and B-cell markers (see panels 1–3 in Supplementary Table S2) was performed for 25 minutes at 4 °C. Surface staining of IL-4 receptor alpha (CD124) with phycoerythrin (PE) was performed for 25 minutes at 37 °C, using an optimization protocol after testing different temperatures. For intracellular and nuclear staining, cells were fixed and permeabilized by using eBioscience Fixation and Permeabilization buffers (Invitrogen) and stained with Granzyme B FITC, Ki67 AF647, and FOXP3 PE.

For intracellular cytokine production, cells were first stimulated with phorbol 12-myristate 13-acetate (20 ng/ml; Sigma-Aldrich, Saint Louis, MO) and ionomycin (1.0 μg/ml; Sigma-Aldrich, Saint Louis, MO) for a total of 4 hours. Golgistop (1/1,500; BD Biosciences, San Jose, CA) was added for the last 3.5 hours of cell culture. Afterward, cells were incubated with the fixable viability dye and surface antibodies (see panels 4–6 in Supplementary Table S2) and then fixed, permeabilized, and intracellularly stained with IFN-γ PE-Cy7, IL-4 BV711 IL-5 PE, TNFα allophycocyanin, IL-13 PerCP-Cy5.5, IL-17a PE, and IL-22 allophycocyanin.

***Experiments 2–3.*** A total of 0.5–1.0 × $10^6$ PBMCs were plated to study actual binding of dupilumab antibodies to IL-4 receptor alpha. Surface staining (see panel 7 in Supplementary Table S2) was performed for 25 minutes at 37 °C, followed by a 25-minute incubation of second antibody (streptavidin-allophycocyanin) at 4 °C.

***Experiment 4.*** For determination of reactivity to IL-4, freshly isolated PBMCs were incubated with titrated amounts of recombinant human IL-4 (PeproTech, Cranbury, NJ) in RPMI + 1% FBS for 15 minutes at 37 °C, followed by immediate fixation for 10 minutes at 37 °C using BD Phosflow Fix Buffer I (BD Biosciences) and staining of surface antigens (see panel 8a + 8b in Supplementary Table S2) for 30 minutes at 4 °C. Subsequently, cells were permeabilized using BD Phosflow Perm Buffer III (BD Biosciences) at 4 °C for 1 hour, followed by intracellular staining of phosphorylated signal transducer and activator of transcription 6 (Y641) for 30 minutes at room temperature.

Stained cells from all experiments were resuspended in PBS containing 2% FBS and 0.1% sodium azide (Sigma-Aldrich). Data acquisition was performed on a FACS LSR Fortessa (BD Biosciences) and data was analyzed using FlowJo Software (Tree Star, Ashland, OR).

***ImageStream.*** PBMCs were thawed and resuspended in PBS (Sigma-Aldrich) (0.5–1.0 × $10^6$ living cells/well). Surface staining of CD3 FITC, CD19 allophycocyanin, and IL-4 receptor alpha PE (panel 9 in Supplementary Table S2) was performed for 25 minutes at 37 °C. Data acquisition was performed on the Amnis ImageStream (Millipore Sigma, Burlington, MA), and on-focus cells were analyzed using Amnis IDEAS software (Millipore Sigma).

***Cell cultures.*** A total of 100,000 PBMCs were plated in a round bottom 96-well plate in RPMI 1640 medium

containing 2mM L-glutamine, 1% penicillin-streptomycin, and 10% FBS. PBMCs were stimulated by 0.1 μg/ml coated anti-CD3 (Invitrogen) and incubated for 72 hours at 37 °C. Supernatants were collected and stored at −80 °C until use.

***Skin biopsies in culture.*** Full-thickness biopsies (3 mm) of lesional and nonlesional skin were collected and incubated in DMEM (×1) + Glutamax (Gibco) containing 10% FBS and 100 U/ml penicillin-streptomycin (Invitrogen). Biopsies were weighted before they were placed in a 48-well plate (Corning, Corning, NY) at 37 °C. Supernatants were collected after 24 hours and immediately cryopreserved at −80 °C until use, as previously prescribed in patients with psoriasis (Gallais Sérézal et al., 2018).

***Multiplex immunoassay.*** Concentrations of 16 cytokines and chemokines were measured in supernatants by Luminex multiplex immunoassay (de Jager et al., 2005) at the Multiplex Core Facility of the Center for Translational Immunology (University Medical Center Utrecht), using an in-house validated panel of analytes, listed in Supplementary Table S1. Uniquely color-coded magnetic beads (MagPlex Microspheres, Luminex, Austin, TX) were conjugated to antibodies specific for the reported analytes and incubated with 50 μl of standard dilutions per sample for 1 hour (continuous shaking in the dark). Samples were diluted in High Performance ELISA buffer (Sanquin, Amsterdam, The Netherlands). Pretreatment of samples included filtration and incubation with HeteroBlock to prevent interference by binding of heterophilic antibodies. Plates were washed (Bio-Plex Pro II Wash Station; Bio-Rad, Hercules, CA), and a corresponding cocktail of biotinylated detection antibodies was added for 1 hour. Repeated washings were followed by a 10-minute streptavidin-PE incubation. Fluorescence intensity of PE was measured using a Flexmap 3D system (Luminex) and analyzed by using BioPlex Manager software version 6.1 (Bio-Rad) using 5-parameter curve fitting (Thijs et al., 2017). Supernatant samples that were above or below the assay limits of detection were given values equivalent to the lower limit divided by two or the upper limit multiplied by two.

***Total IgE ELISA.*** Maxisorp 96-well nunc plates (Thermo Fisher Scientific, Waltham, MA) were coated with 4 μg/ml goat anti-human IgE (catalog number A80-108A, Bethyl Laboratories, Montgomery, TX) for 60 minutes at room temperature (RT). Three washes were performed with PBS containing 0.05% TWEEN20. The plates were incubated with PBS containing 5% FBS for 30 minutes at RT to block nonspecific interactions. After washing, patient samples were added in different dilutions (1:10, 1:100, and 1:1,000) and incubated for 60 minutes at RT. Serum from healthy controls was taken as negative control. Afterward, the detection antibody mouse anti-human IgE biotin (0.1 μg/ml) (BD Biosciences Pharmingen, San Diego, CA) was added and incubated for 60 minutes at RT. Streptavidin−horseradish peroxidase (100 ng/ml) (Sanquin) was used for the detection of biotins and was incubated for 30 minutes at RT. TMB substrate (BioLegend, San Diego, CA) was added to the wells and the reaction was stopped by 1 M sulfuric acid when the wells turned blue. The read-out was performed within 30 minutes on the ELISA reader at 450 nm (BMG Labtech, Ortenberg, Germany). A wavelength of 570 nm was taken to correct for the background of every well. Data were analyzed using Clario Star (BMG Labtech).

### Statistical analysis

Statistical analyses were performed using SPSS (for Windows, version 25.0, SPSS Inc, Chicago, IL) and Prism (version 7.4, GraphPad Software, San Diego, CA). Flow cytometric data were presented with medians. The Wilcoxon signed rank test was used to compare two continuous variables in the same patient. Differences between baseline and healthy control flow cytometric data were assessed by the Wilcoxon rank sum test. $P < 0.05$ were considered statistically significant.

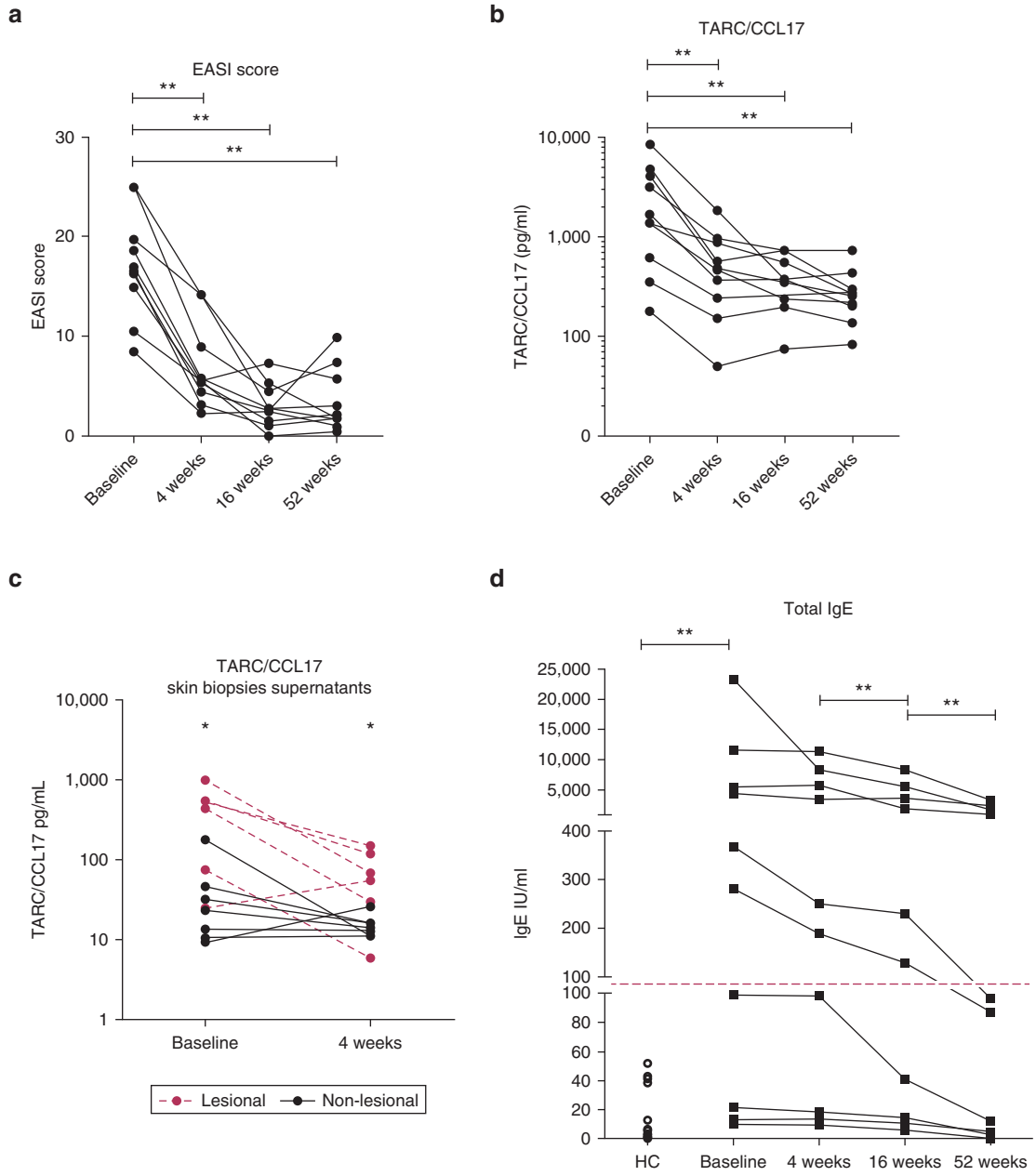
## SUPPLEMENTARY REFERENCES

de Jager W, Prakken BJ, Bijlsma JW, Kuis W, Rijkers GT. Improved multiplex immunoassay performance in human plasma and synovial fluid following removal of interfering heterophilic antibodies. J Immunol Methods 2005;300:124−35.

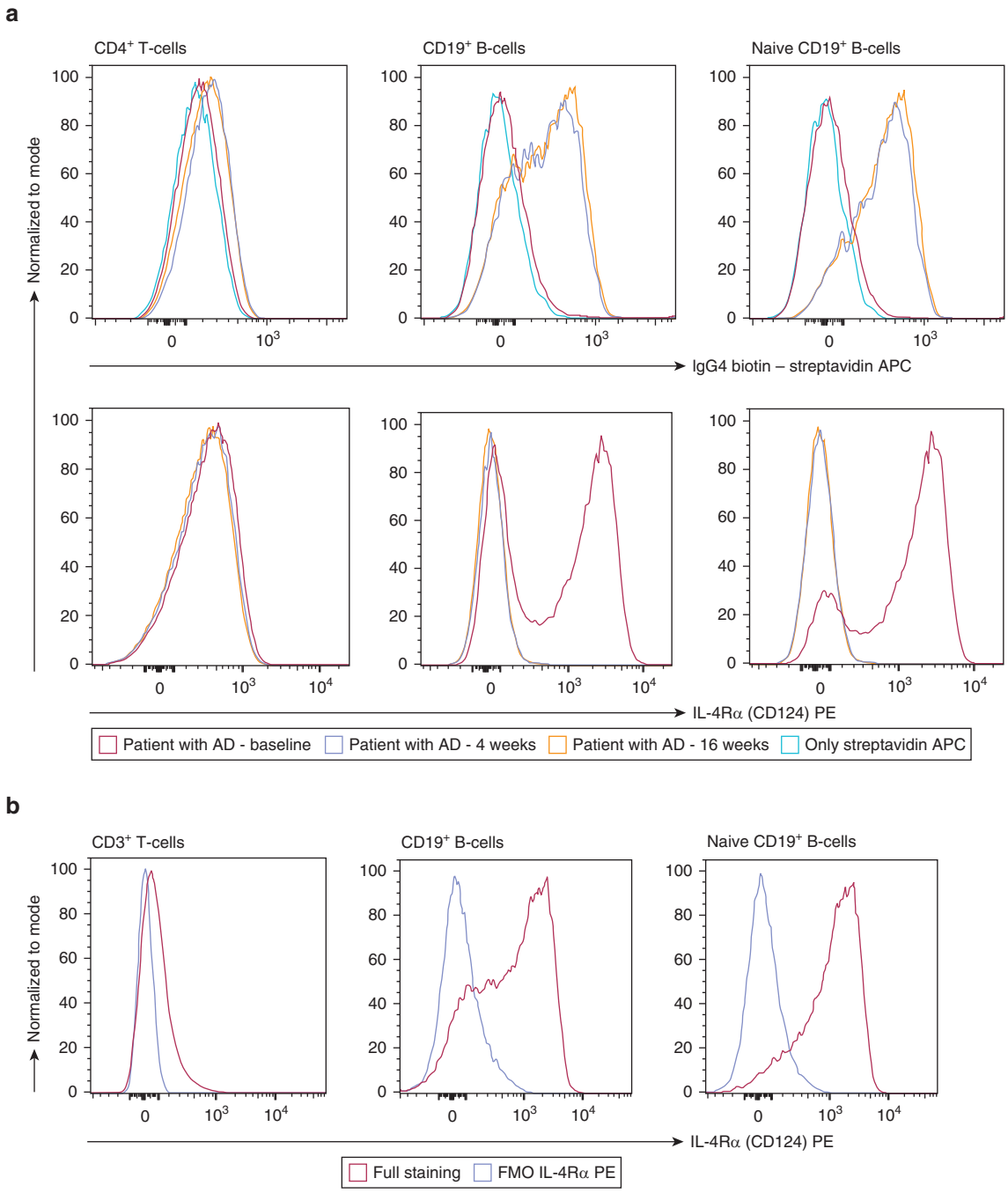
Gallais Sérézal I, Classon C, Cheuk S, Barrientos-Somarribas M, Wadman E, Martini E, et al. Resident T cells in resolved psoriasis steer tissue responses that stratify clinical outcome. J Invest Dermatol 2018;138:1754−63.

Thijs J, Krastev T, Weidinger S, Buckens CF, de Bruin-Weller M, Bruijnzeel-Koomen C, et al. Biomarkers for atopic dermatitis: a systematic review and meta-analysis. Curr Opin Allergy Clin Immunol 2015;15:453−60.

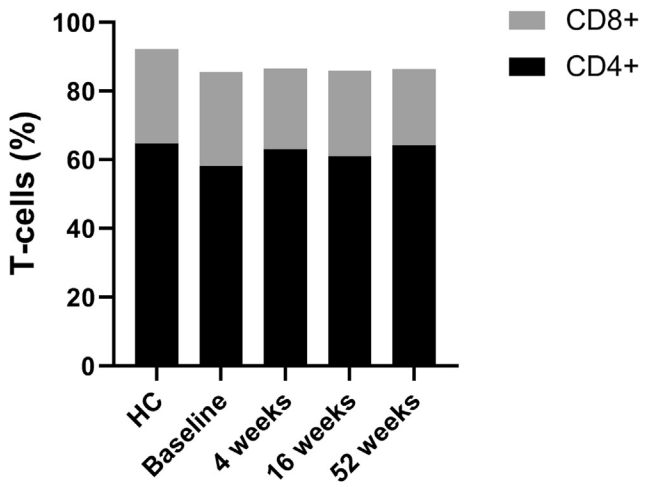
Thijs JL, Strickland I, Bruijnzeel-Koomen CAFM, Nierkens S, Giovannone B, Csomor E, et al. Moving toward endotypes in atopic dermatitis: identification of patient clusters based on serum biomarker analysis [published correction appears in J Allergy Clin Immunol 2018;142:714]. J Allergy Clin Immunol 2017;140:730−7.



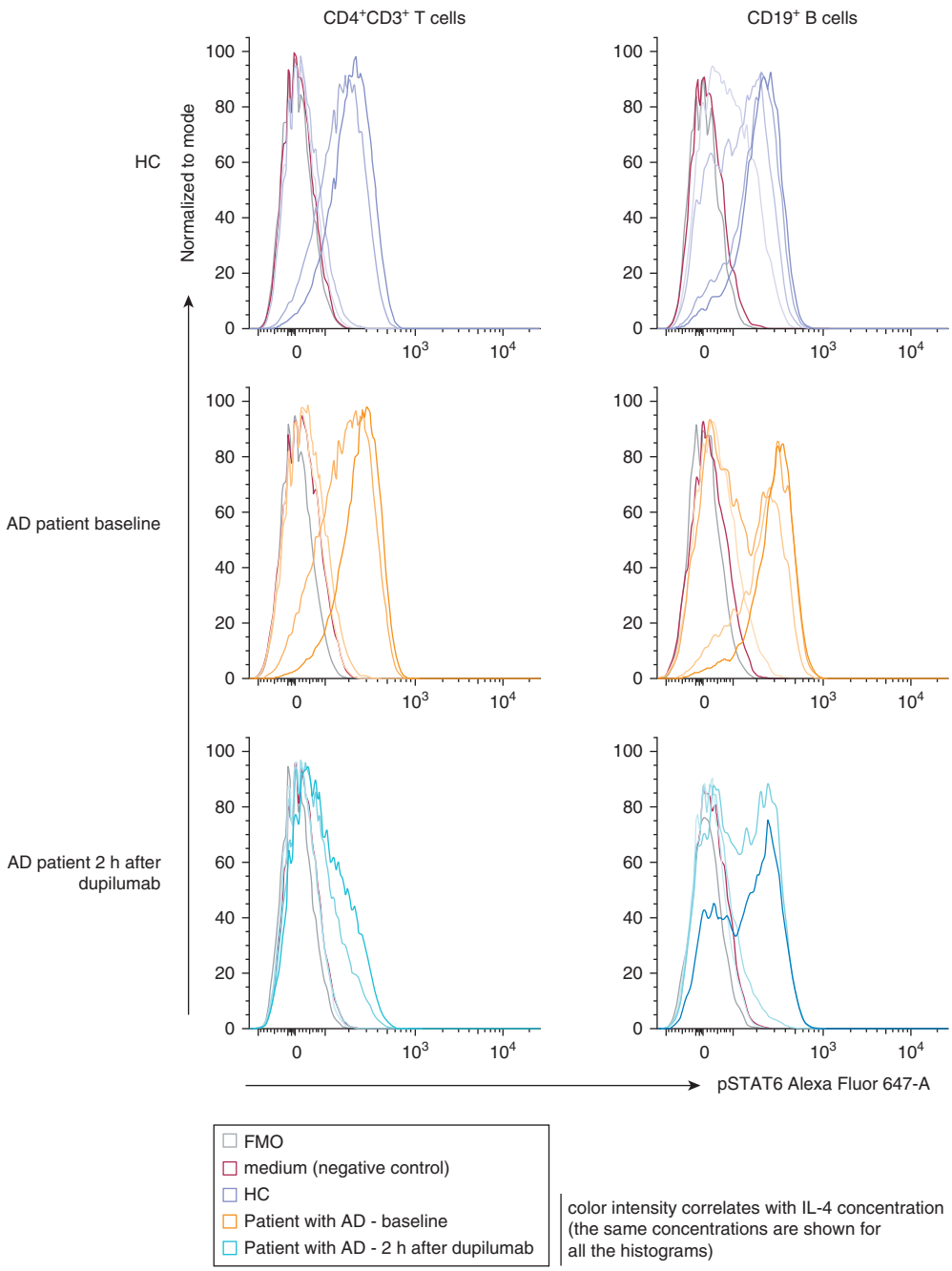

**Supplementary Figure S1. Clinical efficacy of dupilumab treatment.** Clinical efficacy assessed in 10 AD patients treated with dupilumab. Change in EASI score (**a**) and serum TARC/CCL17 levels (**b**) during 52 weeks of dupilumab treatment. Data are shown as median with interquartile range. (**c**) TARC/CCL17 levels in supernatants from 24 hour cultured fresh lesional (red) and nonlesional (blue) skin biopsies. Data are shown as absolute values (pg/mL). Black asterisks, significance of comparison between lesional and nonlesional skin. (**d**) Individual total serum IgE levels in 10 AD patients during 52 weeks of dupilumab treatment, compared to HC. Data are shown as absolute values (IU/mL). Significance levels correspond to the following *P* values: $*P < 0.05$, $**P < 0.01$, and $***P < 0.005$. AD, atopic dermatitis; EASI, Eczema Area Severity Index; HC, healthy control; TARC, thymus and activation-regulated chemokine.



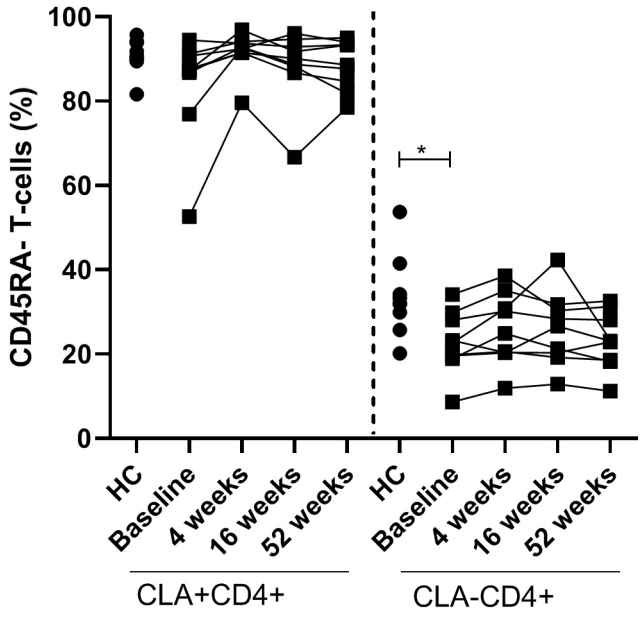

**Supplementary Figure S2. Flow cytometry histogram IL-4Rα and IgG4 (anti-dupilumab).** (**a**) Representative histograms of flow cytometry analysis of IgG4/dupilumab (upper) and IL-4Rα (lower) on CD4+ T-cells, $CD19^+$ B-cells and naive ($CD27^-IgD^+$) $CD19^+$ B-cells from an AD patient before initiation of dupilumab (purple), and after 4 weeks (green) and 16 weeks of dupilumab treatment (blue), and the control staining including only the conjugated secondary antibody streptavidin-APC (yellow). (**b**) Representative histograms of flow cytometry analysis of IL-4Rα on $CD3^+$ T-cells, $CD19^+$ B-cells and naive ($CD27^-IgD^+$) $CD19^+$ B-cells from an AD patient before initiation of dupilumab, presenting the full staining (blue) and the FMO control staining foFMOr IL-4Rα PE (red). AD, atopic dermatitis; FMO, fluorescence minus one.



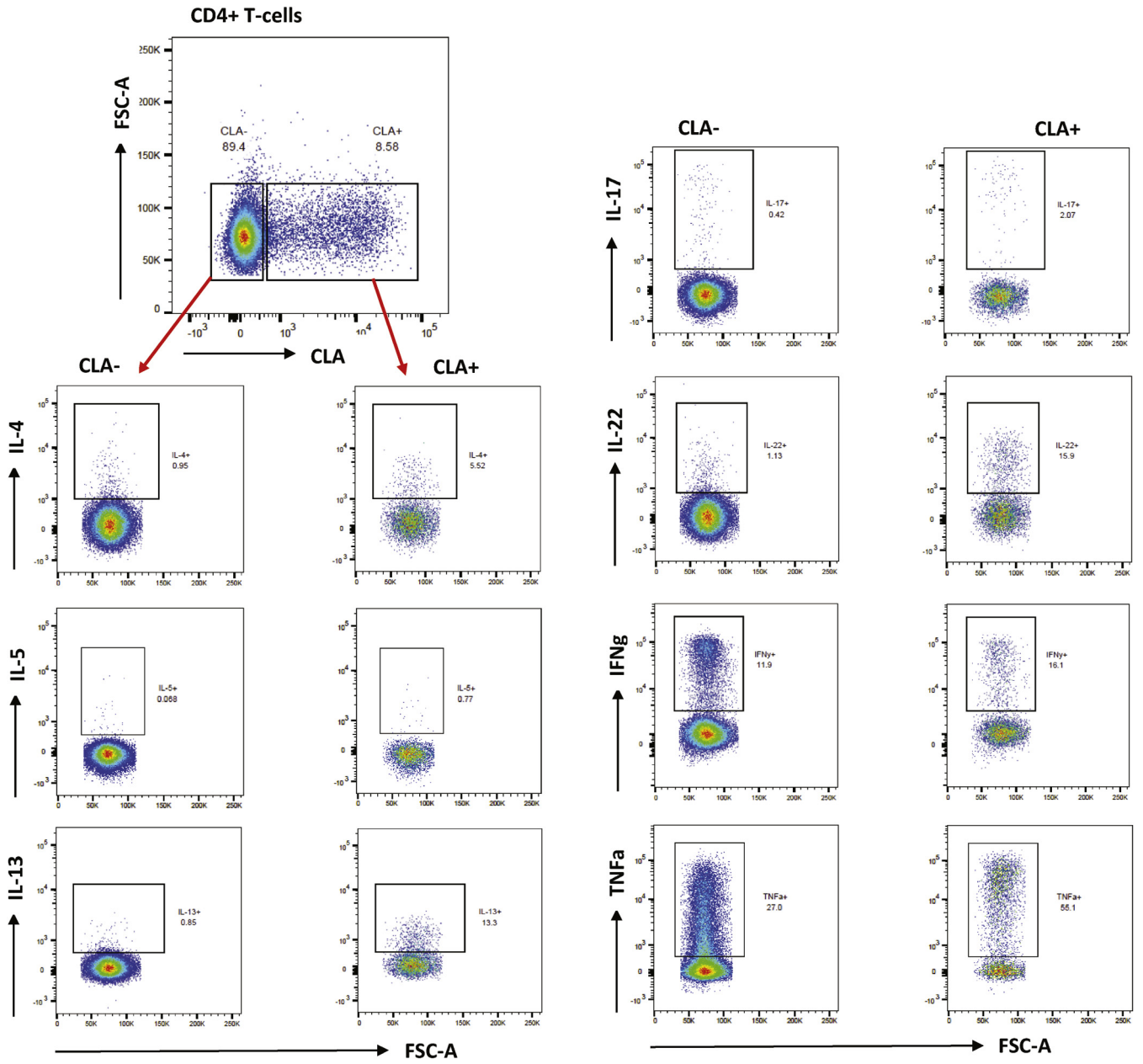

**Supplementary Figure S3. CD4/CD8 ratio.** Ratio of percentage of $CD4^+$ and $CD8^+$ T-cells in PBMCs from AD patients during 52 weeks of dupilumab treatment, compared to healthy controls. AD, atopic dermatitis.



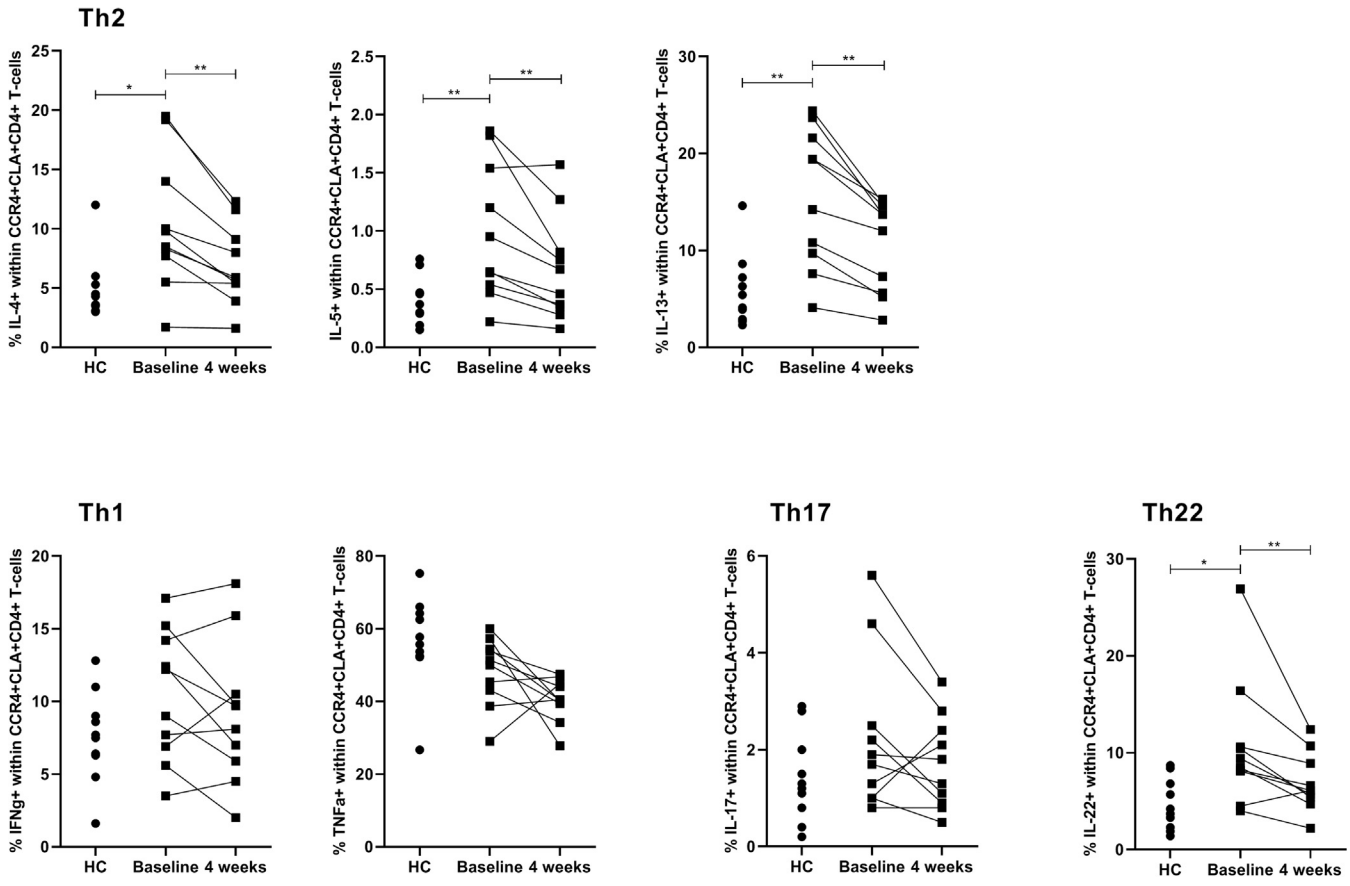

**Supplementary Figure S4. Flow cytometry histogram for pSTAT6 functional assays.** Representative histograms of flow cytometry analysis of intracellular pSTAT6 signaling in response to stimulation with recombinant human IL-4 in vitro in $CD4^+CD3^+$ T-cells, $CD4^-CD3^+$ T-cells and $CD19^+$ B-cells from an AD patient before initiation of dupilumab treatment (pink) and within two hours after the first administration (blue), compared to healthy controls (red), a negative control (orange), and FMO control. Color intensity correlates with different IL-4 concentrations. AD, atopic dermatitis; FMO, fluorescence minus one; pSTAT6, phosphorylated signal transducer and activator of transcription 6.



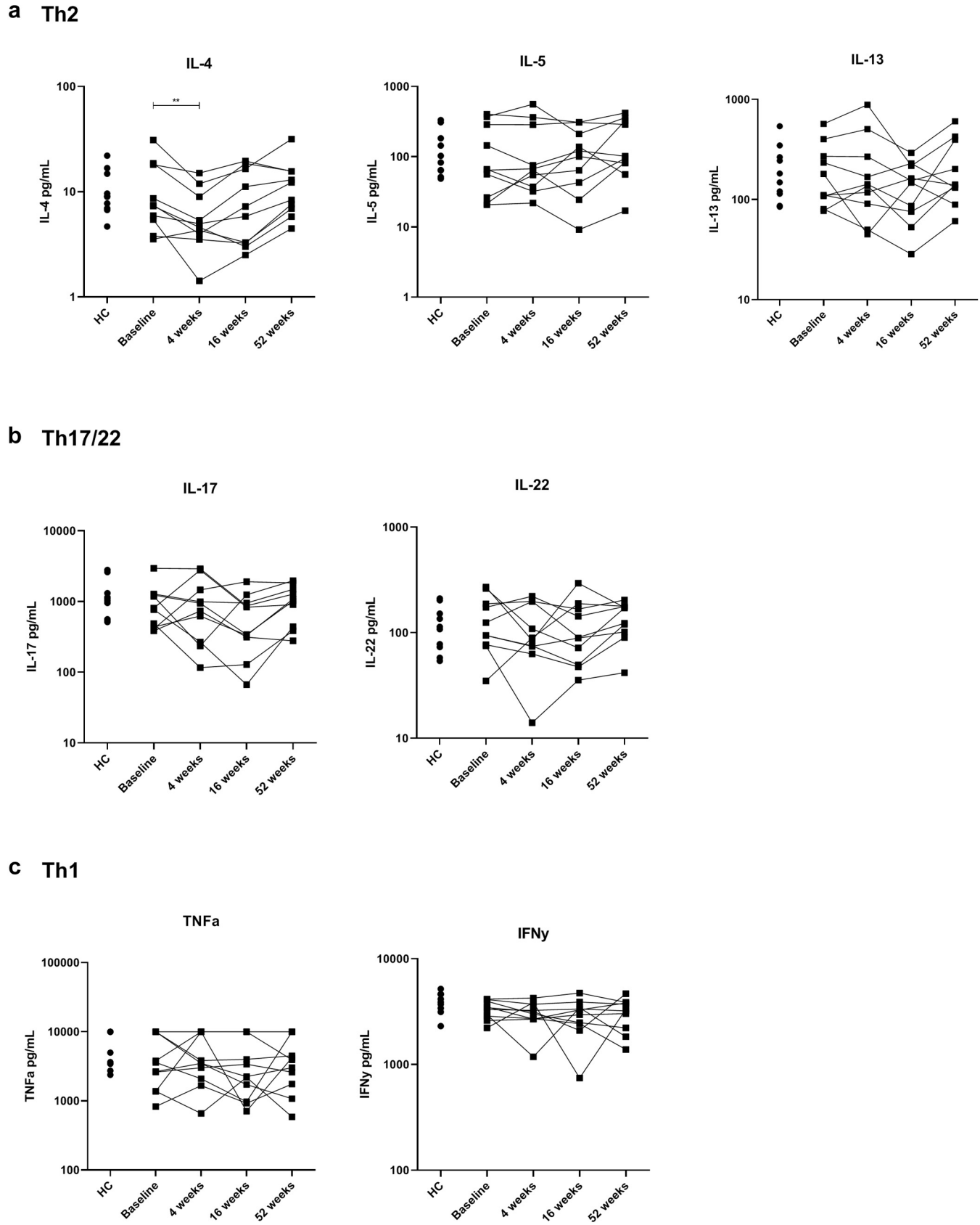

**Supplementary Figure S5. Skin-homing T-cell population consisted mainly of memory T-cells.** Percentages of $CD45RA^{-}$ memory T-cells in skin-homing ($CLA^{+}$) and non skin-homing ($CLA^{-}$) $CD4^{+}$ T-cells in PBMCs from AD patients during 52 weeks of dupilumab treatment, compared to healthy controls. Significance: $^{*}P < 0.05$. AD, atopic dermatitis.



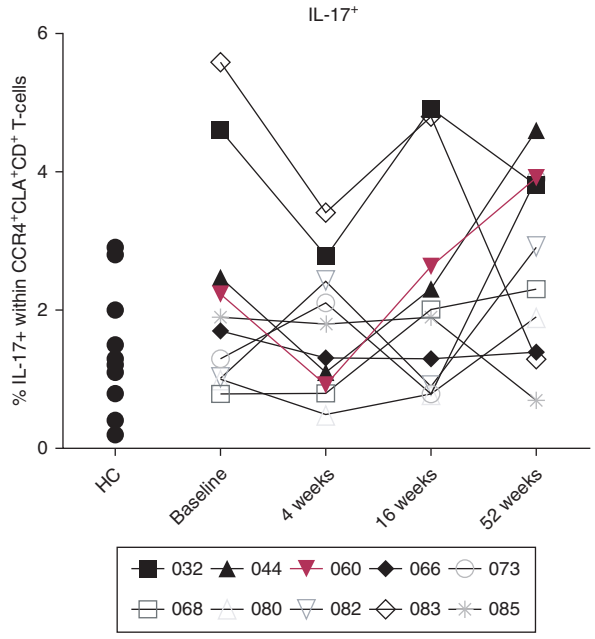

**Supplementary Figure S6. Flow cytometry gating strategy for cytokine-producing T-cells.** Representative gating strategy for non skin-homing ($CLA^-$) and skin-homing ($CLA^+$) T-cell phenotypes (left upper panel), and cytokine producing cells within both populations.




**Supplementary Figure S7. Short-term effect of dupilumab treatment on cytokine producing $CLA^+CCR4^+CD4^+$ T-cells.** Percentages of Th2- (IL-4, IL-5, IL-13), Th1- (IFNγ, TNFα), Th17- (IL-17), and Th22-related (IL-22) cytokine producing skin-homing ($CLA^+CCR4^+$) $CD4^+$ T-cells in PBMCs from 10 AD patients at baseline and after 4 weeks of dupilumab treatment, compared to healthy controls. Significance levels correspond to the following *P* values: $*P < 0.05$, $**P < 0.01$ and $***P < 0.005$. AD, atopic dermatitis.




**Supplementary Figure S8. Cytokine production of total PBMC population after 72 hour of anti-CD3 stimulation.** Levels of (**a**) Th2- (IL-4, IL-5, IL-13), (**b**) Th17/Th22- (IL-17, IL-22), and (**c**) Th1-related (IFNγ, TNFα) cytokines in supernatants from 72 hour anti-CD3 stimulated PBMCs from 10 AD patients during 52 weeks of dupilumab treatment, compared to healthy controls. Significance: $^{**}P < 0.01$. AD, atopic dermatitis.




**Supplementary Figure S9. IL-17 production $CLA^+CCR4^+CD4^+$ T-cells in individual patients.** Percentages of IL-17 producing $CCR4^+CLA^+CD4^+$ T-cells in PBMCs from 10 AD patients during 52 weeks of dupilumab treatment, compared to healthy controls. Patient 060 (marked in red) developed severe rosacea after 40 weeks of treatment. AD, atopic dermatitis.

## Supplementary Table S1. Baseline Characteristics

| Clinical Characteristics | Experiment 1 Full Staining (n = 10) | Experiment 2 IL-4Rα + IgG4 4 + 16 wk (n = 5) | Experiment 3 IL-4Rα + IgG4 2 h (n = 4) | Experiment 4 IL4Rα 2h + STAT-6 Response (n = 5)[1] | Experiment 5 Biopsies (n = 8) |
|---|---|---|---|---|---|
| Age, y,[2] median (IQR) | 50 (46–56) | 47 (25–58) | 44 (37–50) | 38 (38–60) | 45 (30–58) |
| Male, n (%) | 6 (60) | 2 (20) | 5 (100) | 2 (40) | 5 (63) |
| Atopic comorbidities, n (%) | | | | | |
| Allergic asthma | 5 (50) | 2 (40) | 3 (60) | 2 (40) | 5 (63) |
| Allergic rhinitis | 6 (60) | 2 (40) | 3 (60) | 2 (40) | 3 (38) |
| Food allergy | 4 (40) | 2 (40) | 2 (40) | 2 (40) | 1 (13) |
| Allergic conjunctivitis | 8 (80) | 3 (60) | 3 (60) | 1 (20) | 4 (50) |
| No atopic disease | 2 (20) | 2 (40) | 1 (20) | 0 | 2 (25) |
| IgE levels IU/ml, median (IQR) | 342.7 (19.5–7,031.3) | NA | NA | NA | NA |
| Age of onset, n (%) | | | | | |
| Children | 9 (90) | 4 (80) | 2 (40) | 4 (80) | 7 (88) |
| Adolescent | 0 | 0 | 1 (20) | 1 (20) | 0 |
| Adult | 1(10) | 1 (20) | 2 (20) | 0 | 1 (13) |
| Previous use of systemic immunosuppressants for AD, n (%) | | | | | |
| ≥2 Oral immunosuppressants | 6 (60) | 3 (60) | 0 | 1 (20) | 4 (50) |
| Cyclosporine A | 9 (90) | 5 (100) | 4 (80) | 4 (80) | 8 (100) |
| Methotrexate | 3 (30) | 1 (20) | 4 (80) | 2 (40) | 5 (63) |
| Azathioprine | 2 (20) | 2 (40) | 0 | 2 (40) | 3 (38) |
| Mycophenolate mofetil | 3 (30) | 2 (40) | 0 | 0 | 2 (25) |
| EASI score, median (IQR) | 16.8 (13.8–21.1) | 18.6 (15.9–24.6) | 27.0 (10.4–30.7) | 11.4 (6.7–14.6) | 12.2 (9.5–13.9) |
| Weekly average pruritus NRS, median (IQR) | 7.5 (6.8–8.3) | 8.0 (7.5–9.0) | 7.0 (4.0–7.5) | NA | 8.0 (7.0–9.0) |

Abbreviations: AD, atopic dermatitis; EASI, Eczema Area Severity Index; IL-4Rα, IL-4 receptor alpha; IQR, interquartile range; NA, not applicable; NRS, numeric rating scale; STAT, signal transducer and activator of transcription.

[1]Experiment 4 included baseline samples from two patients with AD and samples within 2 hours after first dupilumab administration from three patients.

[2]Age at the moment of sampling.

## Supplementary Table S2. Flow Cytometry Panels

| Antigen/Target | Fluorochrome | Clone | Dilution | Company | Catalog Number |
|---|---|---|---|---|---|
| Panel 1 | | | | | |
| Fixable viability dye | eF506 | — | 1,000 | Thermo Fisher Scientific | 65-0866 |
| CD3 | BV605 | UCHT1 | 100 | BioLegend | 300460 |
| CD4 | BV785 | RPA-T4 | 50 | BioLegend | 300554 |
| CD8 | APC-Cy7 | SK1 | 50 | BD Biosciences | 557834 |
| CD45RA | BV711 | HI100 | 500 | BioLegend | 304138 |
| CLA | PB | HECA-452 | 200 | BioLegend | 321308 |
| IL-4Rα (CD124) | PE | G077F6 | 50 | BioLegend | 355004 |
| Panel 2 | | | | | |
| Fixable viability dye | eF506 | — | 1,000 | Thermo Fisher Scientific | 65-0866 |
| CD3 | BV605 | UCHT1 | 100 | BioLegend | 300460 |
| CD4 | BV785 | RPA-T4 | 50 | BioLegend | 300554 |
| CD8 | APC-Cy7 | SK1 | 50 | BD Biosciences | 557834 |
| CLA | PB | HECA-452 | 200 | BioLegend | 321308 |
| CD25 | PE-Cy7 | M-A251 | 25 | BD Biosciences | 557741 |
| GzmB | FITC | GB11 | 50 | BD Biosciences | 560211 |
| FOXP3 | PE | PCH101 | 50 | Thermo Fisher Scientific | 12-4776 |
| Ki67 | AF647 | B56 | 50 | BD Biosciences | 558615 |
| Panel 3 | | | | | |
| CD3 | AF700 | UCHT1 | 50 | BioLegend | 300424 |
| CD19 | APC-eF780 | HIB19 | 20 | Thermo Fisher Scientific | 47-0199 |
| IgD | BV510 | IA6-2 | 500 | BioLegend | 348220 |
| CD27 | APC | L128 | 50 | BD Biosciences | 337169 |
| IL-4Rα (CD124) | PE | G077F6 | 50 | BioLegend | 355004 |
| Panel 4 | | | | | |
| Fixable viability dye | eF506 | — | 1,000 | Thermo Fisher Scientific | 65-0866 |
| CD3 | BV605 | UCHT1 | 100 | BioLegend | 300460 |
| CD4 | BV785 | RPA-T4 | 50 | BioLegend | 300554 |
| CD8 | APC-Cy7 | SK1 | 50 | BD Biosciences | 557834 |
| CLA | PB | HECA-452 | 200 | BioLegend | 321308 |
| CCR4 | FITC | 205410 | 16.7 | R&D Systems | FAB1567F |
| IL-4 | BV711 | MP4-25D2 | 12.5 | BD Biosciences | 564112 |
| IL-13 | PerCP-Cy5.5 | JES10-5A2 | 50 | Sony Biotechnology | 3109555 |
| IL-17A | PE | eBio64DEC17 | 100 | Thermo Fisher Scientific | 12-7179 |
| IL-22 | APC | IL22JOP | 40 | Thermo Fisher Scientific | 17-7222 |
| IFN-γ | PE-Cy7 | 4S.B3 | 200 | BD Biosciences | 557844 |
| Panel 5 | | | | | |
| Fixable viability dye | eF506 | — | 1,000 | Thermo Fisher Scientific | 65-0866 |
| CD3 | BV605 | UCHT1 | 100 | BioLegend | 300460 |
| CD4 | BV785 | RPA-T4 | 50 | BioLegend | 300554 |
| CD8 | APC-Cy7 | SK1 | 50 | BD Biosciences | 557834 |
| CLA | PB | HECA-452 | 200 | BioLegend | 321308 |
| CCR4 | FITC | 205410 | 16.7 | R&D Systems | FAB1567F |
| IL-5 | PE | JES1-39D10 | 50 | BioLegend | 500904 |
| TNF-α | APC | MAB11 | 200 | BioLegend | 502912 |
| Panel 6 | | | | | |
| Fixable viability dye | eF506 | — | 1,000 | Thermo Fisher Scientific | 65-0866 |
| CD3 | BV605 | UCHT1 | 100 | BioLegend | 300460 |
| CD4 | BV785 | RPA-T4 | 50 | BioLegend | 300554 |
| CD8 | APC-Cy7 | SK1 | 50 | BD Biosciences | 557834 |
| CLA | PB | HECA-452 | 200 | BioLegend | 321308 |
| CCR10 | PE | 314305 | 50 | R&D | FAB3478P |
| CRTH2 | AF647 | BM16 | 20 | BioLegend | 350104 |
| IL-4 | BV711 | MP4-25D2 | 12.5 | BD Biosciences | 564112 |
| IL-13 | PerCP-Cy5.5 | JES10-5A2 | 50 | Sony Biotechnology | 3109555 |
| IFN-γ | PE-Cy7 | 4S.B3 | 200 | BD Biosciences | 557844 |

(*continued*)

## Supplementary Table S2. Continued

| Antigen/Target | Fluorochrome | Clone | Dilution | Company | Catalog Number |
|---|---|---|---|---|---|
| Panel 7 | | | | | |
| CD3 | BV605 | UCHT1 | 100 | BioLegend | 300460 |
| CD4 | BV785 | RPA-T4 | 50 | BioLegend | 300554 |
| CD8 | PE-Cy7 | SK-1 | 200 | BD Biosciences | 335822 |
| CD19 | APC-eF780 | HIB19 | 20 | Thermo Fisher Scientific | 47-0199 |
| IgD | BV510 | IA6-2 | 500 | BioLegend | 348220 |
| CD27 | PerCP-Cy5.5 | O323 | 200 | BioLegend | 302818 |
| IL-4R-α (CD124) | PE | G077F6 | 50 | BioLegend | 355004 |
| IgG4 | Biotin | HP6025 | 200 | Invitrogen | A10663 |
| Streptavidin | APC | — | 100 | Thermo Fisher Scientific | 17-4317 |
| Panel 8a | | | | | |
| CD3 | BV421 | UCHT1 | 200 | BioLegend | 300433 |
| CD4 | PE-Cy7 | RPA-T4 | 200 | BioLegend | 300511 |
| CD19 | APC-eF780 | HIB19 | 20 | Thermo Fisher Scientific | 47-0199 |
| IL-4Rα (CD124) | PE | G077F6 | 50 | BioLegend | 355004 |
| Panel 8b | | | | | |
| CD3 | BV421 | UCHT1 | 200 | BioLegend | 300433 |
| CD4 | PE-Cy7 | RPA-T4 | 200 | BioLegend | 300511 |
| CD19 | PE | HIB19 | 100 | TONBO | 50-0199-T025 |
| pSTAT6 (Y641) | AF647 | 18/P-Stat6 | 20 | BD Biosciences | 562079 |
| Panel 9 - ImageStream | | | | | |
| CD3 | FITC | OKT3 | 25 | BioLegend | 317306 |
| CD19 | APC | HIB19 | 25 | Sony Biotechnology | 2111060 |
| IL-4Rα (CD124) | PE | G077F6 | 20 | BioLegend | 355004 |

Abbreviations: APC, allophycocyanin; CLA, cutaneous lymphocyte antigen; IL-4Rα, IL-4 receptor alpha; PB,Pacific blue; PE, phycoerythrin; pSTAT6, phosphorylated signal transducer and activator of transcription 6.

## Supplementary Table S3. List of Proteins Measured in Supernatants by Luminex Multiplex Immunoassay

| Protein Symbol | Protein Name |
|---|---|
| IL-4 | Interleukin 4 |
| IL-5 | Interleukin 5 |
| IL-13 | Interleukin 13 |
| IL-17 | Interleukin 17 |
| IL-22 | Interleukin 22 |
| IL-31 | Interleukin 31 |
| IL-33 | Interleukin 33 |
| TNF-α | Tumor necrosis factor alpha |
| IFN-γ | Interferon gamma |
| TSLP | Thymic stromal lymphopoietin |
| TARC or CCL17 | Thymus and activated regulated chemokine or Chemokine (C-C Motif) ligand 17 |
| PARC or CCL18 | Pulmonary and activation-regulated chemokine or Chemokine (C-C Motif) ligand 17 |
| Eotaxin-3 or CCL26 | Eotaxin-3 or C-C motif chemokine ligand 26 |
| CTACK or CCL27 | Cutaneous T-cell-attracting chemokine or C-C motif chemokine ligand 27 |
| IP-10 or CXCL10 | Interferon gamma-induced protein 10 or C-X-C motif chemokine 10 |
| sIL-2Rα | Interleukin 2 receptor alpha, soluble |