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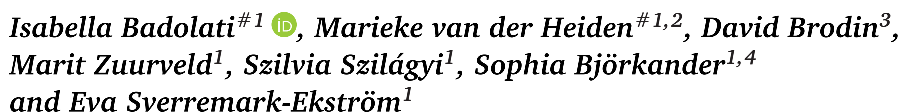
Allergy and inflammation

Basic

Research Article

Staphylococcus aureus-derived factors promote human Th9 cell polarization and enhance a transcriptional program associated with allergic inflammation



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T helper (Th) 9 cells, characterized by robust secretion of IL-9, have been increasingly associated with allergic diseases. However, whether and how Th9 cells are modulated by environmental stimuli remains poorly understood. In this study, we show that *in vitro* exposure of human PBMCs or isolated CD4 T-cells to *Staphylococcus (S.) aureus*-derived factors, including its toxins, potently enhances Th9 cell frequency and IL-9 secretion. Furthermore, as revealed by RNA sequencing analysis, *S. aureus* increases the expression of Th9-promoting factors at the transcriptional level, such as FOXO1, miR-155, and TNFRSF4. The addition of retinoic acid (RA) dampens the Th9 responses promoted by *S. aureus* and substantially changes the transcriptional program induced by this bacterium, while also altering the expression of genes associated with allergic inflammation. Together, our results demonstrate a strong influence of microbial and dietary factors on Th9 cell polarization, which may be important in the context of allergy development and treatment.

Keywords: allergy · environmental stimuli · retinoic acid · *Staphylococcus aureus* · T helper 9 cells

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Introduction

The microbiota and dietary metabolites play a crucial role in the process of differentiation, also called polarization, of naïve CD4+ T helper (Th) cells into each effector subset [1]. Different microbial species are known to induce distinct types of Th profiles, potentially influencing the development of disease [2]. However, previous research in the field has focused on Th1, Th2, and Th17 cells, while little is known about the effects of environmental stimuli on newly emerged Th subpopulations, such as Th9.

Th9 cells, first described about a decade ago, are characterized by the potent secretion of IL-9, a pleiotropic cytokine with both protective and disease-inducing effects. Functionally, Th9 cells promote anti-tumor immunity [3, 4] as well as defense against parasitic infections [5], but have also been associated with the pathogenesis of allergic diseases [6, 7]. The transcription factor network controlling Th9 responses is not yet fully understood, and a master gene regulator has so far not been found, as it has for the other Th subsets [8]. Instead, multiple transcription factors have been suggested as essential for Th9 cell development, including IRF4 [9], PU.1 [10], FOXO1 [11], BATF [12], and STAT6 [13]. Although Th9 and Th2 cells appear to be closely related, and the existence of Th9 as an independent subset has previously been challenged [14], Th9 cells uniquely differentiate in the presence of IL-4 and TGF-β while Th2 polarization relies only on IL-4 [15, 16]. Furthermore, the capacity of Th9 cells to drive allergic inflammation in the skin, gut, and airway mucosa [7], which are environments rich in microbial and dietary compounds, suggests that these factors might be of great importance in regulating the Th9 subset.

Staphylococcus (S.) aureus, a gram-positive bacterium that is commonly found in the human nasopharynx, skin, and gastrointestinal tract, is also a pathogen, which frequently causes infections. The virulence of *S. aureus* is mainly determined by the numerous toxins it is able to secrete, which can act as superantigens and cause potent inflammation [17]. *S. aureus* colonization has been linked to several types of allergic diseases, such as atopic dermatitis [18], food allergy [19], asthma, and allergic rhinitis [20], but the underlying mechanisms of this association remain unclear. Earlier studies have shown that *S. aureus* triggers Th2 cell activation [21], and that the toxins are able to drive somatic hypermutation in B cells towards IgE [22]. In addition, a connection between *S. aureus* and Th9 cells was recently described in a murine model, where staphylococcal enterotoxin B (SEB) strongly activated Th9 cells [23]. However, whether the same can be observed in human settings is still unknown, and the effect of other *S. aureus*-derived factors on Th9 cells warrants further investigation.

Immunity of the skin and even more of the gut, organs where *S. aureus* colonization may particularly occur during infancy [24], is profoundly impacted by retinoic acid (RA), an active metabolite of vitamin A [25, 26]. RA is mainly synthesized by dendritic cells in the intestinal mucosa and influences Th cell differentiation with variable effects depending on its concentration and the surrounding milieu [27]. Interestingly, RA was recently found to inhibit Th9 cells by largely impacting their transcriptome and to ameliorate allergic pathology in mice [28]. However, the interactions of RA with microbial metabolites, including staphylococcal factors, and the potential implications that these may have for allergy development have not been previously explored.

In the present study, we aimed to investigate whether *S. aureus*-derived factors influence Th9 cell polarization, whether RA can modulate the effects induced by this bacterium, and through which mechanisms. Using several different *in vitro* approaches, we show that cell-free supernatant (CFS) from *S. aureus*, and in particular its toxins, promotes Th9 cell polarization in both PBMCs and isolated (naïve) CD4 T-cells, impacting cytokine production as well as transcriptome of the treated cells. RA was able to dampen the *S. aureus* Th9-enhancing effect and to reduce the expression of *S. aureus*-induced genes associated with allergic inflammation. These findings highlight the importance of Th9 cell regulation by microbial and dietary factors and suggest that the interactions between Th9 cells and the environment may play a key role in the development of atopic diseases.

Results

S. aureus-derived factors strongly promote Th9 cell polarization

In order to investigate if the allergy-associated bacterium *S. aureus* influences Th9 cell polarization, we first treated PBMCs with *S. aureus*-CFS for 24 h and then stimulated the cells for 72 h with CD3:CD28 beads combined with IL-4 and TGF-β, previously observed to be optimal conditions for inducing Th9 polarization (Fig. S1B). The *S. aureus*-CFS significantly enhanced both the frequency of IL-9$^+$ IRF4$^+$ cells (Th9) within the CD4$^+$ population and the secretion of IL-9, as compared to the untreated control, under Th9-polarizing conditions (Fig. 1A and B). A significant but less pronounced increase in IL-9 secretion was also observed after activation with polyclonal beads alone (Fig. 1A, right). Similar results upon exposure to *S. aureus*-CFS were observed in isolated total CD4 T-cells (Fig. 1C) and naïve CD4 T-cells (Fig. 1D).

Next, we evaluated the effects of *S. epidermidis*, another *Staphylococcus* species considered both as a human commensal and an opportunistic pathogen [29], on Th9 cell polarization. Different from *S. aureus*-CFS, *S. epidermidis*-CFS did not enhance the frequency of Th9 cells or the secretion of IL-9, as compared to the control (Fig. 1E). *S. epidermidis* does not produce toxins as *S. aureus* does [29], thus we asked whether the toxins could be involved in the Th9-promoting effect observed with the *S. aureus*-CFS. Notably, treatment of PBMCs with toxic shock syndrome toxin-1 (TSST-1) and staphylococcal enterotoxin A (SEA), two frequently studied staphylococcal toxins, significantly increased Th9 cell polarization compared to the untreated samples (Fig. 1F).

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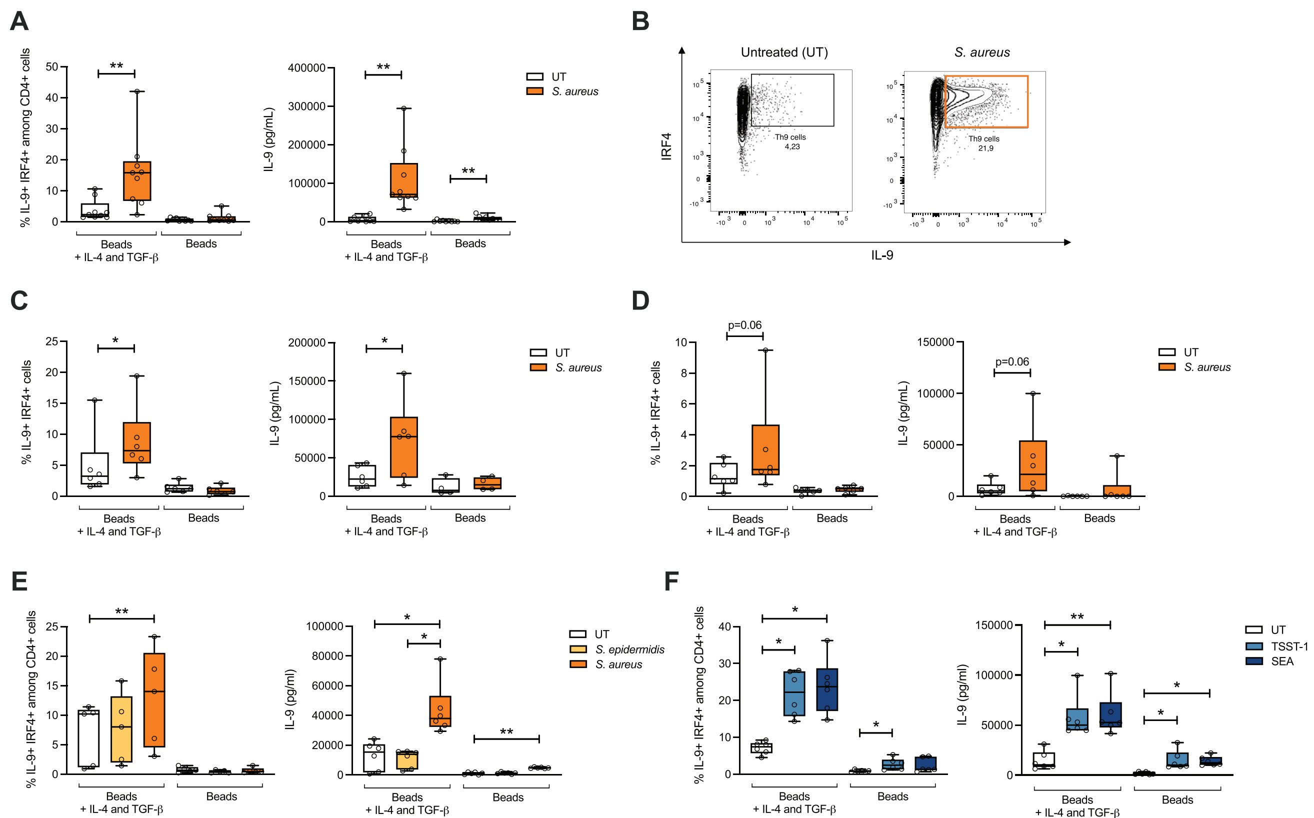

Figure 1. *S. aureus*-derived factors promote Th9 cell polarization. Frequency of IL-9$^+$ IRF4$^+$ (Th9) cells among CD4$^+$ cells (left) and secretion of IL-9 (right) after 24 h treatment with *S. aureus*-CFS (*S. aureus*, orange) or culture medium as control (UT = untreated, white), followed by 72 h stimulation with CD3:CD28 beads together with IL-4 + TGF-β (Th9 polarization) or CD3:CD28 beads only, in PBMCs **(A)**, isolated CD4 T-cells **(C)**, and isolated naïve CD4 T-cells **(D)**. $n = 4$–9, number of independent experiments: 9. In all graphs the min to max boxplots were used, with the line indicating the median. The *S. aureus*-treated samples were compared with the UT samples using the Wilcoxon matched-pairs signed rank test. $^*p < 0.05$, $^{**}p < 0.01$. **(B)** Representative flow cytometry plots of the frequency of Th9 cells among CD4$^+$ cells after inducing Th9 polarizion in PBMCs, preceded by no treatment (left) or *S. aureus*-CFS treatment (right). Frequency of Th9 cells among CD4$^+$ cells (left) and secretion of IL-9 (right) after 24 h treatment of PBMCs with either *S. epidermidis*-CSF (*S. epidermidis*, yellow) and *S. aureus*-CFS (*S. aureus*, orange) **(E)**, the *S. aureus* toxins TSST-1 and SEA (light and dark blue, respectively) **(F)**, or culture medium as control (UT, white) **(E, F)**, followed by 72h stimulation with Th9 polarizing factors or CD3:CD28 beads only. $n = 5$–6, number of independent experiments: 6. The min to max boxplots are shown in each graph, with the line indicating the median. The Friedman test followed by Dunn's multiple comparisons test was used to determine the statistical difference between each treatment. $^*p < 0.05$, $^{**}p < 0.01$.

RA dampens the *S. aureus* Th9-inducing effect and promotes a skew toward IL-5 responses

The dietary metabolite RA was previously shown to inhibit Th9 cell differentiation in a mouse model through the retinoic acid receptor (RAR) alpha [28]. In agreement with these findings, we observed a clear suppression of Th9 cell frequency and IL-9 secretion upon RA treatment in Th9-polarized human PBMCs (Fig. S2A), as well as isolated total CD4 and naïve CD4 T-cells (Fig. S2B and C). Exposure of PBMCs to a pan-RAR inverse agonist (BMS493) led to the opposite result, that is, an increase in IL-9 secretion, confirming the involvement of RARs in this Th9-inhibiting effect of RA (Fig. S2D).

Considering these results, we next investigated whether RA could modulate *S. aureus*-induced Th9 cell polarization. PBMCs were exposed to different combinations of RA and *S. aureus*-CFS before being polarized toward Th9, which led to a marked dampening of Th9 cell frequency as well as IL-9 secretion compared to the *S. aureus*-CFS treatment alone, in a slightly dose-dependent manner (Fig. 2A). This dampening was independent of the order in which the RA and *S. aureus*-CFS treatments were added (Fig. S3A and B). In addition, similar results were obtained in isolated total CD4 and naïve CD4 T-cells, using a fixed concentration of RA (2 μg/mL) in combination with the *S. aureus*-CFS (Fig. 2B).

The *S. aureus*-CFS induced CD4 T-cell proliferation and increased expression of the early T-cell activation marker CD69, as compared to the untreated control (Fig. 2C and 2D). The addition of RA neither dampened the proliferation nor the CD69 expression promoted by *S. aureus*-CFS (Fig. 2C and 2D), but it changed the cytokine profile of the Th9-polarized cells, as shown in the PCA plot (Fig. 2E). Samples treated with both *S. aureus*-CFS

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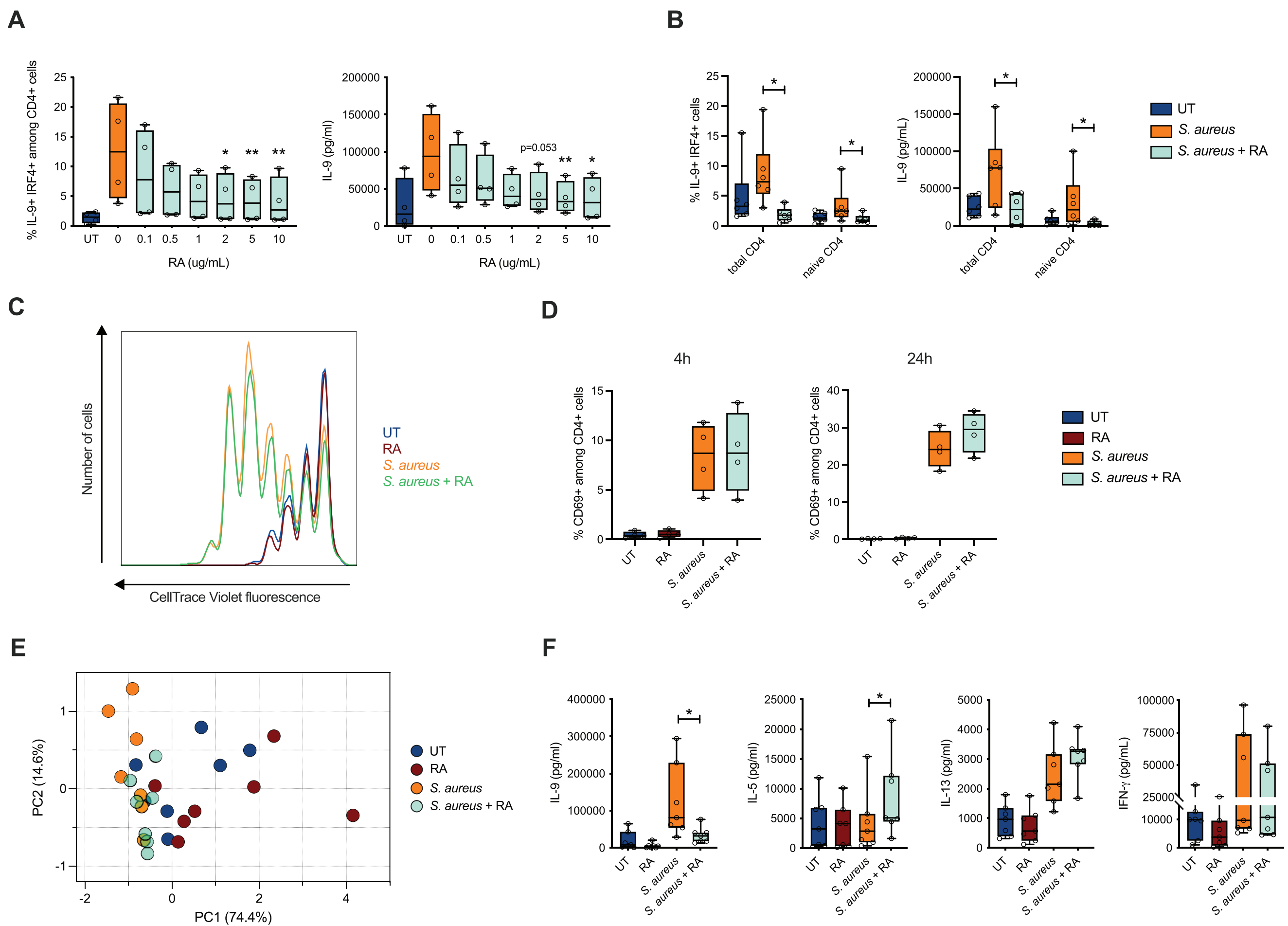

Figure 2. RA dampens the *S. aureus* Th9-inducing effect and promotes a skew toward IL-5 responses. **(A)** Frequency of IL-9$^+$ IRF4$^+$ (Th9) among CD4$^+$ cells (left) and secretion of IL-9 (right) after 24h treatment of PBMCs with culture media as control (UT, blue), *S. aureus*-CSF alone (*S. aureus*, orange), or combined with increasing concentrations of RA (*S. aureus* + RA, green), followed by 72 h of Th9 polarization (CD3:CD28 beads supplemented with IL-4 + TGF-β). $n = 4$, number of independent experiments: 4. The combined *S. aureus*-CFS and RA treatments were compared to the *S. aureus*-CFS only treatment using the Friedman test followed by Dunn's multiple comparisons test. $^*p < 0.05$, $^{**}p < 0.01$. **(B)** Frequency of Th9 cells and secretion of IL-9 in isolated total CD4 T-cells and naïve CD4 T-cells, treated and polarized as described for **(A)**. $n = 6$, number of independent experiments: 3. The Wilcoxon matched-pairs signed rank test was used to determine the statistical difference between the combined *S. aureus*-CFS and RA treatment and the *S. aureus*-CFS only treatment. $^*p < 0.05$. In both A and B, the min to max boxplots were used to show the data, with the line indicating the median. **(C)** Representative plot of live CD4 T-cell proliferation following the different treatments (UT control, RA, *S. aureus*-CFS, and the combination of *S. aureus*-CFS and RA) and Th9 polarization, assessed by CellTrace Violet dye dilution and flow cytometry. **(D)** Frequency of CD69$^+$ among CD4$^+$ cells after 4 h (left) and 24 h (right) treatment of PBMCs with culture media as control (UT, blue), RA (RA, red), *S. aureus*-CFS alone (*S. aureus*, orange) or combined with RA (*S. aureus* + RA, green). $n = 4$, number of independent experiments: 2. The Wilcoxon matched-pairs signed rank test was used to determine the statistical difference between the *S. aureus*-CFS + RA treatment and the *S. aureus*-CFS only treatment. No significant difference was found. Data is displayed as min to max boxplots, with the line indicating the median. **(E)** Principal component analysis (PCA) comparing the cytokine profile of PBMCs polarized towards Th9 that were previously either left untreated (UT), treated with RA, with *S. aureus*-CSF, or with *S. aureus*-CSF together with RA. The PCA plot includes the log transformed data on the secretion of IL-9, IL-5, IL-13, and IFN-γ. The percentage of variance explained by the principal components (PC) 1 and 2 are indicated on the axes. $n = 7$, number of independent experiments: 5. **(F)** Box plots illustrating the cytokine data (IL-9, IL-5, IL-13, and IFN-γ secretion, from left to right) used for the PCA analysis in **(E)**. The Wilcoxon matched-pairs signed rank test was used to determine the statistical difference between the combined *S. aureus*-CFS and RA treatment and the *S. aureus*-CFS only treatment. $^*p < 0.05$.

and RA segregated from those treated with *S. aureus*-CFS only (Fig. 2E). Besides a difference in IL-9 contributing to this segregation, IL-5 levels were altered when combining *S. aureus*-CFS with the RA treatment and were significantly enhanced compared to the microbial treatment alone. Secretion of IL-13, another Th2-associated cytokine, showed a slight tendency to increase upon addition of RA, while the Th1-associated IFN-γ remained unchanged (Fig. 2F).

RA changes the *S. aureus*-induced transcriptional program of Th9-polarized CD4 T cells

To understand the impact of *S. aureus* and RA on Th9 cell polarization in more depth, non-targeted RNA-Seq was performed on isolated CD4 T cells treated with *S. aureus*-CFS and/or RA and polarized to Th9, as previously done. The transcriptional program differed between the four donors, but was clearly affected by

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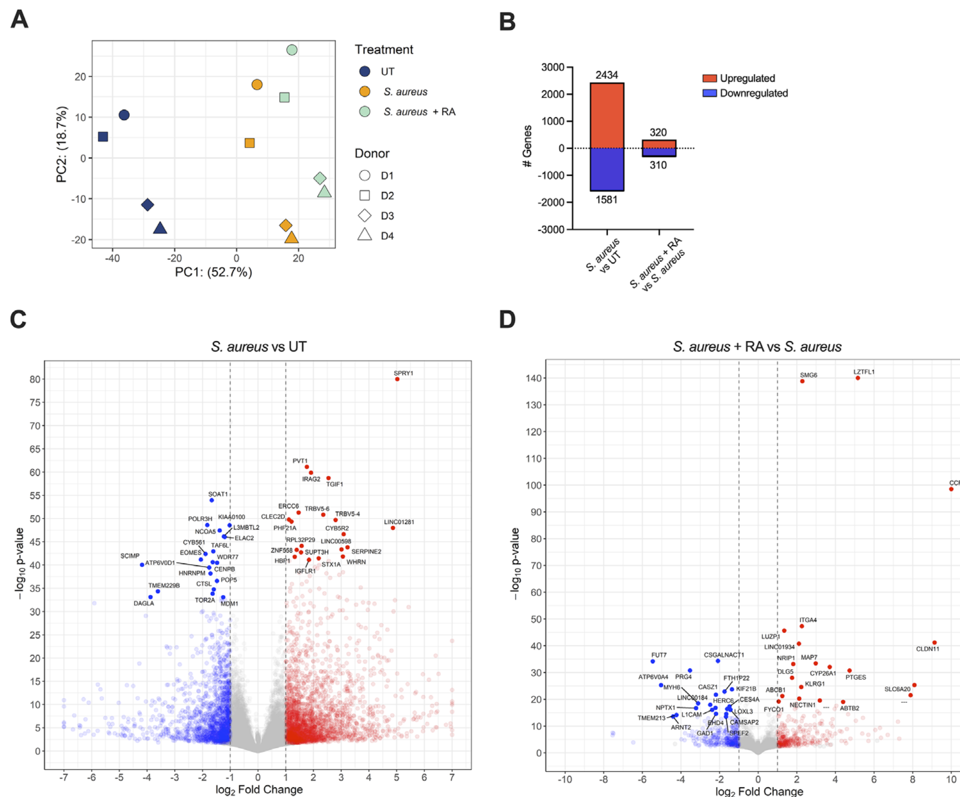

Figure 3. RA changes the S. *aureus*-induced transcriptional program of Th9-polarized CD4 T-cells. **(A)** Principal component analysis (PCA) comparing the transcriptional program of Th9-polarized CD4 T-cells between the different treatment conditions: untreated (UT, blue), treated with S. *aureus*-CSF (orange), or with the combined S. *aureus*-CFS and RA (green). The normalized gene counts of all detectable genes were used to generate the plot, and the percentage of variance explained by the principal components (PC) 1 and 2 is shown on the axes. Data were obtained by RNA-Seq. $n = 4$, indicated by different symbols, number of independent experiments: 2. **(B)** Number of differentially expressed genes (DEGs) between the different treatments of CD4 T-cells polarized toward Th9. Upregulated = $\log_2$-fold change >2 and adj. p-value < 0.05, downregulated = $\log_2$-fold change < −2 and adj. p-value < 0.05. Volcano plots of gene expression in Th9-polarized CD4 T-cells comparing the different treatments as follows: S. *aureus*-CFS versus UT **(C)**, S. *aureus*-CFS + RA versus S. *aureus*-CFS only **(D)**. The $\log_2$-fold change is indicated on the x-axis and the statistical significance ($-\log_{10}$ p-value) on the y-axis. Upregulated genes ($\log_2$-fold change >1, adj. p-value <0.05) are indicated in red, while downregulated genes ($\log_2$-fold change < −1, adj. p-value <0.05) in blue. The top 20 significant genes are labeled in the plots.

the treatments in a similar manner (Fig. 3A). Th9-polarized cells treated with *S. aureus*-CFS markedly deviated from the untreated ones, and the addition of RA induced a further shift in their transcriptome (Fig. 3A). A large number of genes were significantly upregulated ($\log_2$-fold change > 2) and downregulated ($\log_2$-fold change <-2) upon exposure of the samples to *S. aureus*-CFS, as compared to no treatment (Fig. 3B). Of these differentially expressed genes (DEGs), the most significant were highlighted in the volcano plot and included genes involved in T-cell function and proliferation among the upregulated (*TRBV5-6*, *TRBV5-4*, *SPRY1*, *PVT1*), and Th1 cell function, in particular, among the downregulated (*EOMES*, *CTSL*) [30–33] (Fig. 3C). Interestingly, combining RA with the staphylococcal treatment also changed the expression of a substantial number of genes, more than 600, compared to *S. aureus*-CFS alone (Fig. 3B). The addition of RA enriched, for instance, genes belonging to a known cluster on chromosome 3 (*LZTFL1*, *CCR9*, *SLC6A20*, and *FYCO1*), together with common RA targets important for lymphocyte gut-homing (*ITGA4*) [34, 35] (Fig. 3D). In contrast, a gene involved in lymphocyte skin-homing (*FUT7*) could be found among the most significantly downregulated by RA [36] (Fig. 3D).

Gene set enrichment analysis (GSEA) revealed that the combination of *S. aureus*-CFS and RA, as compared to the *S. aureus*-CFS-only treatment, enriched genes involved in retinol metabolism. Instead, pathways associated with T-cell activation and inflammatory responses, such as T-cell receptor signaling and JAK-STAT signaling pathways, appeared to be downregulated (Fig. S4). However, the latter results were not significant when considering the adjusted p-values.

S. *aureus* treatment, alone or combined with RA, influences the expression of Th9-driving factors

In Th9-polarized CD4 T-cells, expression of genes encoding the Th9-associated transcription factors IRF4, FOXO1, BACH2, STAT5A, and STAT5B was enhanced by *S. aureus*-CFS as compared to no treatment, and albeit not significantly, this enhancement appeared to be dampened by the addition of RA (Fig. 4A).

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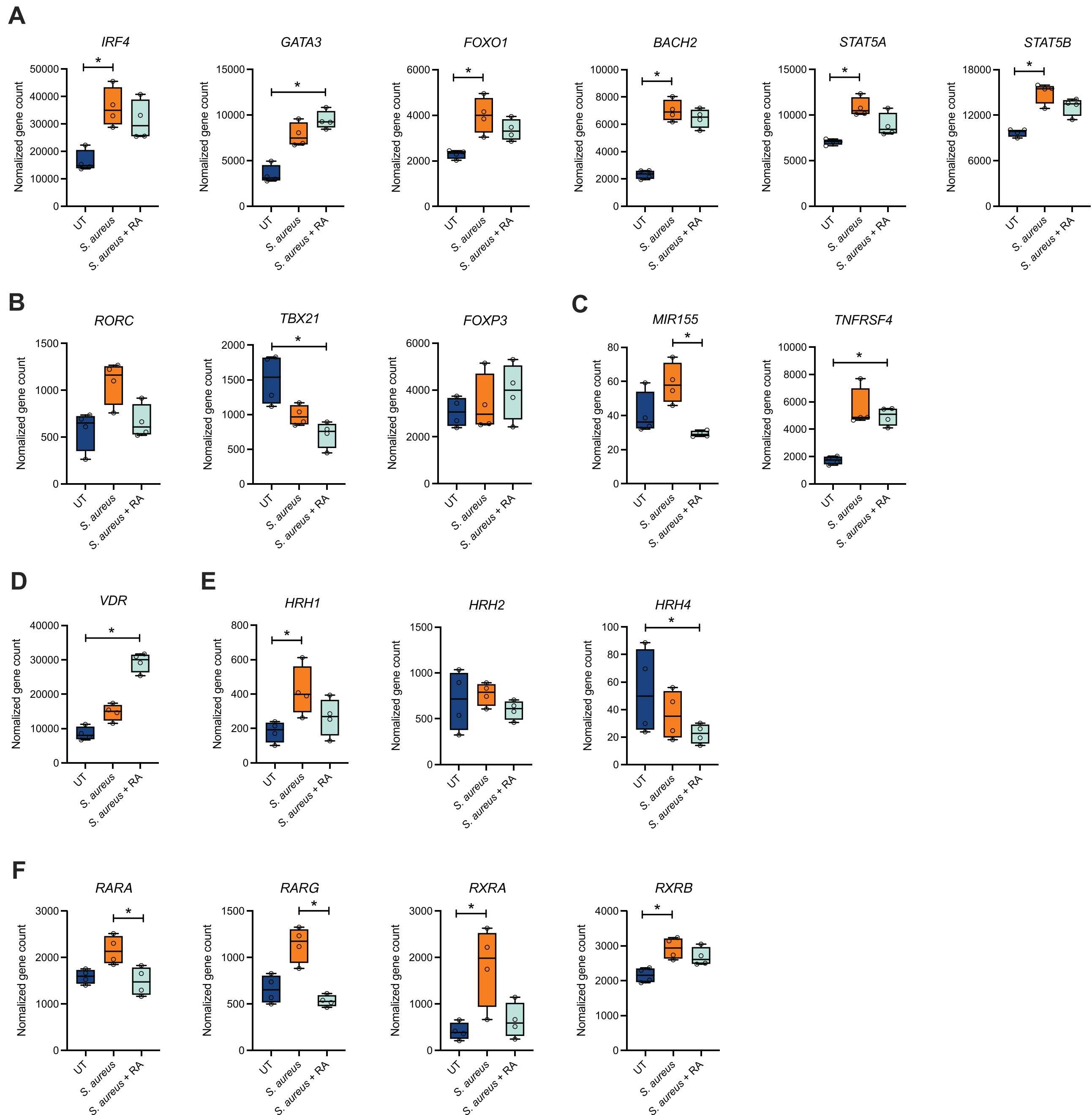

Figure 4. Effects of S. *aureus*, alone, or combined with RA, on Th9-associated transcription factors and regulatory molecules. Normalized gene counts for *IRF4*, *GATA3*, *FOXO1*, *BACH2*, *STAT5A*, and *STAT5B* **(A)**, for *RORC*, *TBX21*, and *FOXP3* **(B)**, as well as *MIR155*, *TNFRSF4* **(C)**, *VDR* **(D)**, *HRH1*, *HRH2* and *HRH4* **(E)**, and *RARA*, *RARG*, *RXRA*, and *RXRB* **(F)**, obtained by RNA-Seq analysis of Th9-polarized CD4 T-cells previously left untreated (UT, blue), treated with *S. aureus*-CFS (orange), or *S. aureus*-CFS together with RA (green). $n = 4$, number of independent experiments: 2. Data is displayed as min to max boxplots with the line indicating the median. The Friedman test followed by Dunn's multiple comparisons test was used to determine the statistical difference between the treatments. $^{*}p < 0.05$.

In contrast, the gene encoding GATA3, linked to both Th2 and Th9 cells, was upregulated to a greater extent by the combined *S. aureus*-CFS and RA treatment (Fig. 4A). Regarding the expression of hallmark transcription factors of other Th subsets, *RORC* (RORγt) was also upregulated by *S. aureus*-CFS in Th9-polarized CD4 T cells and then reduced when combined with RA. A different pattern was instead observed for *TBX21* (Tbet), which was markedly downregulated by both treatments, and *FOXP3*, whose expression did not seem to be affected by any of the treatments (Fig. 4B).

Furthermore, the expression of the genes encoding miR-155 and TNFRSF4 (or OX40 receptor), both known to promote Th9

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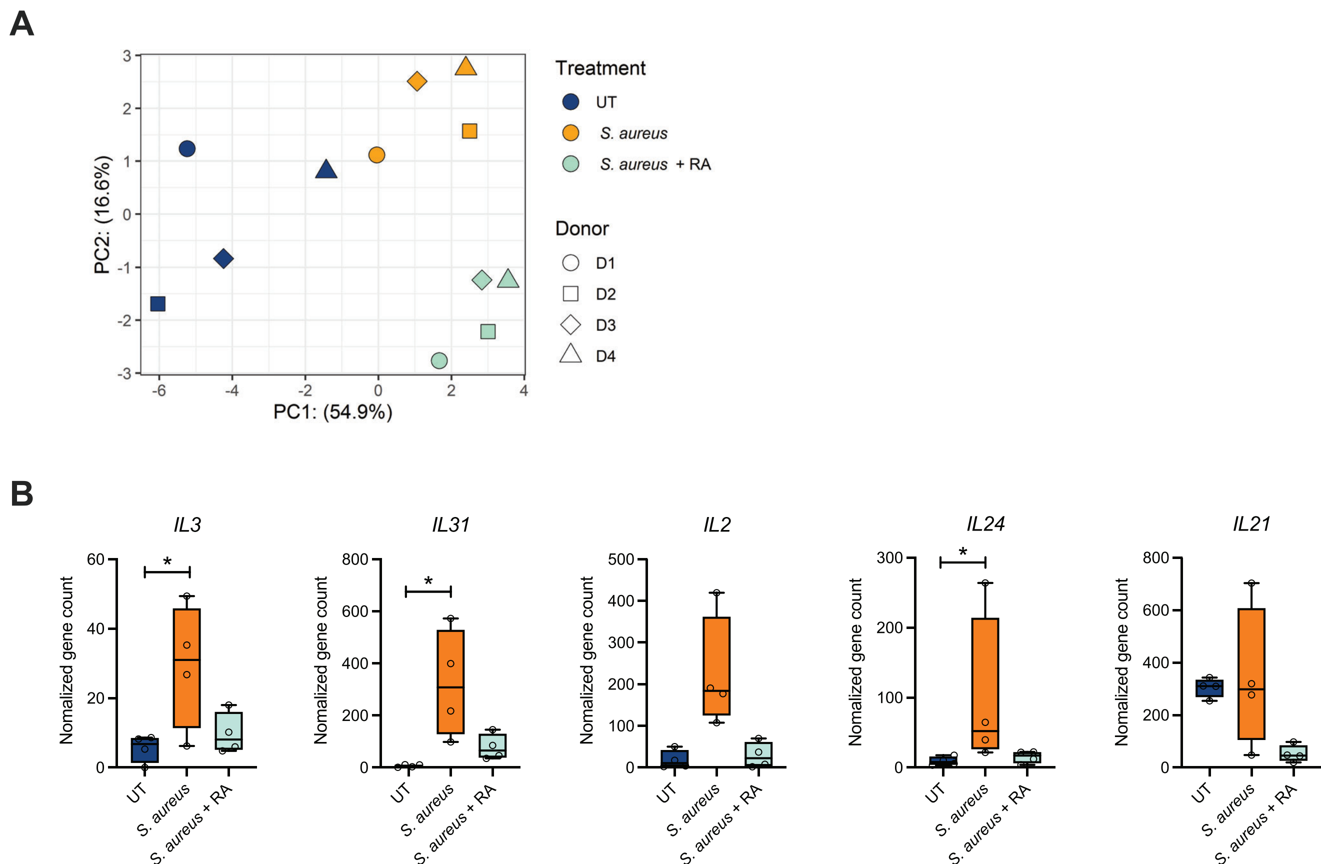

Figure 5. Expression of asthma- and other allergy-related genes is altered by *S. aureus* and RA treatment of Th9-polarized CD4 T-cells. **(A)** Principal component analysis (PCA) plot including normalized counts of 28 asthma-associated genes expressed by the Th9-polarized CD4 T-cells under the different treatment conditions: UT (blue), *S. aureus*-CSF (orange), and *S. aureus*-CFS + RA (green). The percentage of variance explained by the principal components (PC) 1 and 2 is displayed on the axes. $n = 4$, indicated by different symbols, number of independent experiments: 2. **(B)** Normalized gene counts for *IL3*, *IL31*, *IL2*, *IL24*, and *IL21* obtained by RNA-seq analysis of Th9-polarized CD4 T-cells after the same treatments as **(A)**. $n = 4$, number of independent experiments: 2. The min to max boxplots were used to show the data, with the line indicating the median. To determine the statistical difference between the treatments, the Friedman test followed by Dunn's multiple comparisons test was applied. $^{*}p < 0.05$.

cell development [37, 38], was induced by *S. aureus*-CFS and dampened by the addition of RA (Fig. 4C). On the other hand, although being slightly increased by *S. aureus*-CFS, the expression of *VDR*, encoding the vitamin D receptor, was significantly upregulated by the simultaneous addition of RA (Fig. 4D). The treatments also influenced the expression of the allergy-associated and IL-9-promoting histamine receptor genes *HRH1*, *HRH2*, and *HRH4* [39] (Fig. 4E). Lastly, the expression levels of the RA receptor genes *RARA*, *RARG*, *RXRA*, and *RXRB* were enhanced by *S. aureus*-CFS treatment, while the addition of RA led to a decrease (Fig. 4F).

We had previously found that RA changes the cytokine profile of Th9-polarized CD4 T-cells at a protein level; thus, we next examined different Th cytokines at a transcriptional level. In agreement with the results shown in Fig. 2E, adding RA to the staphylococcal treatment suppressed the expression of *IL9*, while upregulating *IL5*, as compared to the *S. aureus*-CFS treatment alone (Fig. S5A). No difference was found between the two treatments in *IL13* and *IFNG* expression (Fig. S5A). However, the transcription of *IL4*, *IL17A*, and *IL10* was reduced by the combination of *S. aureus*-CFS with RA, compared to the *S. aureus*-CFS only treatment, with the most evident effect on *IL10* (Fig. S5B).

Expression of allergy-related genes is altered by prior exposure of Th9 cells to S. *aureus* and RA

In light of the obtained results, we finally asked whether a mechanistic link could be found between *S. aureus*, RA, Th9 cells and allergy. We performed further analysis of our RNA-Seq data and looked into genes involved in allergic diseases, including asthma. Based on the expression of 28 asthma-associated genes, found in the KEGG pathway database and shown in Table S1, Th9-polarized CD4 T-cells that had been treated with *S. aureus*-CFS or *S. aureus*-CFS plus RA evidently separated from the untreated control and also highly differed from each other (Fig. 5A). Moreover, we noted that the expression of single factors known to play a role in allergic inflammation and/or T-cell activation, namely IL-3 [40], IL-31 [41], IL-2 [42], IL-24 [43], and IL-21 [44], followed a similar pattern, with *S. aureus*-CFS inducing upregulation

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as compared to the untreated sample, and RA strongly reducing this effect (Fig. 5B).

Discussion

Since the discovery of Th9 cells in 2008, substantial evidence has emerged supporting their involvement in the pathogenesis of allergies and other chronic inflammatory diseases [6, 7]. However, the influence of environmental factors, such as microbes and dietary metabolites, on Th9 cell development is just beginning to be explored, and our understanding of how Th9 cells are regulated is still limited. Here, we show that CFS from a well-known human pathobiont, *S. aureus*, strongly promotes Th9 cell polarization *in vitro*, while the vitamin A metabolite RA dampens the Th9-stimulation induced by this bacterium. Furthermore, we demonstrate that RA changes the *S. aureus*-induced transcriptional program of Th9-polarized CD4 T-cells, downregulates Th9-driving factors known to act at transcriptional, post-transcriptional, and protein levels, and alters the expression of genes involved in atopic diseases, possibly attenuating the allergy-inducing potential of *S. aureus*.

Although the staphylococcal toxin SEB has been previously described to trigger Th9 expansion in a mouse cancer model [23], and an association between methicillin-resistant *S. aureus* (MRSA) pneumonia and Th9 cells has recently been found [37, 45], we are the first to demonstrate a direct enhancement of human Th9 cell polarization upon exposure to *S. aureus*-derived factors. This *S. aureus* Th9-inducing effect was strongest in whole PBMC cultures but was also clear in isolated total CD4 T-cells and naïve CD4 T-cells. A previous study by Schlapbach *et al.* showed that *S. aureus* induces IL-17 rather than IL-9 responses, which would be in contrast with our results [46]. However, a different experimental procedure and the use of whole heat-killed bacteria as a treatment instead of bacterial CFS (the secretome) could explain this divergence. Our findings suggest a major role for the *S. aureus*-produced toxins in the promotion of Th9 polarization, given that both purified SEA and TSST-1 showed potent Th9-enhancing effects. This was further supported by the fact that another staphylococcal strain unable to secrete toxins, namely *S. epidermidis*, had no effect on Th9 polarization in our model. Importantly, besides being the strongest known T-cell mitogens, *S. aureus* toxins have been associated with an increased risk of allergic sensitization and disease severity, especially in allergies affecting the airways [47]. The exact mechanism by which toxins trigger allergic inflammation is not yet fully understood, but we here propose a role for Th9 cells, which should be further explored in clinical settings. Besides the toxins, we cannot exclude the involvement of other components of the *S. aureus*-CFS in the Th9-promoting effect. For instance, serine protease-like (Spl) proteins, also produced by *S. aureus* and shown to contribute to allergy development [48, 49], may be implicated and warrant future detailed examination.

Moreover, our data expand the knowledge about the immunomodulatory potential of RA on Th9 cell polarization in several ways. We show that RA's suppressive effect is still visible when a potent Th9-enhancer such as *S. aureus* is present, and we prove that this dampening occurs at the cellular, protein, and transcriptional levels. In fact, RA not only dampened the Th9 cell frequency and IL-9 secretion but also altered the *S. aureus*-induced gene expression of the Th9-polarized cells. While *S. aureus*-derived compounds substantially increased the levels of genes encoding key Th9-driving factors, such as IRF4, FOXO1, BACH2, STAT5, miR-155, and TNFRSF4 [9, 11, 50, 51, 37, 38], addition of RA led to a reduction of their expression. Notably, these factors favor the development of Th9 cells through different modes of action, being transcriptional regulators (IRF4, FOXO1, BACH2, and STAT5), microRNAs (miR-155), and T-cell costimulatory receptors (TNFRSF4), suggesting that the effects of the treatments on Th9 polarization are the result of multiple parallel mechanisms. Furthermore, pathways associated with T-cell activation and inflammatory responses were downregulated by RA, which could be expected given the general tolerogenic properties of this metabolite [25]. Interestingly, we found that the expression of the vitamin D receptor gene (*VDR*) was significantly enhanced by the combined *S. aureus*-CFS and RA treatment. Knowing that calcitriol, or vitamin D_3, has earlier been shown to have an inhibitory effect on Th9 cell differentiation [52], we could hypothesize that RA activates calcitriol signaling to concertedly regulate Th9 polarization.

From our data, we cannot draw any conclusions about the mechanism underlying RA's ability to dampen the Th9 polarization promoted by *S. aureus*. However, the enhanced expression of RA receptors upon staphylococcal treatment could indicate that the *S. aureus*-CFS makes CD4 T-cells more susceptible to the RA present in the environment and consequently more susceptible to RA's Th9-suppressive capacity. Whether this implies that RA is uniquely able to negate the *S. aureus*-induced response remains an open question. In the future, it would be relevant to explore whether other dietary metabolites previously proposed to have immunomodulatory effects, such as short-chain fatty acids, also possess inhibiting capacities on Th9 cells in human settings [53].

Remarkably, the combination of RA with the *S. aureus*-CFS induced a shift towards IL-5 responses in the Th9-polarized cells, both at the secretion and gene expression levels. Furthermore, the *GATA3* gene was enriched by the addition of RA, while genes encoding IL-13 and IL-4, other common type 2 cytokines, were not affected in a similar way. These results reflect the complexity of RA's influence on Th2 polarization, with different studies showing conflicting results [27]. From our transcriptome analysis, we found upregulation of the *LZTFL1* gene following treatment of the cells with *S. aureus*-CFS plus RA, as compared to *S. aureus*-CFS alone. An increased expression of *LZTFL1* has been previously reported in CD4 T-cells when exposed to RA in combination with a TCR trigger and has been strongly associated with secretion of IL-5 [54]. This might explain the enhanced IL-5 levels we detected in our experimental setup, in which the *S. aureus*-derived factors likely served as the additional TCR stimulants. However, future investigations should elucidate the biological relevance of RA-promoted IL-5 in this particular Th9-polarizing environment.

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Aside from the evident effects on Th9-related factors, *S. aureus*-CFS also had a broader impact on allergy-associated genes. Upon staphylococcal treatment, the Th9-polarized cells highly expressed *IL3*, which is essential for induction of eosinophil differentiation and promotes survival, migration, and activation of this granulocyte type [40]. The genes encoding IL-31 and IL-24, both implicated in the pathogenesis of multiple atopic disorders, including lung and skin allergies [41, 43], were also upregulated. Although these cytokines have not been described as produced by Th9 but rather by Th2 cells, the fact that they were enhanced under Th9-polarizing conditions strengthens the hypothesis of a high plasticity between Th2 and Th9 cells and shows how heterogeneous responses from the Th9 subset are. Importantly, the expression of all the aforementioned factors, with the addition of the IL-21 and histamine receptor encoding genes, was dampened when combining RA with the *S. aureus*-CFS. This observation is in line with studies indicating a protective effect of RA in allergy and asthma [55, 56]. A more comprehensive investigation using *in vivo* experiments will help to clarify the outcome of staphylococcal and RA Th9-induction and regulation, respectively.

We also recognize that our study has some weaknesses and raises questions that remain to be addressed. Besides the fact that we used an *in vitro* system, we investigated the influence of microbial and dietary compounds on Th9 polarization in blood-derived CD4 T-cells, which may not fully resemble CD4 T-cells at mucosal sites [57]. In addition, we were limited in the number of donors included in the RNA-Seq analysis, which affected the statistical power of our results, as well as the depth of the conclusions we could draw. Next, it would also be of value to study the modulation of Th9 cells in allergic individuals and whether this differs from a healthy context.

In conclusion, we show that *S. aureus*-derived factors strongly enhance Th9 cell polarization, while RA has the capacity to dampen this effect, even at a transcriptional level. These results highlight the importance of microbial and dietary metabolites present in the surrounding milieu during human CD4 T-cell polarization and propose novel possible mechanisms of Th9 cell regulation, which may be further explored for clinical purposes in diseases such as allergy and asthma.

Materials and methods

Human subjects and isolation of peripheral blood mononuclear cells

Peripheral blood samples were obtained from healthy, anonymous volunteers donating blood (ages 18–65). Given that the individuals could not be traced back, no ethical permit from the Swedish ethical review authority was needed for this project.

The venous blood samples were resuspended in RPMI-1640 supplemented with 20 mM HEPES (both from HyClone Laboratories, Inc., Logan, UT, USA), and peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque gradient centrifugation (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Cells were washed and then either frozen in freezing medium containing 40% RPMI-1640, 50% fetal calf serum (FCS), and 10% dimethyl sulfoxide (both Sigma Aldrich, St Louis, MO, USA), and stored in liquid nitrogen, or used fresh (as described below).

Isolation of T-cell subsets and monocytes

Freshly isolated PBMCs were used to enrich total and naïve CD4 T-cells, as well as monocytes. Total CD4 T-cells were separated using the EasySep Human CD4^{+} T-cell isolation Kit, naïve CD4 T-cells were separated using the EasySep Human Naïve CD4+ T-cell Kit II, and monocytes were separated using the EasySep Human Monocyte Enrichment Kit (all STEMCELL Technologies, Vancouver, BC, Canada). Briefly, 5 x 10^{7} PBMCs/mL were resuspended in PBS containing 2% FCS and 1 mM EDTA (Invitrogen, Grand Island, NY, USA) and then incubated with an antibody cocktail, followed by the addition of magnetic particles. The cells of interest were recovered by negative selection using an EasySep magnet and resuspended in a cell culture medium. The purity of the obtained naïve and total CD4^{+} T-cells was assessed with flow cytometry before starting the cell culture and resulted to be of 98–99%.

Processing and *in vitro* stimulation and polarization of PBMCs and isolated T-cell subsets

Frozen PBMCs were thawed, washed with RPMI-1640, counted, and assessed for viability using Trypan Blue. Cells were diluted to a concentration of 1×10^{6} PBMCs/mL in cell culture medium, containing RPMI-1640 supplemented with 20 mM HEPES, 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (all from HyClone Laboratories), and seeded at 500,000 cells/well in flat-bottom 48-well plates.

The isolated T-cell subsets and monocytes were counted, assessed for viability using Trypan Blue, and diluted to a concentration of 0.5×10^{6} cells/mL in cell culture medium. The total CD4 T-cells and naïve CD4 T-cells were seeded at 100.000 cells/well while autologous monocytes, used for T-cell support, were added at 20,000 cells/well in flat-bottom 96-well plates. For the RNA sequencing (RNA-Seq) experiments, 500,000 isolated total CD4 T cells combined with 100,000 autologous monocytes were seeded per well in flat-bottom 48-well plates. At the end of the cultures, monocytes were rarely present among the cells subjected to further analyses (Fig. S1A).

Following 1 h rest at 37°C and 5% CO_2, different treatments were added to the PBMCs or isolated CD4 T-cell cultures: 2.5% *S. aureus* 161:2 cell-free supernatant (CFS) (kind gift from Åsa Rosengren, The National Food Agency, Uppsala, Sweden), 2.5% *S. epidermidis* KX293A1 CFS (own isolate, unpublished), 20 ng/mL of the staphylococcal toxins TSST-1 (Toxin Technology Inc, Sarasota, FL, USA) and SEA (kindly provided by Jenny Schelin and Karin Lindkvist, Lund University, Sweden), 2 μg/mL (except from

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Table 1. Schematic overview of the flow cytometry antibodies used

Panel	Marker	Fluorochrome	Clone	Staining	Company
Th9	CD4	APC-H7	RPA-T4	surface	BD Biosciences
	CD14	FITC	M5E2	surface	BD Biosciences
	GATA3	BV421	16E10A23	intracellular	BioLegend
	FoxP3	PE	259D/C7	intracellular	BD Biosciences
	IRF4	PE- Cy7	IRF4.3E4	intracellular	BioLegend
	IL-9	AF647	MH9A4	intracellular	BioLegend
Proliferation	CD4	PerCP Cy5.5	SK3	surface	BioLegend
Activation	CD4	FITC	RPA-T4	surface	BD Biosciences
	CD69	PerCP	L78	surface	BD Biosciences
Purity	CD3	APC-H7	SK7	surface	BD Biosciences
	CD4	PE	RPA-T4	surface	BioLegend
	CCR7	BV421	G043H7	surface	BioLegend
	CD45RA	PerCP Cy5.5	HI100	surface	BioLegend
	CD45RO	FITC	UCHL1	surface	BD Biosciences

the dosage experiment, Fig. 2A) all-trans-RA (Sigma–Aldrich), 1 μM of the pan-RAR inverse agonist BMS493 (Sigma–Aldrich), or culture medium as control. For some experiments (Figs. 2–5, and Figs. S3–S5), the *S. aureus*-CFS and RA treatments were combined at the aforementioned final concentrations. Following 24 h incubation, cells were washed and resuspended in fresh culture medium. Furthermore, CD3:CD28 Dynabeads (Thermo Fisher Scientific, Waltham, MA, USA) at a 2:1 cell:bead ratio were added, with or without the following recombinant human (rh) cytokines (all from PeproTech Inc., Rocky Hill, USA): 50 ng/mL rhIL-12, 50 ng/mL rhIL-4, and 10 ng/mL rhTGF-β1. For Th9 cell polarization, performed in all experiments, a combination of rhIL-4 and rhTGF-β1 was used. In addition, 2 ng/mL rhIL-2 was added to the isolated total and naïve CD4 T-cell cultures. Except for the kinetics experiment (Fig. S1B and C), cells were incubated with beads and polarizing cytokines for 72 h at 37°C and 5% CO_2. In the last 4 h, a Protein Transport Inhibitor containing Monensin (BD Biosciences, San Jose, CA, USA) was also added. After incubation, cell culture supernatants were collected and stored at −20°C for downstream applications, while cells were processed for flow cytometry analysis.

Generation of bacterial cell-free supernatants

Both *S. aureus* 161:2 and *S. epidermidis* KX293A1 were grown in BHI broth (Merck, Darmstadt, Germany) for 72 h at 37°C as still culture. The bacteria were then centrifuged at 3400 × *g* and the supernatants were separated from the pellet. After that, the supernatants were sterile-filtered (0.2 μm) and stored at −20°C until used.

Flow cytometry

For all flow cytometry analyses, the cells were transferred to 96-well V-shaped staining plates and washed with PBS. Cells were stained with LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Life Technologies, Carlsbad, CA, USA) diluted in PBS, and cell surface Fc receptors were blocked using 10% human serum in FACS wash buffer, consisting of PBS with 0.1% BSA (Roche Diagnostics GmbH, Mannheim, Germany), and 0.1% EDTA (Invitrogen). Subsequently, cells were surface-stained with different combinations of antibodies in FACS wash buffer, see Table 1. After extracellular staining, cells were washed and either fixed with 4% paraformaldehyde (Purity, Activation, and Proliferation panels) or fixed and permeabilized with the True-Nuclear Transcription Factor Buffer Set (Th9 panel) (BioLegend, San Diego, CA, USA), according to the manufacturer's instructions. Intracellular blocking was performed using 10% human serum, and then cells were stained intracellularly with antibodies diluted in permeabilization buffer, see Table 1. The stained cells were washed, resuspended in FACS wash buffer, and analyzed with the FACSVerse instrument using the FACSuite software (both BD Biosciences). Th9 cells were defined as IL-9$^+$ IRF4$^+$ cells among CD4$^+$ cells (for a complete gating strategy, see Fig. S1A). Fluorescence-minus-one (FMO) and isotype controls were used for gating.

To analyze cell proliferation, PBMCs were stained with the CellTrace™ Violet Cell proliferation kit (Invitrogen) according to the manufacturer's instructions. Once labeled, the cells were treated and polarized according to the protocol described above.

ELISA

Sandwich ELISA kits for IL-9 (R&D systems Inc, Minneapolis, MN, USA), IL-5, IL-13, and IFN-γ (all from Mabtech AB, Nacka, Sweden) were used to measure cytokine concentrations in cell culture supernatants. All ELISAs were performed following the manufacturer's instructions. The optical density was measured with a microplate reader at 450 nm (IL-9) or 405 nm (IL-5, IL-13, and IFN-γ) and analyzed with the SoftMax Pro 5.2 rev C software (Molecular Devices Corp, San Jose, CA, USA).

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RNA extraction and RNA-Seq

RNA from co-cultured CD4 T-cells and monocytes was isolated with the mirVana^{TM} miRNA Isolation kit (Ambion by Life Technologies, Carlsbad, CA, USA) following the protocol for total RNA extraction, as provided by the manufacturer. The obtained RNA was subjected to quality control with Agilent TapeStation (Agilent Technologies, Santa Clara, CA, USA). Libraries were prepared using the Illumina Stranded mRNA Prep Ligation protocol (Illumina, San Diego, CA, USA), which included the steps of mRNA isolation, cDNA synthesis, ligation of anchors, amplification, and indexing of the libraries. The yield and quality of the libraries were assessed using Qubit (Thermo Fisher Scientific, Waltham, MA, USA) and the Agilent TapeStation. After normalizing and pooling the libraries, sequencing was performed on the Illumina NextSeq 550 platform, v2 75 cycles, high output, single-end mode.

RNA-Seq data analysis

Base-calling and demultiplexing were performed using Illumina bcl2fastq (v2.20). Sequence data quality was assessed using FastQC (v0.11.8). Reads were aligned to the Ensembl GRCh38 reference genome using STAR (v2.6.1d). Counts for each gene were estimated using featureCounts (v1.5.1). The Bioconductor package DESeq2 (v1.34) was used for count normalization and donor-paired treatment group comparisons, generating $\log_2$-fold changes, Wald test *p*-values, and *p*-values adjusted for multiple testing. RStudio 1.3.5033 (RStudio Inc., Boston, MA, USA) was used to perform the downstream analyses and realize the figures, of which details are explained in the respective figure legends. Gene set enrichment analysis (GSEA) was performed to identify enriched gene sets using the Bioconductor package FGSEA and the Kyoto Encyclopedia of Genes and Genomes (KEGG) functional database.

Statistical analysis

The Wilcoxon matched-pairs signed rank test and the Friedman test followed by Dunn's multiple comparisons test were used to determine the differences between two or more differently treated groups, respectively. A p-value <0.05 was considered significant. $*p < 0.05$. $**p < 0.01$. Bar graphs display the median with 95% confidence interval (CI), while box plots show the median as the central line, with boxes covering the 25th to 75th percentile and whiskers indicating min-max values. Graphpad Prism V8-9 was used for statistical analysis and data presentation (GraphPad Software, La Jolla, CA, USA).

The PCA analyses were performed in RStudio 1.3.5033. The data were reduced into two principal components, of which the amount of variance in the data explained by each component was mentioned as a percentage on the axes. The parameters used in the PCA are mentioned in the figure legends.

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Conflict of interest: The authors declare no commercial or financial conflict of interest.

Author contributions: MvdH, SB, ESE, and IB conceptualized the study. IB and MvdH performed all experiments and analyzed the data. MZ and SS performed the initial experiments leading to this study. DB performed bioinformatics analysis and contributed to figure realization. IB, MvdH, and ESE wrote the manuscript, and all authors critically revised the manuscript.

Data and code availability: The RNA-Seq data that were generated for this study have been submitted to the Gene Expression Omnibus (GEO) and will be accessible from the date of publication through the GEO accession number GSE206288.

Ethics statement: Peripheral blood was obtained from healthy, anonymous blood donors. No ethical permit from the Swedish ethical review authority was required for this project given that the individuals could not be traced back. The participants gave an informed consent for research in general when donating the blood.

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