ALLERGY

Sodium chloride is an ionic checkpoint for human T_H2 cells and shapes the atopic skin microenvironment

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The incidence of allergic diseases has increased over the past 50 years, likely due to environmental factors. However, the nature of these factors and the mode of action by which they induce the type 2 immune deviation characteristic of atopic diseases remain unclear. It has previously been reported that dietary sodium chloride promotes the polarization of T helper 17 (T_H17) cells with implications for autoimmune diseases such as multiple sclerosis. Here, we demonstrate that sodium chloride also potently promotes T_H2 cell responses on multiple regulatory levels. Sodium chloride enhanced interleukin-4 (IL-4) and IL-13 production while suppressing interferon-γ (IFN-γ) production in memory T cells. It diverted alternative T cell fates into the T_H2 cell phenotype and also induced de novo T_H2 cell polarization from naïve T cell precursors. Mechanistically, sodium chloride exerted its effects via the osmosensitive transcription factor NFAT5 and the kinase SGK-1, which regulated T_H2 signature cytokines and master transcription factors in hyperosmolar salt conditions. The skin of patients suffering from atopic dermatitis contained elevated sodium compared to nonlesional atopic and healthy skin. These results suggest that sodium chloride represents a so far overlooked cutaneous microenvironmental checkpoint in atopic dermatitis that can induce T_H2 cell responses, the orchestrators of atopic diseases.

INTRODUCTION

Naïve T helper (T_H) cells can differentiate into functionally diverse subsets of effector T cells that are tailored to specific antimicrobial responses. They respond to polarizing cues such as T cell receptor (TCR) signal strength, costimulation, and cytokine signaling to differentiate into distinct T cell subsets (*1*). Still, much remains unknown with respect to how signals are recognized and integrated to determine cell fate and function. Microenvironmental factors in the tissues are thought to provide additional cues that drive T_H cell differentiation. Recently, ionic checkpoints such as sodium chloride (NaCl) and potassium have been demonstrated to modulate T cell responses. In particular, NaCl has been shown to boost T_H17 cell differentiation from naïve T cell precursors with implications for the pathogenesis of multiple sclerosis under high-salt diet conditions (*2*–*4*). Moreover, elevated concentrations of potassium following tumor cell necrosis have been shown to provide a tumor-permissive microenvironment by paralyzing cytotoxic T cell functions (*5*). Therefore, the ionic composition of the microenvironment represents a relatively unexplored determinant of T cell polarization and T cell effector functionalities.

Epidemiological studies provide robust support for a rapid increase in the incidence of allergic and autoimmune diseases over the past 50 years. This is thought to be due to environmental nongenetic changes of yet unclear origin (*6*). More than 100 years ago, a renowned pediatric reference book claimed the improvement of atopic dermatitis upon dietary salt restriction based on clinical observations but without any mechanistic evidence (*7*). Considering the steady increase in the dietary consumption of processed food and thus of NaCl over the past 50 years, we set out to investigate a potential link between NaCl-mediated ionic signaling and enhanced T_H2 cell responses, which are the culprits in the pathogenesis of allergic diseases. Our data demonstrate the existence of an ionic determinant of T_H2 immunity, which challenges the current cytokine-focused concept of T cell polarization and proposes an alternative target for type 2 immunity in allergic diseases.

RESULTS

NaCl enhances T_H2 cell effector functions

Recent reports have demonstrated a strong impact of NaCl on increasing T_H17 cell differentiation from naïve T cell precursors in polarizing cytokine conditions (*2*, *3*). However, NaCl is particularly enriched in peripheral barrier tissues such as the skin, which naïve T cells do not enter during their regular recirculation route (*8*). Therefore, we sought to determine whether NaCl could also regulate T cell responses on the memory and effector T cell level, which would support a role of ionic signals for in situ immunomodulation.

We purified $CD4^+$ $CD45RA^-$ memory T cells from the blood of healthy adult donors and analyzed interleukin-17 (IL-17) expression on the single-cell level after expansion with CD3 and CD28 antibodies in the absence (low NaCl) or presence (high NaCl) of additional 50 mM NaCl, which reflects physiological NaCl concentrations in peripheral blood and skin, respectively (*9*). Addition of NaCl beyond 50 mM decreased cell viability and resulted in cell death (fig. S1).

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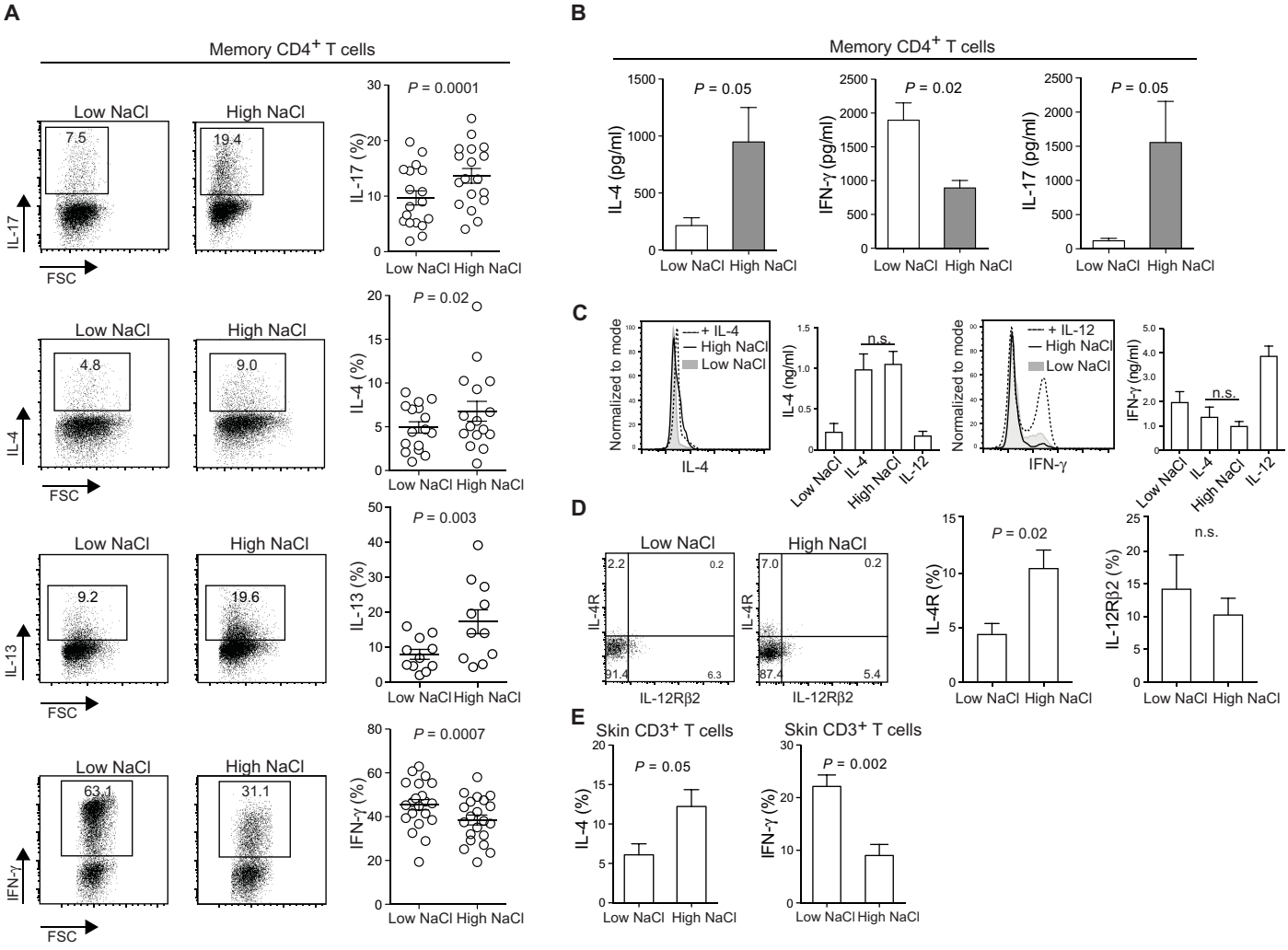

Fig. 1. NaCl enhances T_H2 and suppresses T_H1 cell responses in memory T cells. (**A** to **D**) Human memory T_H cells were sorted from fresh peripheral blood mononuclear cells (PBMCs) as $CD4^+CD14^-CD45RA^-$ T cells by flow cytometry and stimulated for a total culture period of 5 days in the presence (high) or absence (low) of additional 50 mM NaCl with anti-CD3 and anti-CD28 monoclonal antibody (mAb) for 48 hours. (A) Intracellular staining and flow cytometry [fluorescence-activated cell sorter (FACS)] on day 5 after phorbol 12-myristate 13-acetate (PMA) and ionomycin restimulation. FACS staining of an individual experiment (left) and cumulative data is shown, with each circle representing one donor (IL-17, $n = 18$; IL-4, $n = 17$; IL-13, $n = 11$; IFN-γ, $n = 22$). (B) ELISA of cell culture supernatants analyzed on day 5 ($n = 3$). (C) Flow cytometry and ELISA of T cells stimulated as in (A) and (B) and treated in IL-4 or IL-12 cytokine microenvironments (FACS panels are representative of three experiments; ELISA, $n = 3$). $P = 0.007$ (IL-4) and $P = 0.003$ (IFN-γ), Kruskal-Wallis test. Dunn's multiple comparisons post hoc test was performed for comparisons between IL-4 and high NaCl. (D) IL-12RB expression as shown by FACS. One representative experiment and cumulative data ($n = 5$) are shown. (**E**) Skin $CD3^+$ T cells obtained after abdominoplasties were sorted and characterized by flow cytometry according to fig. S4 and stimulated and analyzed as in (A) ($n = 3$). Paired Student's *t* tests were used for comparisons between two groups. n.s., not significant.

NaCl strongly enhanced IL-17 expression in memory T_H cells in the absence of exogenous polarizing cytokines (Fig. 1A). In line with a general enforcement of the T_H17 cell signature, it also increased IL-22, a cytokine involved in the repair of barrier tissues, as well as other T_H17 cell–associated signature molecules such as the master transcription factor ROR-γt, the chemokine receptor CCR6, and the antimicrobial T_H17 cytokine IL-26 (fig. S2) (*10*–*12*). Memory T cells display a complex cytokine expression pattern. We therefore extended our analysis to other T_H cell lineage–defining cytokines. Unexpectedly, we observed up-regulation of the T_H2 signature cytokine IL-4 by both intracellular cytokine staining and enzyme-linked immunosorbent assay (ELISA) even in the presence of IL-4–neutralizing antibodies (Fig. 1, A and B, and figs. S3 and S4). The T_H2 signature was corroborated by IL-13 up-regulation (Fig. 1A). Interferon-γ (IFN-γ) expression was down-regulated in accordance with the reciprocal regulation of T_H2 and T_H1 pathways. This T_H2 bias was further supported by the up-regulation of IL-4, IL-13, and IL-5 and down-regulation of IFN-γ in T_H cell clones upon restimulation in high NaCl conditions (fig. S5). NaCl acted as an equally efficient signal for T_H2 cytokine up-regulation and IFN-γ suppression as exogenous IL-4 (Fig. 1C), which has so far been considered to be the principal T_H2-polarizing factor (*13*). NaCl also up-regulated IL-4R expression on memory T_H cells, which supports a feedback amplification of the T_H2 axis (Fig. 1D). IL-12Rβ2 expression was unaffected. The T_H2-promoting effect was restricted to ionic osmolytes containing sodium and was strongest for NaCl among all tested tonicity signals. A T_H2 skewing could not be induced by nonionic

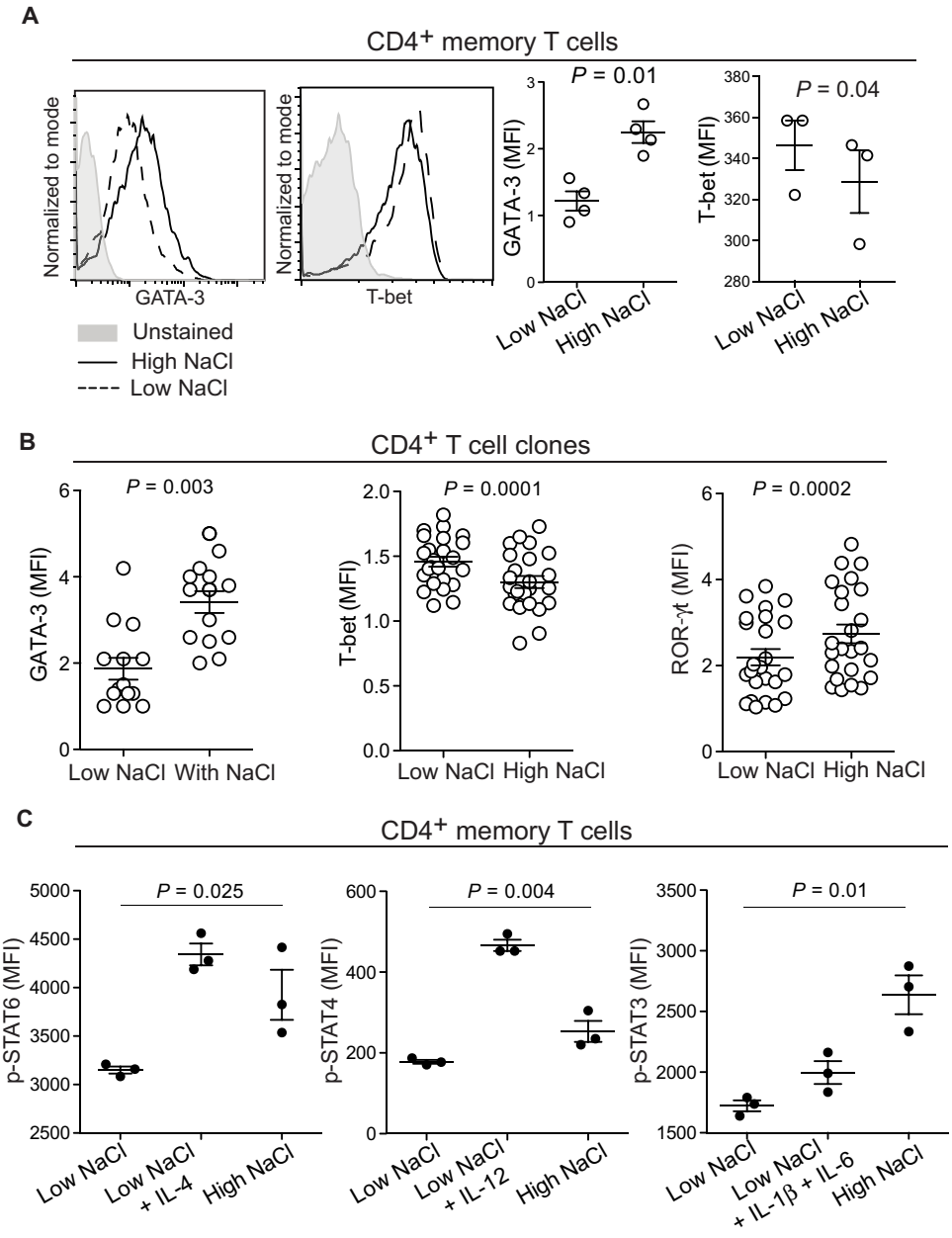

Fig. 2. NaCl induces the transcriptional activation of T_H2 and suppression of T_H1 programs. (**A**) Human memory T cells were stimulated with anti-CD3 and anti-CD28 mAb for 48 hours of a 5-day culture period in the presence (high) or absence (low) of additional 50 mM NaCl before intracellular staining for transcription factors on day 5. Representative flow cytometry analyses (left) and cumulative data (right) are shown, with each circle indicating one donor and experiment (GATA-3, $n = 4$; T-bet, $n = 3$). MFI, mean fluorescence intensity. (**B**) T cell clones were generated from $CD4^+$ $CD45RA^-$ memory T cells during a 14-day culture period with irradiated allogeneic feeder cells and phytohemagglutinin and were restimulated and analyzed as in (A). T cell clones were randomly selected from growing cultures for restimulation experiments. Each circle represents an individual T cell clone (GATA-3, $n = 14$; T-bet, $n = 24$; ROR-γt, $n = 24$). Paired Student's t test was used for comparison between two groups (A and B). (**C**) Memory T cells were stimulated as in (A) and (B) in low or high NaCl conditions or in the presence of recombinant polarizing cytokines in low NaCl conditions for 5 days before restimulation for 30 min with IL-4, IL-12, and IL-6. Phosphorylation of STAT molecules was assessed after intracellular staining and flow cytometry ($n = 3$). $P = 0.025$ (p-STAT6), $P = 0.004$ (p-STAT4), and $P = 0.01$ (p-STAT3), Kruskal-Wallis test.

osmolytes such as urea or mannitol, which were tested at various concentrations (fig. S6, A and B).

We also extended our investigations on the impact of NaCl signals to the CD8 T cell compartment. IL-17 was up-regulated in CD8 memory T cells under elevated NaCl conditions, although to a much lower extent than in CD4 memory T cells. IL-4 production, however, was not increased in CD8 T cells in contrast to CD4 T cells (fig. S7).

We next tested the response of T cells isolated from healthy human skin to restimulation with NaCl. Peripheral tissues contain heterogeneous memory T cell populations, which differ from those in the blood (*14*). Their functional plasticity and response to immunomodulatory factors remain poorly defined. Skin-resident T cells are particularly prone to exposure to ionic signals exerted by fluctuating concentrations of NaCl, because the skin is known to act as a reservoir for excess dietary NaCl (*15*). To characterize the identity of skin T cells, we phenotyped them according to their differential expression of CD69 and CD103, as both markers correlate with tissue residency (*16*). This confirmed the heterogeneous composition of skin memory T cells, which is distinct from memory T cell subsets in the blood (fig. S8). We found robust IL-4 up-regulation with concomitant IFN-γ down-regulation in skin-resident T cells upon stimulation with NaCl (Fig. 1E). In addition, we also observed up-regulation of the skin-homing chemokine receptor CCR8 by NaCl, which correlates with higher IL-4 and lower IFN-γ expression (fig. S9, A and B), as well as with skin T cell residency (*17*, *18*). Therefore, NaCl also exerts T_H2-promoting effects on cutaneous memory T cell subsets, which reside at sites of relatively elevated NaCl concentrations in vivo. Overall, these findings demonstrate that ionic signals exerted by NaCl can directly alter human memory T cell functions

in the absence of exogenous cytokine signals, with potent T_H2-promoting functions that may match the T_H2-polarizing potential of IL-4.

NaCl induces transcriptional activation of the T_H2 program

To further corroborate that NaCl can promote the T_H2 identity, we analyzed the expression of master transcription factors after T cell stimulation in the presence of increased NaCl concentrations (Fig. 2). GATA-3, the master transcription factor of T_H2 cells, was highly up-regulated in memory T cells upon TCR stimulation in the presence of additional NaCl, whereas T-bet, the master transcription factor of T_H1 cells, was down-regulated (Fig. 2A). We also generated T cell clones to avoid the selective outgrowth of undefined T cell subpopulations and subjected each clone individually to restimulation in the presence or absence of additional NaCl. GATA-3 was highly up-regulated in memory T cell clones upon NaCl stimulation, whereas T-bet was down-regulated, again supporting the T_H2-promoting role of NaCl (Fig. 2B). In accordance with higher IL-17 expression (Fig. 1A and fig. S5B), ROR-γt expression was also enhanced upon NaCl stimulation (Fig. 2B).

Signal transducers and activators of transcription proteins (STATs) have crucial roles in transmitting cytokine signals and in specifying T_H cell polarization (*19*). For that reason, we investigated the effect of NaCl on lineage-specifying STAT molecules (Fig. 2C). Memory T_H cells were stimulated polyclonally with anti-CD3 and anti-CD28 in the presence or absence of additional NaCl for 5 days or with the polarizing cytokines IL-4, IL-12, or IL-6 and IL-1β. On day 5, T cells were briefly restimulated with IL-4, IL-12, or IL-6 for 30 min to induce STAT6, STAT4, or STAT3 phosphorylation, respectively. T cells that were stimulated in the presence of NaCl displayed stronger STAT6 phosphorylation upon IL-4 exposure than control T cells cultured in the absence of exogenous NaCl. STAT4 phosphorylation, however, was unaffected by NaCl stimulation. STAT3 was strongly up-regulated by NaCl in memory T_H cells (Fig. 2C). Together, these results argue that NaCl promotes the T_H2 and T_H17 cell signature also on the level of the transcriptional STAT gatekeepers.

To provide a more global view on the T_H cell polarization bias induced by NaCl, we performed next-generation mRNA sequencing to analyze the gene expression changes of memory T_H cells stimulated in the presence of low versus high NaCl concentrations. Gene set enrichment analysis of the differentially expressed genes using T_H1- and T_H2-associated gene sets corroborated the strong NaCl-induced T_H2 bias also on a transcriptome-wide level (fig. S10).

NaCl skews distinct T_H cell subsets to acquire T_H2 properties

We next wanted to determine whether distinct T_H cell fates displayed plasticity to adopt the T_H2 phenotype upon exposure to NaCl. We therefore isolated fully differentiated human T_H1, T_H2, and T_H17 cell subsets ex vivo from peripheral blood according to their differential expression of chemokine receptor surface markers, which, according to our previous work, reliably identifies the respective T_H cell lineages (*11*, *20*). After 5 days of stimulation with CD3 and CD28 mAb, the T cell subsets maintained their characteristic signature cytokine profiles as expected. In the presence of additional NaCl, T_H2 cells further up-regulated IL-4, whereas their low baseline IFN-γ production was further down-regulated, promoting their T_H2 identity. T_H2 cells also up-regulated IL-17, although overall IL-17 expression remained relatively low. T_H1 cells did not alter their low IL-4 or IL-17 production upon restimulation with NaCl but substantially down-regulated IFN-γ. T_H17 cells also increased IL-4 production, while down-regulating IFN-γ and increasing IL-17 (Fig. 3A). To corroborate plasticity as the underlying process of T_H2 phenotype acquisition, we generated T cell clones and subjected individual clones to restimulation in the presence of additional NaCl. This approach confirmed that homogenous clonal T cell populations could acquire or increase IL-4 production while at the same time expressing decreased IFN-γ and increased IL-17 on the clonal level (Fig. 3B). We also observed that IL-4–negative T cell clones could acquire the ability to produce IL-4 in response to restimulation with NaCl (Fig. 3, B and C). This de novo acquisition of cytokine expression also applied to individual IL-17–negative T cell clones, which up-regulated IL-17 expression upon restimulation with NaCl (Fig. 3C).

Because memory T cells are long-lived and can be reactivated in changing microenvironmental contexts by their cognate antigens, we investigated the stability of NaCl-induced functional changes in human T_H cells. To this end, we stimulated memory T_H cells in high NaCl conditions for 5 days before transferring them into low NaCl conditions for another 5 days of anti-CD3 and anti-CD28 restimulation and vice versa. The data demonstrate that the up-regulation of IL-4 is dependent on the presence of high NaCl concentrations because increased IL-4 expression could not be maintained upon restimulation in low NaCl conditions. Repetitive restimulation compromised overall IL-4 production. This was partially counteracted if restimulation occurred in high NaCl conditions (Fig. 3D).

It has previously been shown that epigenetic mechanisms regulate the stability and plasticity of T cell programs (*21*). We therefore determined whether ionic signaling by NaCl could epigenetically imprint the T_H2 program in human T_H cells. To this end, we analyzed DNA methylation signatures of selected regulatory regions at several candidate loci in response to NaCl using targeted ultradeep sequencing of bisulfite-converted DNA. Our data suggest that the increase in T_H2 effector functions is not exerted by methylation changes at the analyzed regions (Fig. 3E and table S1). Together, NaCl-mediated ionic signaling most likely preserved the flexibility of T cell effector functions and provided an acute adaptation to the microenvironment, in line with the functional re-adaptation to low NaCl conditions shown in the restimulation experiments (Fig. 3D).

NaCl promotes T_H2 cell differentiation from naïve T cell precursors in the absence of polarizing cytokines

Given the T_H2-promoting effects of NaCl on memory and effector T cell subpopulations, we next sought to determine whether it could exert direct polarizing effects on the naïve T cell compartment. Although NaCl has previously been demonstrated to promote human T_H17 cell differentiation from naïve T cell precursors, this only occurred in the presence of exogenous polarizing cytokines (*2*). We therefore isolated human naïve T cells to high purity and stimulated them in the presence or absence of additional NaCl. We observed a significant increase of IL-4 and IL-13 production upon stimulation with NaCl in the absence of polarizing cytokines (Fig. 4A). This also occurred in the presence of IL-4–neutralizing antibodies (fig. S11). As expected, IFN-γ could be induced by polyclonal stimulation. It was suppressed, instead, in the presence of exogenous NaCl, in line with the reciprocal T_H1-T_H2 regulation pattern. Also, IL-17–producing cells could be slightly, although significantly, induced by NaCl, even in the absence of exogenous polarizing cytokines, whereas polyclonal stimulation alone had no IL-17–promoting effect. These findings were supported by quantifying cytokine release by ELISA (Fig. 4B). Similar to memory cells, naïve cells selectively up-regulated GATA-3 expression, but not T-bet and ROR-γt, upon NaCl stimulation (Fig. 4C). Under

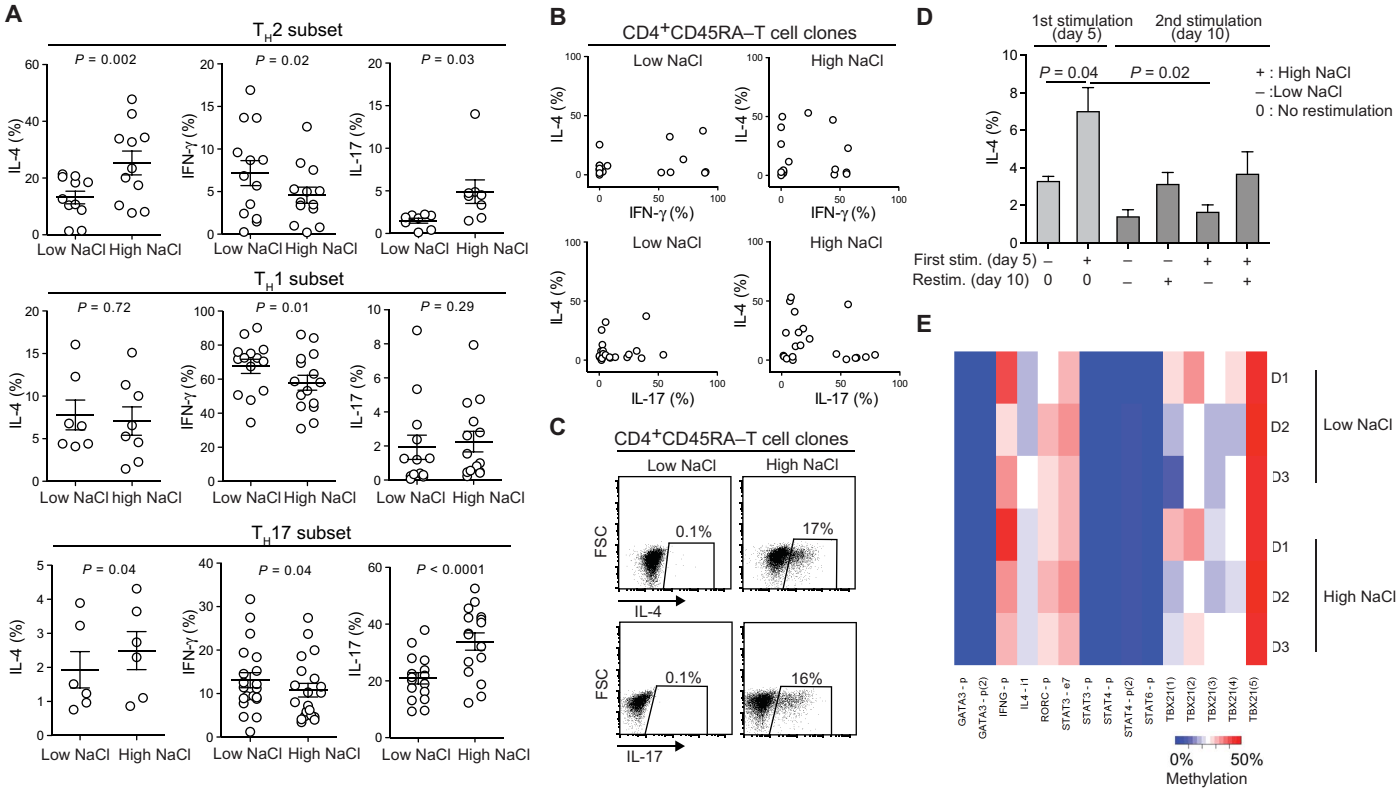

Fig. 3. NaCl reprograms distinct T_H cell subsets to acquire T_H2 properties. (**A**) Polarized human memory T_H cell subsets were sorted ex vivo according to the differential expression of chemokine receptor surface markers and restimulated in the presence (high) or absence (low) of additional 50 mM NaCl with CD3 and CD28 mAb for 48 hours of a 5-day culture period. Cytokine expression was determined by intracellular cytokine staining and flow cytometry. Paired Student's *t* test was used for comparison between two groups. (**B**) Random T cell clones that were generated from memory T cells were stimulated as in (A), and their cytokine coexpression pattern was measured in the presence of low versus high NaCl concentrations. Every circle represents a unique T cell clone. (**C**) Individual T cell clones that were generated from memory T cells were selected on the basis of being negative for IL-4 or IL-17 expression. They were restimulated in the presence of low versus high NaCl concentrations for 5 days before intracellular staining. Two individual clones from one experiment and blood donor ($n = 3$) are shown. (**D**) Intracellular cytokine straining of human $CD4^+$ $CD45RA^-$ memory T cells after stimulation as in (A). The same T cell cultures were then split and restimulated for another 5 days in low and high NaCl conditions before intracellular cytokine staining and flow cytometric analysis following PMA and ionomycin stimulation on day 10 ($n = 4$). Paired Student's *t* test was used for comparison between two groups. (**E**) Heatmap with average CpG methylation as measured by deep sequencing of bisulfite PCR amplicons for indicated regions (p, promoter; I, intron; e, exon; numbers in brackets indicate amplicons for different subregions of the respective genes; D1 to D3, donors 1 to 3; comparison of matched low versus high NaCl conditions for D1 to D3 by Student's *t* test with false discovery rate–adjusted *P* values).

T_H17-polarizing conditions, the NaCl-induced IL-4 up-regulation in human naïve T cells was abrogated (fig. S12).

To further corroborate a role for hypersalinity in the enhancement of type 2 immunity, we tested its effects on murine T cell polarization. Naïve T cells derived from the spleen and lymph nodes from C57BL/6 mice were stimulated with CD3 and CD28 mAb in the presence of distinct polarizing cytokines and cytokine-blocking antibodies that are known to reliably polarize T_H1, T_H2, T_H17, and T_{reg} (T regulatory) responses in vitro. Naïve T cell priming in the presence of additional 40 mM NaCl strongly enhanced T_H2 cell priming with recombinant IL-4 with respect to IL-4 and GATA-3 expression. The presence of additional NaCl also induced slight IL-4 and GATA-3 up-regulation in several non-T_H2 cell conditions despite the presence of IL-4 antagonistic cytokines such as IL-12 and transforming growth factor–β (TGF-β) (fig. S13, A to C). Increased IL-4 concentrations were also detected in the culture supernatant of T_H2 cells that were treated with additional NaCl (fig. S13D).

Together, these findings demonstrate that NaCl acts as a T_H2 cell–polarizing signal during the priming of naïve T cells from both humans and mice as assessed by multiple readouts. Moreover, this occurred in the absence of exogenous T_H2-polarizing cytokines such as IL-4 as well as independent of autocrine IL-4 signaling and, with mouse cells, even despite the presence of T_H2-counteracting cytokines.

SGK-1 and NFAT5 mechanistically link NaCl signaling with the T_H2 program

Having established that NaCl has considerable effects on the induction and enhancement of the T_H2 cell program, we sought to investigate the underlying molecular mechanisms. We tested whether the nuclear factor of activated T cells 5 (NFAT5), which is known to be an osmosensitive transcription factor (*22*), is induced in human memory T_H cells upon polyclonal activation in the presence of NaCl. NFAT5 was significantly up-regulated as assessed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Fig. 5A). Serum- and glucocorticoid-regulated kinase 1 (SGK-1) is also known to be regulated by tonicity signals and to be a downstream target of NFAT5 (*23*). Accordingly, short hairpin RNA (shRNA)–mediated silencing of *NFAT5* down-regulated *SGK1* transcript abundance in low and high NaCl

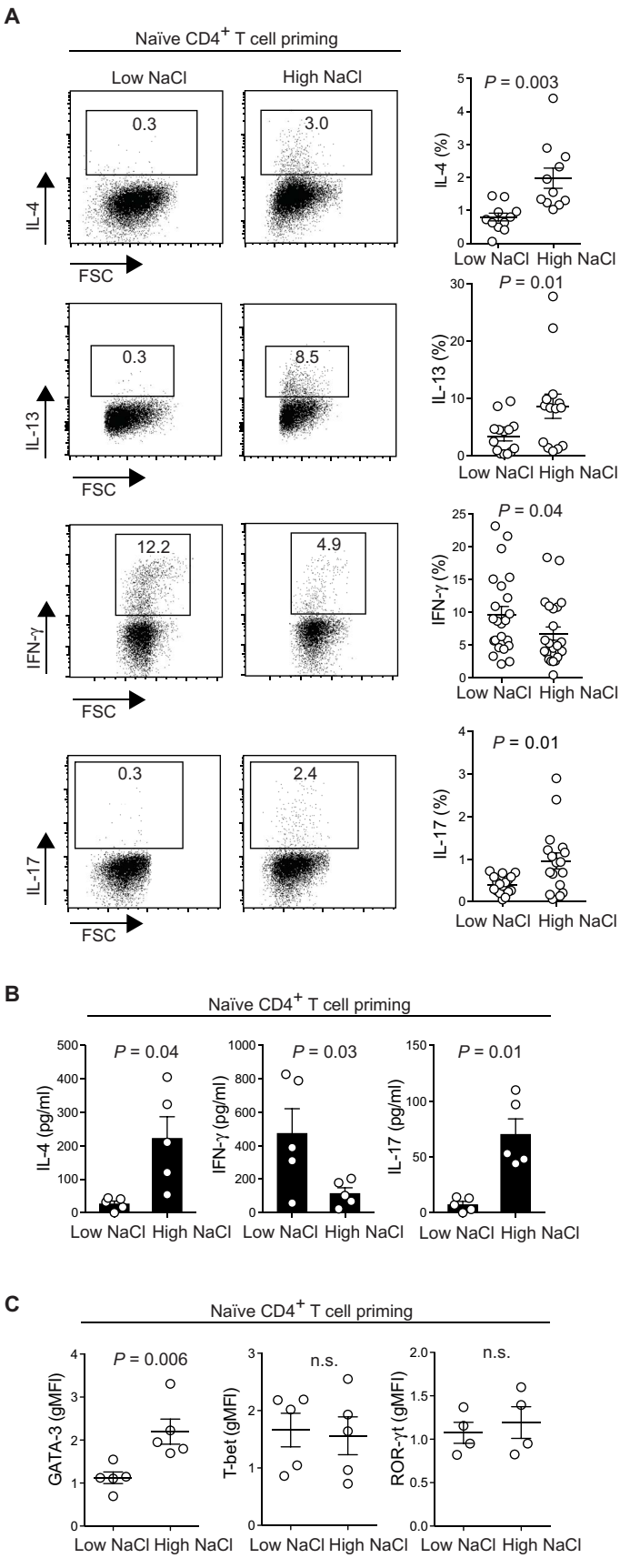

Fig. 4. NaCl promotes T_H2 cell differentiation from naïve T cell precursors in the absence of polarizing cytokines. (**A**) Cytokine expression was determined by flow cytometry after PMA and ionomycin restimulation for 5 hours. Left: Cytokine expression in one individual blood donor. Right: Cumulative data of all donors. (**B**) Supernatants from the cultures in (A) were tested by ELISA and normalized to cell numbers by counting beads. (**C**) Transcription factor expression was determined by flow cytometry after treatment as in (A) and (B). Each circle represents a separate blood donor. Paired Student's *t* test was used for comparison between two groups. gMFI, geometric MFI.

conditions, whereas silencing of *SGK1* had no impact on *NFAT5* expression (fig. S14). Similar to NFAT5, SGK-1 was more abundant in CD3- and CD28-stimulated memory T_H cells in the presence of additional NaCl (Fig. 5A). We found that these osmosensitive molecules directly control the T_H2 program in hyperosmolar conditions because shRNA-induced silencing of NFAT5 and SGK-1 abrogated NaCl-induced IL-4 up-regulation and IFN-γ suppression (Fig. 5B). This was further corroborated by GATA-3 and T-bet expression, which reached baseline expression following NFAT5 and SGK-1 silencing in hypersalinic conditions (Fig. 5C). shRNAs directed against NFAT5 and SGK-1 did not regulate IL-4 or IFN-γ production or GATA3 or TBX21 expression in low NaCl conditions (Fig. 5B and fig. S15). Together, these data demonstrate a role of osmosensitive transcription factors for the regulation of the T_H2 program in hyperosmotic tissue microenvironments.

Atopic dermatitis skin lesions contain increased sodium concentrations

Last, we aimed to identify whether NaCl is associated with the pathogenesis of allergic diseases in humans. Despite intense and long-term research efforts, it still remains elusive how the T_H2 bias is initiated in allergies (*24*). To this end, we investigated NaCl concentrations in the skin of adult patients suffering from atopic dermatitis (also known as atopic eczema; table S2), a T_H2-driven chronic inflammatory skin disease with a high prevalence and strongly increasing incidence in industrialized nations (*25*). Neutron activation analysis (NAA) allows for precise probing of element content in an organic matrix (*26*).

Lesional skin of patients suffering from moderate to severe atopic dermatitis (mean SCORing Atopic Dermatitis = 63) displayed strongly increased concentrations of sodium compared to matched nonlesional patient skin (30-fold) (Fig. 6A and table S2). We did not observe any difference in the sodium content between nonlesional skin of atopic dermatitis patients and skin from age-matched healthy donors. No sodium enrichment was detected in the inflamed skin of age-matched patients suffering from moderate to severe psoriasis (mean Psoriasis Area and Severity Index = 25), indicating that NaCl accumulation is not a general phenomenon of the inflamed skin (Fig. 6B).

DISCUSSION

Adaptive immune cells integrate cues from the tissue microenvironment that tailor their phenotype and function for antigen-specific host defense and tolerance (*27*). IL-4 has so far been considered a crucial cytokine for the final commitment and maintenance of T_H2 immunity (*28*). We demonstrate here an IL-4–independent T_H2 polarization pathway that is mediated by NaCl. NaCl was effective both

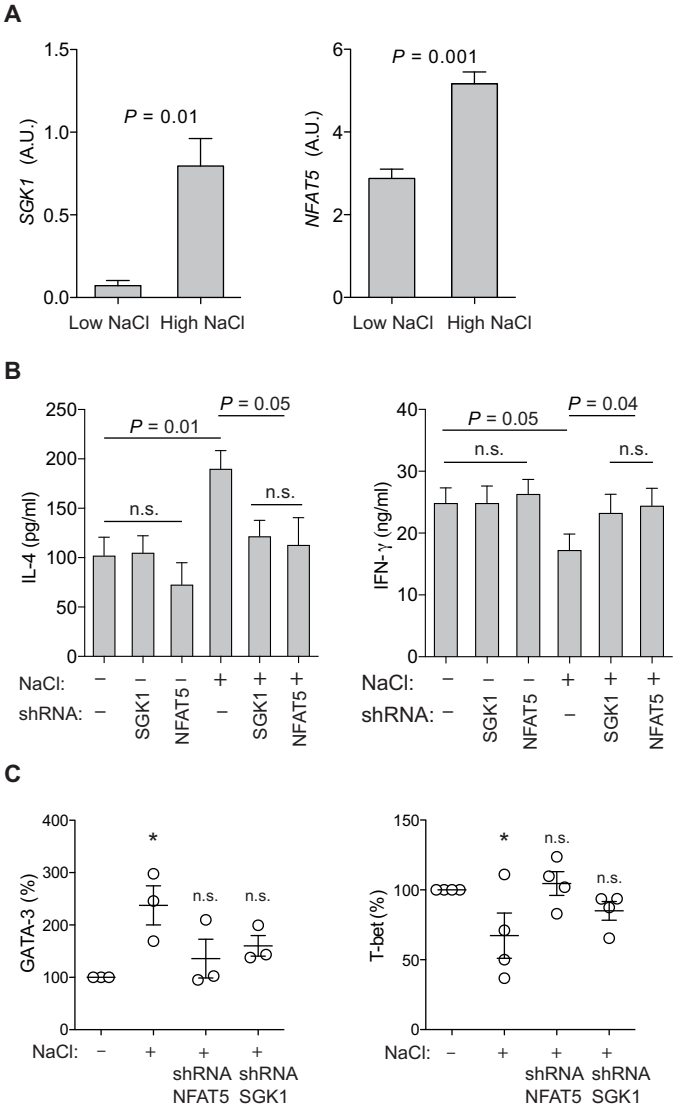

Fig. 5. SGK-1 and NFAT5 mechanistically link salt signaling with the T_H2 program. (**A**) qRT-PCR of memory T_H cells. Paired Student's *t* test was used for comparison between two groups. A.U., arbitrary units. (**B**) Cytokine production in supernatants was determined by ELISA after stimulation in low- or high-salt conditions for 5 days and shRNA-mediated silencing of SGK1 or NFAT5 (n = 3 to 6). The control conditions indicated as (–) contain scrambled shRNA. For IL-4 and IFN-γ, $P \leq 0.05$ (mixed-effects analysis for multiple comparisons). Unpaired Student's *t* test was used for comparison between two groups. (**C**) Intracellular staining and flow cytometry of the master transcription factor GATA-3 ($P \leq 0.05$, Kruskal-Wallis test) and T-bet ($P = 0.12$, Kruskal-Wallis test) after stimulation in low- or high-salt conditions for 5 days and shRNA-mediated silencing of NFAT5 and SGK1. Dunn's post hoc test was not significant (n.s.) for comparison between low NaCl conditions with scrambled shRNA and high NaCl conditions with shRNA for NFAT5 or SGK1. *$P \leq 0.05$, Dunn's post hoc test for comparison with low NaCl conditions containing scrambled shRNA.

during the naïve T cell priming phase as well as at the memory T cell stage and could also divert alternative T_H cell fates into a T_H2 phenotype. NaCl not only biased the T_H1-T_H2 dichotomy toward T_H2 cell responses on multiple regulatory levels but also promoted effector T_H17 cell responses. Our studies have identified NFAT5 and its downstream target SGK-1 as molecular regulators of the T_H2 cell program in hyperosmolar NaCl conditions. The skin of patients with atopic dermatitis, a T_H2-driven disease, was highly enriched in NaCl. Together, these results revealed NaCl as an ionic checkpoint for type 2 immunity and to be associated with the pathogenesis of atopic dermatitis. Therefore, targeting NaCl-induced tonicity signaling could serve as a therapeutic strategy for the treatment of allergic diseases.

Previous studies demonstrated that NaCl promoted the differentiation of T_H17 cells in polarizing cytokine conditions with implications for the pathogenesis of multiple sclerosis (*2*–*4*). Our data support and extend these conclusions not only on the naïve but also on the memory T cell level, even in the absence of exogenous polarizing cytokines. Although T_H2-associated regulatory factors such as GATA-3 and T_H2 effector cytokines were assessed in these previous studies as well, this occurred in T_H17-polarizing cytokine conditions, which, according to our investigations, have abrogated the ability of NaCl to directly promote T_H2 cells (*2*). This suggests that the cytokine microenvironment modulates the impact of NaCl for T cell differentiation and function.

Although our data are in accordance with the NaCl-induced support of T_H17 cell differentiation, they challenge the notion of NaCl-induced T_H17 cell pathogenicity, which requires IL-17 to be coexpressed with IFN-γ on the single-cell level (*2*, *11*, *29*, *30*). The T_H1/IFN-γ down-regulation that we demonstrate is in agreement not only with the concomitant reciprocal up-regulation of T_H2 cell responses but also with the previous observation that osmotic shrinkage of T cells by NaCl blunts IFN-γ expression (*31*). Overall, our data demonstrate considerable plasticity of T cell functions in response to NaCl with an overall skewing toward the T_H2 fate.

mTORC2 (mammalian target of rapamycin complex 2) acts as an upstream activator of SGK-1 (*32*). Our results are in accordance with previous reports that demonstrated a role for mTORC2 in the commitment to the T_H2 lineage but which did not identify which upstream signals differentially engaged the mTORC1 versus mTORC2 pathway (*33*, *34*). T cell–specific deletion of the mTORC2-specific adaptor Rictor abrogated the activation of SGK-1 (*33*), resulting in reduced allergic asthma as well as increased antitumor and antiviral immune responses in mouse models. In addition, the Wnt antagonist Dickkopf-1 promoted T_H2 cell responses via the mTOR pathway and SGK-1 in mouse models of house dust mite–induced asthma or *Leishmania major* infection (*35*). Downstream targets of SGK-1 signaling following mTORC2 activation were shown to be JunB and the long isoform of the transcription factor TCF-1 for the T_H2 versus T_H1 fate, respectively (*32*). These previous insights into the control of T_H cell fates by mTOR complexes in mice corroborate our findings in the context of hypertonic NaCl-induced T cell signaling. In particular, they propose NaCl as an upstream regulator of mTORC2 and thus of T_H2 cell differentiation.

We found highly elevated concentrations of sodium in the affected skin of atopic dermatitis patients (eczema) compared to unaffected control skin, which is in line with the T_H2-mediated pathogenesis of this chronic inflammatory disease (*36*). Previous gene profiling studies reported the up-regulation of genes encoding negatively charged glycosaminoglycans in lesional atopic skin, which can promote and thus potentially explain the accumulation of sodium ions in the skin by non-covalent binding (*15*, *37*, *38*). Genes involved in glycosaminoglycan deposition (i.e., β1,3-glucuronosyltransferase-I) are downstream targets of NFAT5 (*39*), which we have reported here to be the transcriptional regulator of the high-salt response.

The profound barrier dysfunction in atopic dermatitis promotes microbial dysbiosis (*40*–*42*). The atopic skin is heavily colonized by

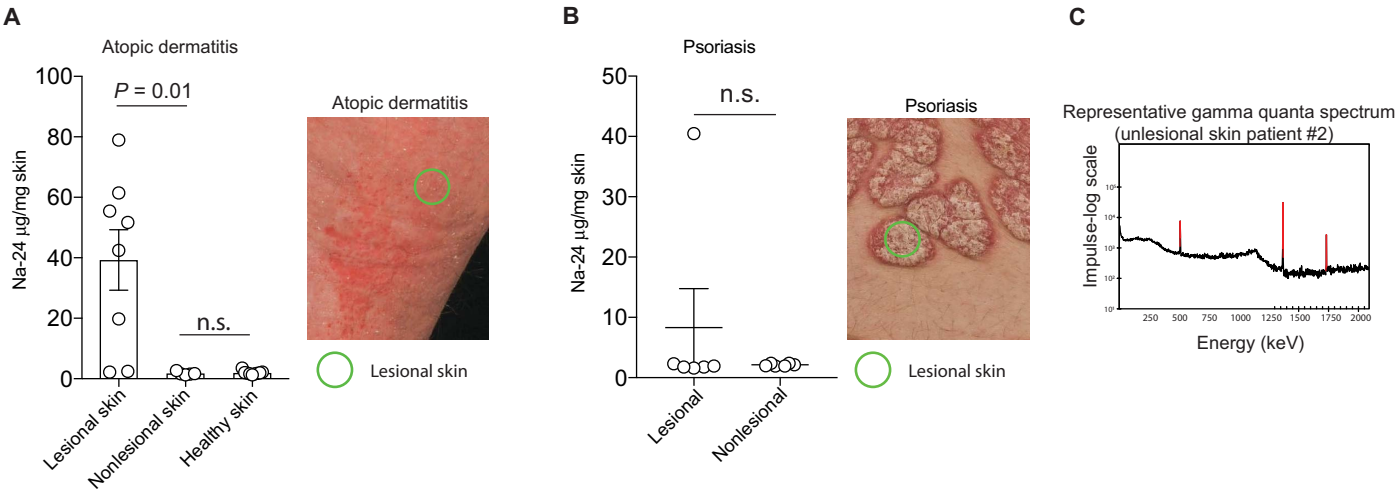

Fig. 6. The skin of atopic dermatitis patients has elevated sodium content. (**A**) NAA of 4-mm skin punch biopsies. The punch biopsies were taken from lesional and nonlesional skin of atopic dermatitis patients and of healthy controls (patients undergoing plastic surgery) (table S2, patient information). The concentration of sodium is shown (mean ± SEM). The right panel illustrates the clinical presentation of a representative patient with atopic dermatitis. (**B**) NAA from lesional and nonlesional skin of psoriasis patients. Right: Clinical presentation of a representative patient with psoriasis. (**C**) Representative spectrum of gamma quanta emission. The spectrum has been recorded after a thermal neutron irradiation with 4.3×10^{16} cm^{-2} and a counting time of 30 min 1 day after the end of exposure. Unpaired Student's *t* test was used for comparison between two groups.

Staphylococcus aureus, which correlates significantly with the severity of atopic skin inflammation, whereas skin colonization with other bacteria is profoundly reduced in atopic dermatitis (*42*, *43*). *S. aureus* is known to be tolerant to high NaCl concentrations and to outgrow other bacteria in elevated NaCl conditions (*42*, *44*, *45*). Therefore, NaCl accumulation could provide a rationale for the microbial dysbiosis of atopic dermatitis, which has so far remained enigmatic.

A limitation of the study is that our investigations have been performed with peripheral human T cells in vitro and that we have not firmly established the causality of sodium deposition in the atopic skin and T_H2-mediated disease pathogenesis in vivo. An epithelial sodium sensor, Na_x, has recently been shown to respond to perturbations of sodium homeostasis in the skin that is characterized by an atopic barrier defect (*46*). Although previous work has not investigated the direct link of Na_x with the immune system, sodium sensing has been implicated in fibroblast-associated pathogenesis of atopic dermatitis because the in vivo knockdown of Na_x in mice resulted in improvement of atopic dermatitis (*46*). Its downstream target ENaC, the major sodium channel in epithelial cells, is regulated by NFAT5 and SGK-1 (*46*–*48*), which we identified herein to regulate the T_H2 bias. In combination, these murine and our human findings mechanistically link evidence of sodium deposition in the atopic skin with its impact on the pathogenesis of atopic dermatitis via the T_H2 axis. This could finally provide a mechanistic rationale for the early observational clinical studies and therapeutic recommendations by Finkelstein (*7*) who published in his renowned pediatric reference book in 1912 that dietary salt restriction improves atopic dermatitis. Together, a reductionist model could be envisioned for the pathogenesis of atopic dermatitis, which integrates the phenomenon of NaCl deposition in the atopic skin, the NaCl-induced T_H2 bias of atopic immune deviation, and the hegemony of NaCl-resistant *S. aureus* (fig. S16).

In conclusion, our investigations have revealed NaCl as an ionic checkpoint for human type 2 immunity with a potential clinical relevance for atopic dermatitis. Future studies into the compartmentalization and dynamic regulation of NaCl and other osmolytes in distinct human tissues will have to be conducted to unravel the full repertoire of tonicity-mediated immunomodulation in health and disease.

MATERIALS AND METHODS

Study design

This is an experimental study involving human blood from healthy donors or C57BL/6 mice. We also collected fresh skin samples from randomly selected healthy donors undergoing elective abdominoplasty or skin biopsies from atopic dermatitis and psoriasis patients as well as their matched blood (table S2). The purpose of the study was to study the effect of NaCl on T cell functions by performing in vitro functional assays and to establish an association with the pathogenesis of T cell–mediated skin diseases by blinded NaCl measurements in skin samples using ex vivo NAA. Replication is indicated in the figures and legends. The ethics approval was obtained from the Institutional Review Board of the Technical University of Munich (195/15s, 491/16 S, and 146/17S) and the Charité-Universitätsmedizin Berlin (EA1/221/11). All work was carried out in accordance with the Declaration of Helsinki for experiments involving humans. Primary data are reported in data file S1.

Cell purification and sorting

PBMCs were isolated by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare). $CD4^+$ T cells were isolated from fresh PBMC by positive selection with CD4-specific microbeads (Miltenyi Biotec) using autoMACS Pro Separator. T_H cell subsets were sorted to at least 98% purity as follows: T_H1 subset, $CXCR3^+CCR4^-CCR6^-CD45RA^-CD25^-CD14^-$; T_H2 subset, $CXCR3^-CCR4^+CCR6^-CD45RA^-CD25^-CD14^-$; and T_H17 subset, $CXCR3^-CCR4^+CCR6^+CD45RA^-CD25^-CD14^-$. Memory T_H cells were isolated as $CD3^+CD14^-CD4^+CD45RA^-$ lymphocytes, and naïve T cells were isolated as $CD3^+CD14^-CD4^+CD45RA^+CD45RO^-$ $CCR7^+$ lymphocytes to a purity of over 98%. The antibodies used for sorting by flow cytometry were identical to those we have described previously (*11*, *30*). Cells were sorted with BD FACSAria III (BD Biosciences).

T cells from fresh healthy human skin (abdominoplasties) were isolated after 16-hour digestion of the epidermis and dermis with collagenase IV (0.8 mg/ml; Gibco).

Cell culture

Human T cells were cultured in RPMI 1640 medium supplemented with 2 mM glutamine, 1 % (v/v) nonessential amino acids, 1 % (v/v) sodium pyruvate, penicillin (50 U/ml), streptomycin (50 μg/ml; all from Invitrogen), and 10 % (v/v) fetal calf serum (Biochrom). Hypersalinity (+NaCl) was induced by increasing NaCl concentrations by 50 mM NaCl compared to baseline cell culture medium including supplements (NaCl, Sigma-Aldrich). In some indicated experiments, T cell cultures were performed in the presence of recombinant cytokines (IL-6, 50 ng/ml; IL-12, 10 ng/ml; IL-4, 10 ng/ml; TGF-β, 5 ng/ml; IL-1β, 10 ng/ml; IL-23, 50 ng/ml; all from R&D Systems) or neutralizing antibodies (anti–IL-4, 10 μg/ml; BD Biosciences). Naïve T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) according to standard protocols and analyzed by gating on $CFSE^{low}$ cells. T cells were stimulated with plate-bound anti-CD3 (2 μg/ml; clone TR66) and anti-CD28 (CD28.2, 2 μg/ml; both BD Biosciences) for 48 hours before transfer into uncoated wells for another 3 days for a total culture period of 5 days unless indicated otherwise in the legends. T cell clones were generated in nonpolarizing conditions as described previously following single-cell deposition with flow cytometry–assisted cell sorting or by limiting dilution (*49*).

Cytokine and transcription factor analyses

Intracellular cytokine and transcription factor staining was performed as described before (*20*). Cells were stained with anti-cytokine antibodies (IL-4, IL-13, IFN-γ, IL-17A, and IL-5, all from BioLegend) and with antibodies against transcription factors (GATA-3 and ROR-γt, eBioscience; T-bet, BioLegend) or surface markers (IL-4R, R&D Systems; IL-12Rβ2, Miltenyi Biotec; CCR8, BioLegend) and were analyzed with BD LSRFortessa (BD Biosciences), CytoFLEX (Beckman Coulter), or MACSQuant Analyzer (Miltenyi Biotec). Flow cytometry data were analyzed with FlowJo software (TreeStar) or with Cytobank (Cytobank Inc.). Cytokines in culture supernatants were measured by ELISA (R&D Systems) or by Luminex (eBioscience) according to standard protocols after restimulation of cultured T cells with PMA (50 nM; Sigma-Aldrich) and plate-bound anti-CD3 (1 μg/ml, TR66) for 8 hours or as indicated in the respective figure legends. Counting beads (CountBright Absolute Counting Beads, Thermo Fisher Scientific) were used to normalize for cell numbers if analysis of cumulative supernatants obtained from 5-day cell cultures was performed.

Gene expression analysis

The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used for complementary DNA (cDNA) synthesis according to the manufacturer's protocol. Transcripts were quantified by real-time PCR with predesigned TaqMan gene expression assays (SGK1, Hs00985033_g1; NFAT5, Hs00232437_m1; TBX21, Hs00203436_m1; GATA3, Hs00231122_m1; RORC2, Hs01076112_m1; 18S, Hs03928985) and reagents (Applied Biosystems). mRNA abundance was normalized to the amount of 18*S* ribosomal RNA and is expressed as arbitrary units (A.U.). Transcriptome-wide gene expression analysis was performed as outlined in Supplementary Materials and Methods.

Lentiviral gene silencing

Bacterial stocks containing lentiviral vectors with shRNA targeted against *SGK1* and *NFAT5* were purchased from Sigma-Aldrich. All vectors were amplified and purified using the MaxiPrep or MidiPrep Kit (Qiagen) according to the manufacturer's instructions. Lentiviral particles were generated in human embryonic kidney (HEK) 293 cells. Presorted human memory T cells (5×10^4) were activated on anti-CD3/CD28–coated plates for 12 hours before transduction with supernatants from cultures with pooled lentiviral particles against *SGK1* and *NFAT5*, without shRNA insertion or with scrambled shRNA. After 48 hours, cells were washed and selected with puromycin (Sigma-Aldrich). Gene expression was measured by quantitative real-time PCR 4 days after transduction.

Quantification of skin sodium concentrations

NAA at the Forschungsneutronenquelle Heinz Maier-Leibniz (FRM II) in Munich was used to detect chemical elements in 4-mm frozen skin punch biopsies (lesional and nonlesional). The emission of several gamma quanta of characteristic energy upon decay of isotopes was measured after neutron irradiation. The intensity of the characteristic gamma radiation allowed the determination of the activity (given in becquerels) of the emitter. The activity A at the end of the neutron exposure is connected to the number N of atoms of the mother isotope in the sample by means of the following equation:

$$A = N^*\sigma^*\phi^*\left(1 - \exp\left[-\ln(2) * t/\frac{T1}{2}\right]\right)$$

where ϕ is the thermal neutron flux density in $1/(cm^2s)$, σ is the activation cross section of the isotope in cm^2, t is the irradiation time, and $T_{1/2}$ is the half-life of the radioisotope in equal units.

Statistical analysis

Student's two-tailed paired and unpaired *t* tests as well as Kruskal-Wallis tests with Dunn's multiple comparisons post hoc tests were used for statistical comparisons between two or more groups, respectively, and their use was indicated in the respective figure legends, with error bars indicating the SEM. *P* values of 0.05 or less were considered significant. Analyses were performed using Prism 6 (GraphPad).

SUPPLEMENTARY MATERIALS

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Materials and Methods

Fig. S1. The viability of human T cells is preserved over a wide range of NaCl concentrations.

Fig. S2. T_H17 cell signature molecules are up-regulated upon stimulation of memory T_H cells with NaCl.

Fig. S3. NaCl increases memory T_H2 cell responses independently of exogenous or autocrine IL-4.

Fig. S4. NaCl induces up-regulation of IL-4, IL-17, as well as IL-4 and IL-17 double-positive T cells.

Fig. S5. NaCl enhances T_H2 and suppresses T_H1 cell responses in T_H cell clones.

Fig. S6. Comparison of different osmolytes demonstrates NaCl to be the most potent inducer of IL-4 and suppressor of IFN-γ in memory T cells.

Fig. S7. Memory CD8 T cells show stable IL-4 and IFN-γ expression upon stimulation with NaCl but up-regulate IL-17.

Fig. S8. Skin T cells are distinct from blood T cells and differ in their expression of the tissue residency markers CD69 and CD103.

Fig. S9. NaCl induces the up-regulation of the skin-homing chemokine receptor CCR8, which enriches for T_H2-associated cytokines.

Fig. S10. Transcriptome-wide enrichment of a T_H2 cell signature by NaCl.

Fig. S11. NaCl induces T_H2 cell polarization independently of exogenous or autocrine IL-4.

Fig. S12. T_H17-polarizing cytokine conditions abrogate the T_H2-promoting effect of NaCl.

Fig. S13. Mouse T cells differentiate into T_H2 cells in response to NaCl.

Fig. S14. SGK-1 is a downstream target of NFAT5.

Fig. S15. GATA-3 and T-bet are not regulated by NFAT5 and SGK-1 in low NaCl conditions.

Fig. S16. Model.

Table S1. Selected regions and primer sequences used for DNA methylation analyses.

Table S2. Patient information.
Data file S1. Primary data.
References (*50*–*54*)

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Author contributions: J. Matthias performed experiments with human cells and analyzed and interpreted the data. J. Maul performed all experiments with murine cells and, together with D.B., analyzed and interpreted the data. R.N., H.M., Y.-Y.C. and D.S. performed experiments and analyzed the data. H.G., F.J., K.E., D.R., and H.M. performed the measurements of sodium concentrations by NAA and analyzed and interpreted the data. G.G. and J.W. performed the epigenetic experiments and analyzed and interpreted the data. G.G. and A.W. performed next-generation RNA sequencing (RNA-seq). K.N. processed and analyzed the RNA-seq data (ID: PRJNA503839). S.D. and P.K. performed further bioinformatic analyses with transcriptomic datasets provided by K.N. N.G.-S., K.E., T.B., and S.G. provided skin biopsies from atopic dermatitis patients and characterized and scored the patient samples. C.E.Z. conceived the study, supervised the experiments, interpreted the data, and wrote the manuscript. All authors reviewed and approved the manuscript. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** All data related to this study are present in the paper or the Supplementary Materials.

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Sodium chloride is an ionic checkpoint for human T_H2 cells and shapes the atopic skin microenvironment

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Salting T cells' game

Sodium chloride can encourage T_H17 differentiation in CD4 T cells. Matthias *et al*. now show that sodium chloride can also promote T_H2 responses, which are central in allergic diseases. They measured effects of different concentrations of sodium chloride on memory and naïve human T cells as well as mouse cells. Collectively, higher sodium chloride enforced a T_H2 transcriptional and phenotypic program. Interestingly, lesional skin from atopic dermatitis patients, but not psoriasis patients, had elevated sodium. These findings suggest that sodium chloride could be a potential player in the progression of allergy in the skin.