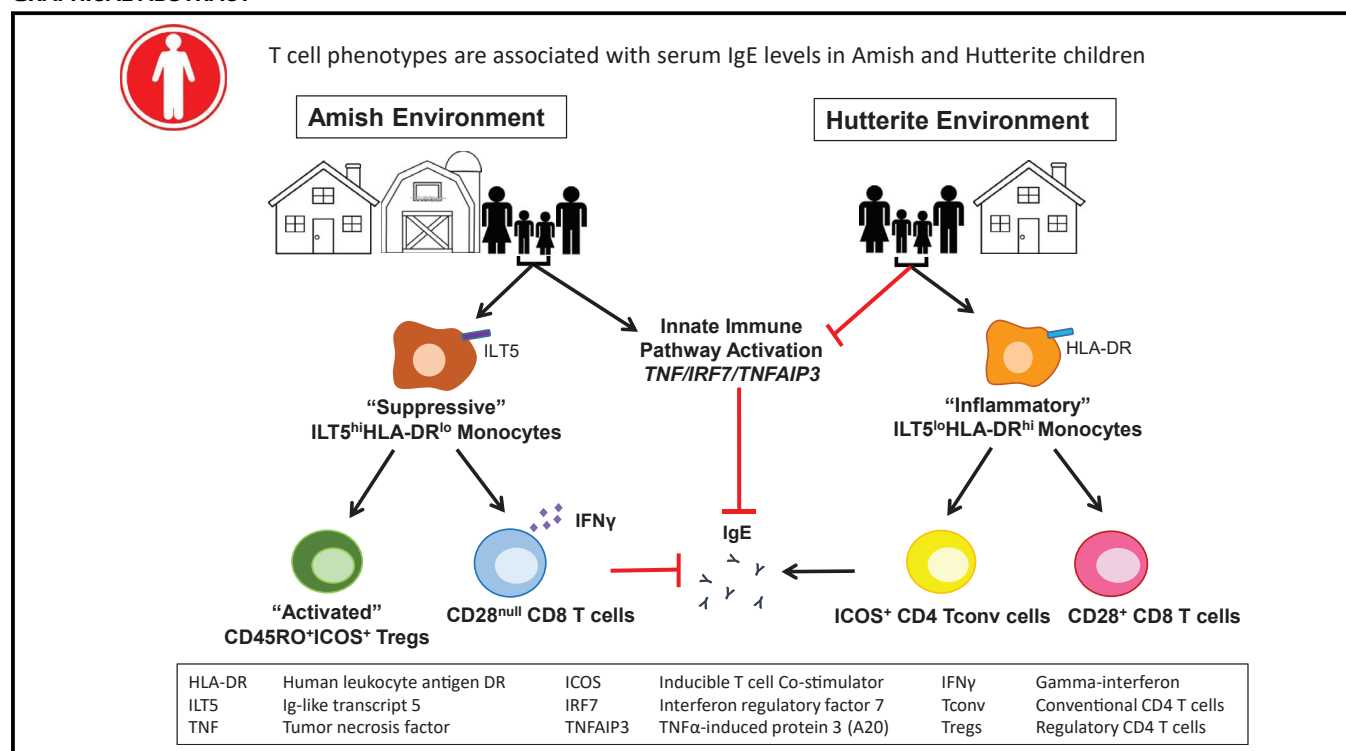


T-cell phenotypes are associated with serum IgE levels in Amish and Hutterite children



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GRAPHICAL ABSTRACT



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Objectives: Amish children raised on traditional farms have lower atopy and asthma risk than Hutterite children raised on modern farms. In our previous study we established that the Amish environment affects the innate immune response to decrease asthma and atopy risk. Here we investigated T-cell phenotypes in the same Amish and Hutterite children as in our earlier study to elucidate how this altered innate immunity affects adaptive T cells.

Methods: Blood was collected from 30 Amish and 30 Hutterite age- and sex-matched children; cells were cryopreserved until analysis. Flow cytometry was used to analyze cell subsets. Atopy was determined based on allergen-specific and total IgE levels. **Results:** Children exposed to Amish farms had increased activated regulatory CD4⁺ T-cell phenotypes, whereas conventional CD4 T cells expressed lower levels of costimulation molecules and other activation markers. The increase in numbers of circulating activated regulatory CD4⁺ T cells was associated with an increase in inhibitory receptors on monocytes in Amish, but not Hutterite, children. Strikingly, the Amish children had a higher proportion of CD28^{null} CD8 T cells than the Hutterite children ($P < .0001$, nonparametric t test), a difference that remained even after accounting for the effects of age and sex (conditional log regression exponential $\beta = 1.08$, $P = .0053$). The proportion of these cells correlated with high T-cell IFN- γ production ($r_s = 0.573$, $P = .005$) and low serum IgE levels ($r_s = -0.417$, $P = .025$). Furthermore, CD28^{null} CD8 T-cell numbers were increased in Amish children, with high expression of the innate genes *TNF* and TNF- α -induced protein 3 (*TNFAIP3*) in peripheral blood leukocytes.

Conclusion: Amish children's blood leukocytes are not only altered in their innate immune status but also have distinct T-cell phenotypes that are often associated with increased antigen exposure. (J Allergy Clin Immunol 2019;144:1391-401.)

Key words: Asthma, atopy, adaptive immunity, T-cell activation, CD4 T cells, CD8 T cells

Asthma is a complex disease with a strong environmental component. Asthma and allergic sensitization are reduced among children raised on farms compared with those from nonfarming and urban environments.¹ Moreover, our previous work demonstrated that Amish school-aged children raised on traditional farms had a 4-fold lower risk of asthma compared with Hutterite children raised on modern mechanized farms,²⁻⁴ despite having otherwise similar lifestyles and ancestry. Notably, this difference in asthma prevalence was paralleled by differences in the proportions and phenotypes of innate peripheral blood leukocytes (PBLs) from these children. In particular, we showed that Amish children had significantly more monocytes with an inhibitory phenotype and more immature neutrophils compared with Hutterite children. In contrast, the proportions of regulatory CD4⁺ T (Treg) cells were not different between Amish and Hutterite children. Therefore we speculated that profound differences in innate immunity in Amish children might affect other T-cell phenotypes.

Continuous exposure to microbial products affects innate immune function. Repeated exposures to LPS induce "endotoxin tolerance" or anergy in macrophages and monocytes that renders these cells unresponsive to further stimulation.⁵⁻⁸ This phenomenon is detrimental in patients with chronic infections or sepsis. It is also associated with reduced T-cell responses, possibly because

Abbreviations used

DN:	Double negative
FoxP3:	Forkhead box P3
ICOS:	Inducible T-cell costimulator
ILT3:	Immunoglobulin-like transcript 3
ILT5:	Immunoglobulin-like transcript 5
IRF7:	Interferon regulatory factor 7
PBL:	Peripheral blood leukocyte
PD-1:	Programmed cell death protein 1
TNFAIP3:	TNF- α -induced protein 3
Treg:	Regulatory CD4 ⁺ T

of low HLA-DR expression.⁹ Monocytes from Amish children also had suppressed HLA-DR levels and increased inhibitory receptor expression,² but how this phenotype correlated with changes in adaptive T-cell immunity in these children had not been explored.

In this study we investigated whether T-cell populations were phenotypically distinct between the same 30 Amish and 30 Hutterite schoolchildren who participated in our earlier study. We found many differences in the costimulatory molecules expressed by Amish and Hutterite children: Amish conventional CD4 T cells expressed less CD28 or inducible T-cell costimulator (ICOS), and CD4⁺ regulatory T (Treg) cells from Amish children expressed significantly greater levels of CD45RO and ICOS, a phenotype consistent with enhanced suppressive function.^{10,11}

Additionally, Amish children carried a significantly expanded distinctive CD28^{null}CD8⁺ T-cell population, and the proportions of these cells correlated positively and negatively with IFN- γ secretion by PBLs and serum IgE levels, respectively. Within the Amish, CD28^{null}CD8⁺ T-cell numbers were increased in children with high expression of the innate genes interferon regulatory factor 7 (*IRF7*), *TNF*, and TNF- α -induced protein 3 (*TNFAIP3* [A20]) in their PBLs. Overall, these results suggest that profound differences in T-cell immunity between Amish and Hutterite children might contribute to their distinct asthma and atopy risk.

METHODS

Study participants and sample collection

The 30 Amish and 30 Hutterite schoolchildren (6-14 years old) were age and sex matched, as previously described.² None of the 30 Amish children had asthma, whereas 6 of the 30 Hutterite children had asthma. Written consent was obtained from the parents, and written assent was obtained from the children. The study was approved by the institutional review boards at the University of Chicago and St Vincent Hospital, Indianapolis, Indiana.

Blood was collected for cell analyses and serum IgE measurements, as previously reported.² To obtain PBLs, whole blood was lysed with ACK lysis buffer (150 mmol/L ammonium chloride, 10 mmol/L potassium carbonate, and 0.1 mmol/L EDTA), and the remaining leukocytes were cryopreserved in 90% FBS/10% dimethyl sulfoxide. Cells were kept in liquid nitrogen storage for approximately 6 months before thawing for flow cytometric experiments.

Flow cytometry

Frozen PBLs were thawed, washed in RPMI containing deoxyribonuclease I (0.02 mg/mL), and resuspended in FACS buffer (PBS containing 0.1% sodium azide and 1% BSA). Approximately 3×10^5 cells in 100 μ L per sample were incubated for 10 minutes with pooled human IgG (FcX; BioLegend,

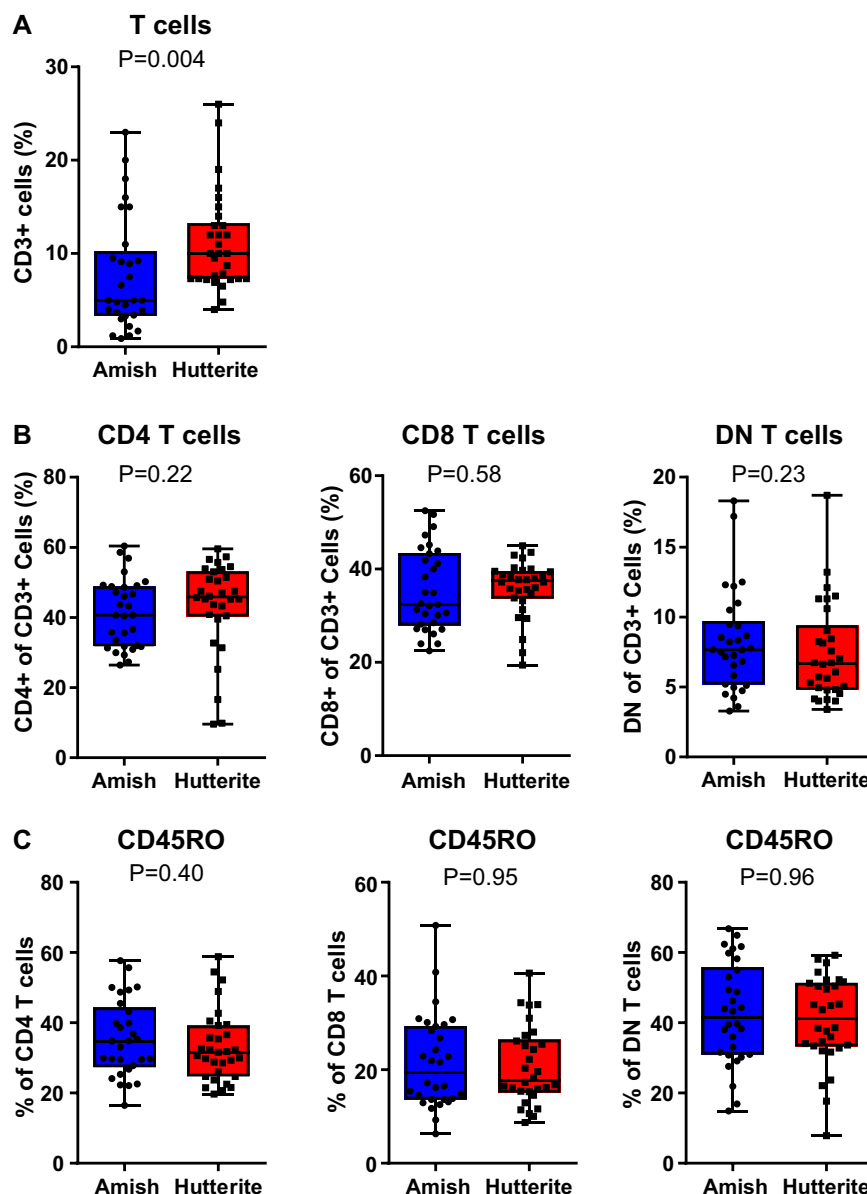


FIG 1. T-cell proportions in Amish and Hutterite children. **A**, Percentage of CD3⁺ cells of total PBLs. **B**, Proportion of CD3⁺ cells that are CD4⁺, CD8⁺, or DN. **C**, CD45RO expression on each subset was measured as a marker of effector and/or memory T cells. Values shown on each graph are *P* values from nonparametric Mann-Whitney tests, with *P* values of less than .0025 required for Bonferonni-corrected significance.

San Diego, Calif) to block nonspecific antibody binding before staining with fluorescently conjugated antibodies (see Table E1 in this article's Online Repository at www.jacionline.org). For surface phenotyping, flow cytometric data were acquired immediately after staining on an LSRFortessa (BD Biosciences, San Jose, Calif), and the data were analyzed with FlowJo software (Tree Star, Ashland, Ore). For forkhead box P3 (FoxP3) staining, cells were surface stained as described above before performing FoxP3 staining according to the manufacturer's instructions (FoxP3 Fix/Perm Kit; eBioscience, San Diego, Calif). T-cell subsets were gated as shown in Figs E1 and E2 in this article's Online Repository at www.jacionline.org.

IFN- γ measurement

Whole blood was drawn directly into TruCulture (Myriad RBM, Austin, Tex) collection tubes. One milliliter of whole blood was drawn into 2 different

tubes: one containing TruCulture media plus anti-CD3 and anti-CD28 antibodies and one containing TruCulture media alone. Whole blood samples in the TruCulture tubes were incubated upright in a dry heat block at 37°C for 30 hours.

After incubation, supernatants from TruCulture tubes were flash-frozen for cytokine studies by using the provided Seraplast valve. Cell samples from Amish subjects were processed in the laboratory at the University of Chicago, and those from Hutterite subjects were processed on site in a makeshift laboratory in the Oaklane Colony. The same persons processed both sets of samples. Supernatants from both Amish and Hutterite subjects were thawed on the same day, and IFN- γ levels were quantified by using a multiplex assay (Millipore Sigma, Burlington, Mass). T-cell IFN- γ was defined as the difference between IFN- γ levels measured in the anti-CD3/CD28 sample and the control media-alone sample for each child.

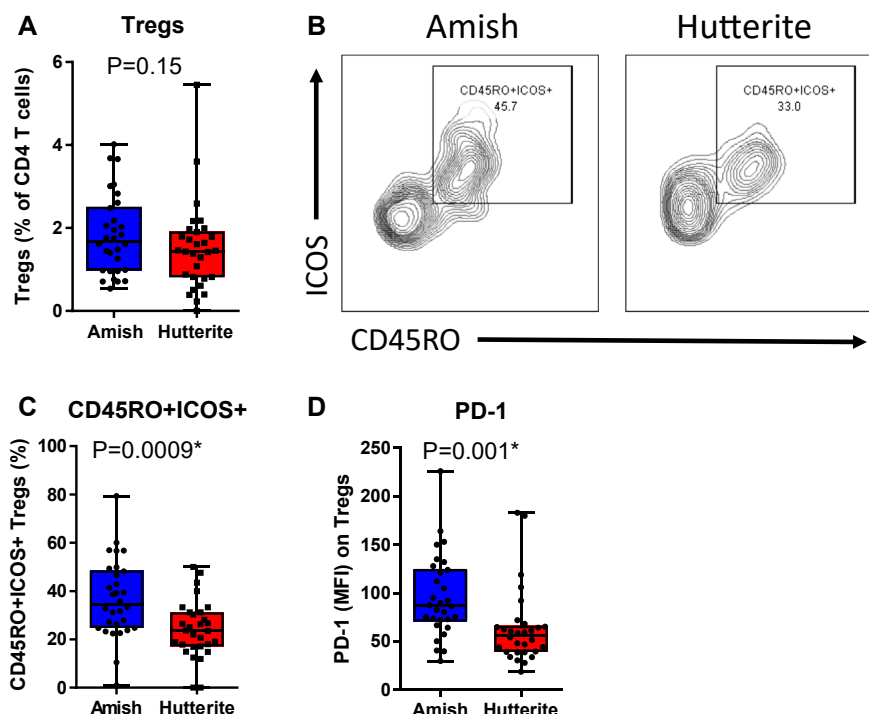


FIG 2. Amish and Hutterite schoolchildren have a similar proportion of Treg cells, but Treg cells from Amish children express increased markers of activation. Treg cells were identified by size, negative for a dead cell stain, and $CD3^+CD4^+FoxP3^+CD127^{lo}$. **A**, Proportion of Treg cells in Amish and Hutterite children of total CD4 T cells. **B** and **C**, Percentage of activated Treg cells identified as $CD45RO^+ICOS^+$ cells of total Treg cells in the 2 groups. **D**, PD-1 expression on Treg cells. Values shown on each graph are *P* values from nonparametric Mann-Whitney tests, with *P* values of less than .0025 required for Bonferroni-corrected significance. Significant *P* values are denoted with asterisks. *MFI*, Mean fluorescence intensity.

Statistical analyses

Two-group comparisons of continuous variable data were analyzed by using a nonparametric Mann-Whitney test. A Bonferroni-corrected *P* value for *t* tests was calculated based on the number of comparisons generated. Correlations were computed by using linear regression, followed by a nonparametric Spearman test for significance. Additional details for each figure can be found in the corresponding legend. All analyses and graphs were generated by using Prism graphing software (GraphPad Software, La Jolla, Calif).

RESULTS

Amish children have similar proportions of circulating T cells compared with Hutterite children

We examined cell phenotypes in a cohort of children from 2 farming communities: the Amish, who have a low incidence of asthma and atopy, and the Hutterites, who have a greater incidence.^{3,12} The overall prevalence of asthma in Amish children aged 6 to 12 years is 5.2%, whereas in Hutterite children aged 6 to 10 years, the prevalence is 21.3%. Allergic sensitization defined by skin prick tests is 7.2% and 33.3% in Amish and Hutterite children, respectively. In our study none of the 30 Amish children and 6 (20%) of the 30 Hutterite children had asthma. Two Amish children and 9 Hutterite children had allergen-specific IgE levels of greater than a 3.5 kU_A/L threshold. Demographic information was previously published² and is shown in Table E1. Amish children had reduced total IgE levels and eosinophil percentages compared with Hutterite children. Total IgE levels were correlated with the sum of specific IgE levels, as well as eosinophil percentages, in all children, although significance was greater in

Hutterite children, who were more likely to have increased IgE levels and eosinophil counts (see Fig E3 in this article's Online Repository at www.jacionline.org). Amish children had a trend toward a reduction in the proportion of $CD3^+$ T cells of total PBLs compared with Hutterite children (*P* = .004, with *P* < .0025 required for Bonferroni-corrected significance; Fig 1, A). No differences were found in the percentage of T cells defined as $CD4^+$, $CD8^+$, or double negative (DN) for both markers (Fig 1, B) or in the percentage of each T-cell subset expressing the effector/memory marker CD45RO (Fig 1, C).

Treg cells from Amish children have an activated phenotype that correlates with upregulation of immunoglobulin-like transcript 5 on monocytes

The percentage of Treg cells ($CD3^+CD4^+FoxP3^+CD127^{lo}$ cells) was similar in the Amish and Hutterite children, despite their disparate asthma and atopy risk (Fig 2, A).² However, the percentage of $CD45RO^+ICOS^+$ Treg cells was significantly increased in Amish children compared with Hutterite children ($36.8\% \pm 16.2\%$ vs $24.2\% \pm 11.8\%$, respectively; *P* = .0009; Fig 2, B and C). These markers are characteristic of activated Treg cells, with more suppressive function in both human subjects and mice.^{10,13,14} Greater levels of the inhibitory molecule programmed cell death protein 1 (PD-1) were also observed on the Treg cells from Amish children (*P* = .001; Fig 2, D).

Inducible Treg cells are polarized from naive T cells through interactions with specific antigen-presenting cell populations.

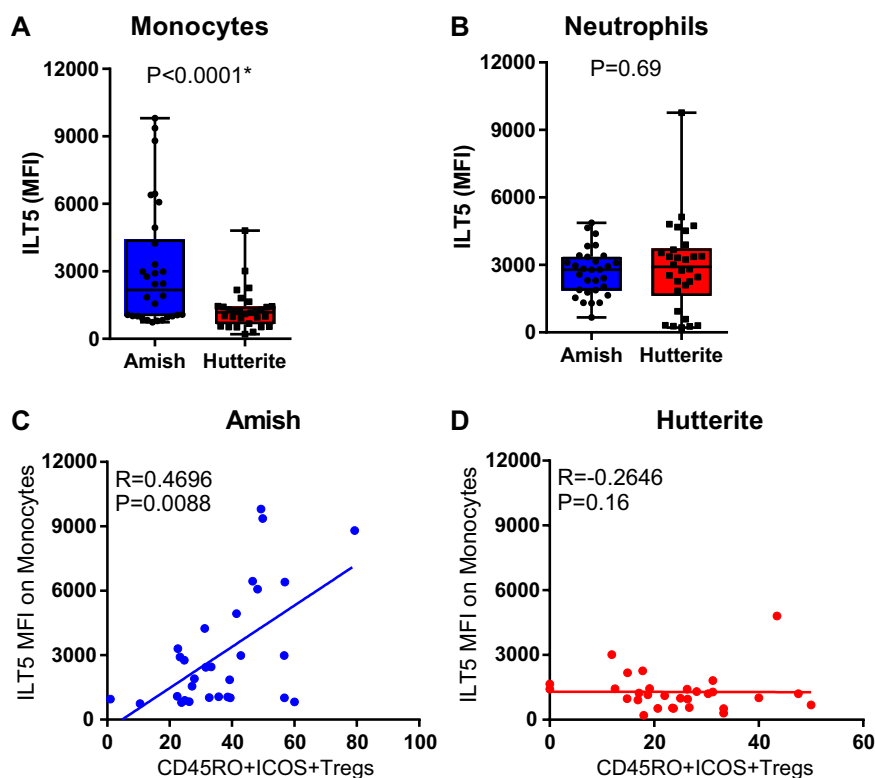


FIG 3. Activation of Treg cells correlates with expression of the inhibitory receptor ILT5 on monocytes. Expression of ILT5 on monocytes (**A**) and neutrophils (**B**) in Amish children. Values shown on each graph are *P* values from nonparametric Mann-Whitney tests, with *P* values of less than .0025 required for Bonferroni-corrected significance. Significant *P* values are denoted with asterisks. **C**, Correlation of ILT5 expression with the percentage of activated CD45RO⁺ICOS⁺ Treg cells in Amish or **D**, Hutterite children. The *R* value is the Spearman rho correlation coefficient and associated *P* value. *MFI*, Mean fluorescence intensity.

Monocytes are one potential antigen-presenting cell that can drive Treg cell expansion. Similar to the observed proportions of Treg cells, the overall proportion of monocytes (CD14⁺CD66b⁺ cells) did not differ between the Amish and Hutterite children.²

As previously reported, monocytes from the Amish children had decreased surface expression of HLA-DR (*P* < .001) and increased surface expression of the inhibitory molecules immunoglobulin-like transcript 3 (ILT3; *P* = .003) and immunoglobulin-like transcript 5 (ILT5; *P* = .005) compared with monocytes from Hutterite children.² Little is known about the function of ILT5, but it can be expressed by both antigen-presenting cells and granulocytes. In Amish children ILT5 was expressed at a significantly greater level on monocytes compared with the levels seen in Hutterite children (*P* < .001; Fig 3, A), whereas neutrophils from both groups of children expressed similar levels (*P* = .69; Fig 3, B). In the monocytes from Amish children ILT5 expression positively correlated with CD45⁺ICOS⁺ Treg cell counts (*r_s* = 0.4696, *P* = .0088; Fig 3, C and D). No association was observed in Hutterite children (*r_s* = -0.26, *P* = .16). Although ILT3 has been associated with induction of Treg cells,^{15,16} we found a negative correlation between ILT3 expression on monocytes and activated CD45RO⁺ICOS⁺ Treg cells in Amish children (*r_s* = -0.421, *P* = .021) and no correlation between these cell phenotypes in

Hutterite children (*r_s* = 0.041, *P* = .8276; see Fig E4 in this article's Online Repository at www.jacionline.org).

CD4 conventional T cells from Amish children express a distinct costimulatory molecule profile indicative of reduced activation

The suppressive phenotypes identified in monocytes and Treg cells from Amish children can function to prevent effector T-cell responses. Therefore we examined the activation state of conventional CD4 T cells in Amish and Hutterite children. Expression of CD28, a critical costimulatory molecule expressed by T cells to facilitate T-cell proliferation and cytokine secretion, was similar in both groups (Fig 4, A). The IL-7 receptor (CD127), which promotes memory T-cell proliferation, showed a trend toward lower expression on CD4 T cells from Amish children (Fig 4, B).

We next examined cell-surface expression of ICOS and PD-1, 2 CD28 family members with costimulatory and coinhibitory function, respectively. A dramatic reduction in numbers of ICOS⁺ CD4 T cells was present in Amish children (10.7% ± 5.1% ICOS⁺ CD4 T cells in Amish subjects compared with 18.7% ± 7.6% in Hutterite subjects, *P* < .0001; Fig 4, C), whereas a nonsignificant trend was observed for greater PD-1 expression on CD4 T cells from Amish children (Fig 4, D).

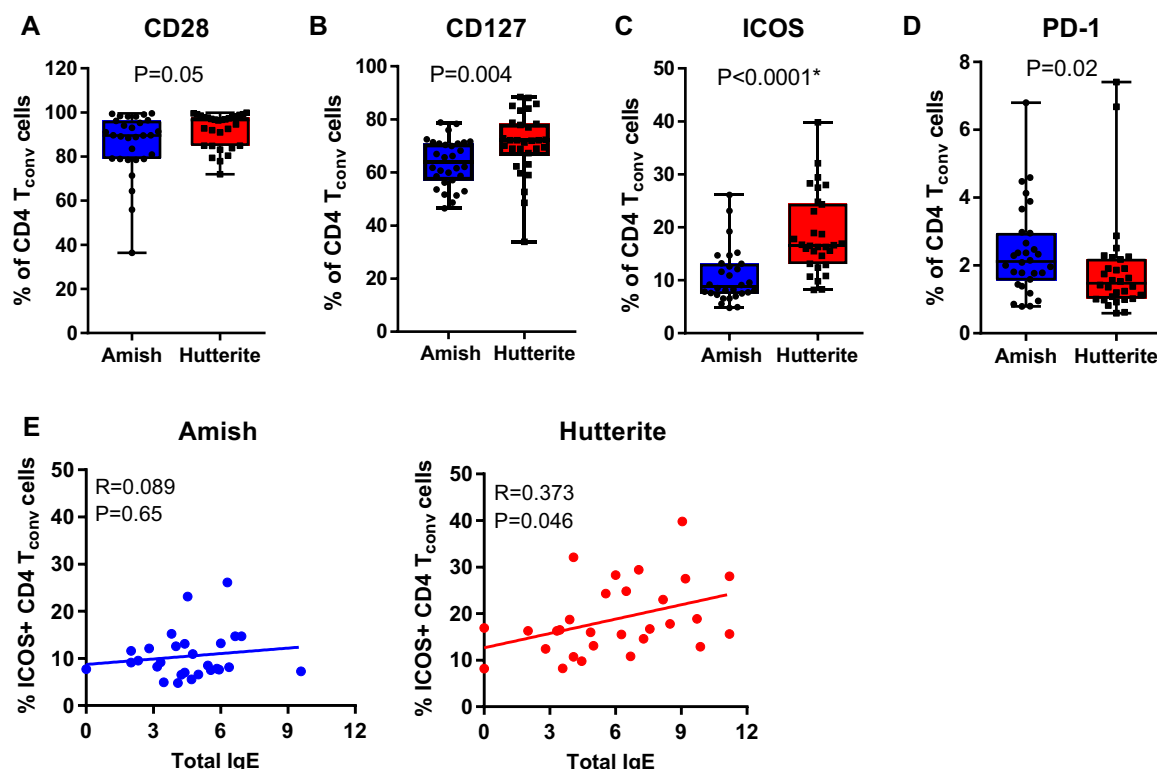


FIG 4. Conventional (non-Treg) CD4 T cells in the Amish exhibit low activation with a specific reduction in ICOS level. **A-D**, Expression of CD28 (Fig 4, A), CD127 (Fig 4, B), ICOS (Fig 4, C), and PD-1 (Fig 4, D) on CD4 T cells from Amish and Hutterite children. Values shown on each graph are *P* values from nonparametric Mann-Whitney tests, with *P* values of less than .0025 required for Bonferroni-corrected significance. Significant *P* values are denoted with asterisks. **E**, ICOS expression on CD4 T cells was correlated with circulating IgE levels in either Amish (left) or Hutterite (right) children. The *R* value is the Spearman rho correlation coefficient and associated *P* value.

A similar phenotype was observed in DN T cells (see Fig E5 in this article's Online Repository at www.jacionline.org).

Our previous studies in a cohort that included both Hutterites and non-Hutterites revealed that single nucleotide polymorphisms in the *ICOS* promoter were associated with allergic sensitization and IgE levels,¹⁷ and ICOS expression is positively correlated with T_H2 responses in mice.¹⁸ Consistent with the role of ICOS in T_H2 responses, the percentage of ICOS⁺ CD4 T cells positively correlated with total IgE levels in Hutterite children but not in Amish children, who had both low ICOS and low IgE levels (Fig 4, E).

CD8 T cells from Amish children are characterized by the presence of CD25^{hi} cells and increased numbers of CD28^{null} cells

Like CD4 T cells, CD8 T cells also play an important role in asthma pathogenesis. CD8 T-cell expression of leukotriene B₄ receptor 1 is associated with asthma severity, and bronchial CD8 T cells from patients with asthma produce IL-13.^{19,20} We examined the phenotype of CD8 T cells in Amish children. Examination of the total CD8 T-cell population demonstrated that CD8 T cells from Amish children expressed greater ICOS and lower CD127 than CD8 T cells from Hutterite children, a phenotype characteristic of effector and/or memory cells (see Fig E6, A and B, in this article's Online Repository at www.jacionline.org).^{21,22} No difference was observed in PD-1 expression (see Fig E6, C).

On further analysis of CD8 T cells, we discovered that PBLs from Amish children contained a unique subset of CD25^{hi}CD127^{lo} CD8 T cells (Fig 5, A-C). This subset was found in 26 of the 30 Amish children (defined as having 1% or greater CD25^{hi} of CD8 T cells) but only 7 of 30 Hutterite children. Median numbers of CD25^{hi} CD8 T cells were 5.30 (interquartile range, 1.89-13.75) and 0.26 (interquartile range, 0.08-1.08) in the Amish and Hutterite children, respectively ($P < .0001$). Because CD25^{hi} CD4 T cells are mainly composed of FoxP3⁺ Treg cells, we assessed whether CD25^{hi} CD8 T cells had a similar regulatory phenotype. However, unlike CD25^{hi} CD4 T cells, this subset did not express FoxP3 or ICOS and instead expressed high levels of PD-1, a marker of exhaustion (Fig 5, D).

Strikingly, the majority of CD8 T cells from Amish children lacked the costimulatory molecule CD28 (CD28^{null} CD8 T cells, $P < .0001$; Fig 6, A and B). A significant difference remained in a conditional regression model to remove the effects of age and sex (conditional log regression exponential $\beta = 1.08$, $P = .0053$; see Table E1). Levels of CD28^{null} CD8 T cells negatively correlated with IgE levels in Amish but not Hutterite children ($r_s = -0.417$, $P = .025$ and $r_s = 0.061$, $P = .75$ in Amish and Hutterite children, respectively; Fig 6, C). CD28^{null} CD8 T cells have been associated with chronic antigen exposure, cytomegalovirus infection, aging, and cancer.²³⁻²⁵

Because CD28^{null} CD8 T cells are generally associated with IFN- γ -mediated responses, we investigated whether the proportion of these cells correlated with IFN- γ production by T cells. No difference was observed between IFN- γ production in Amish

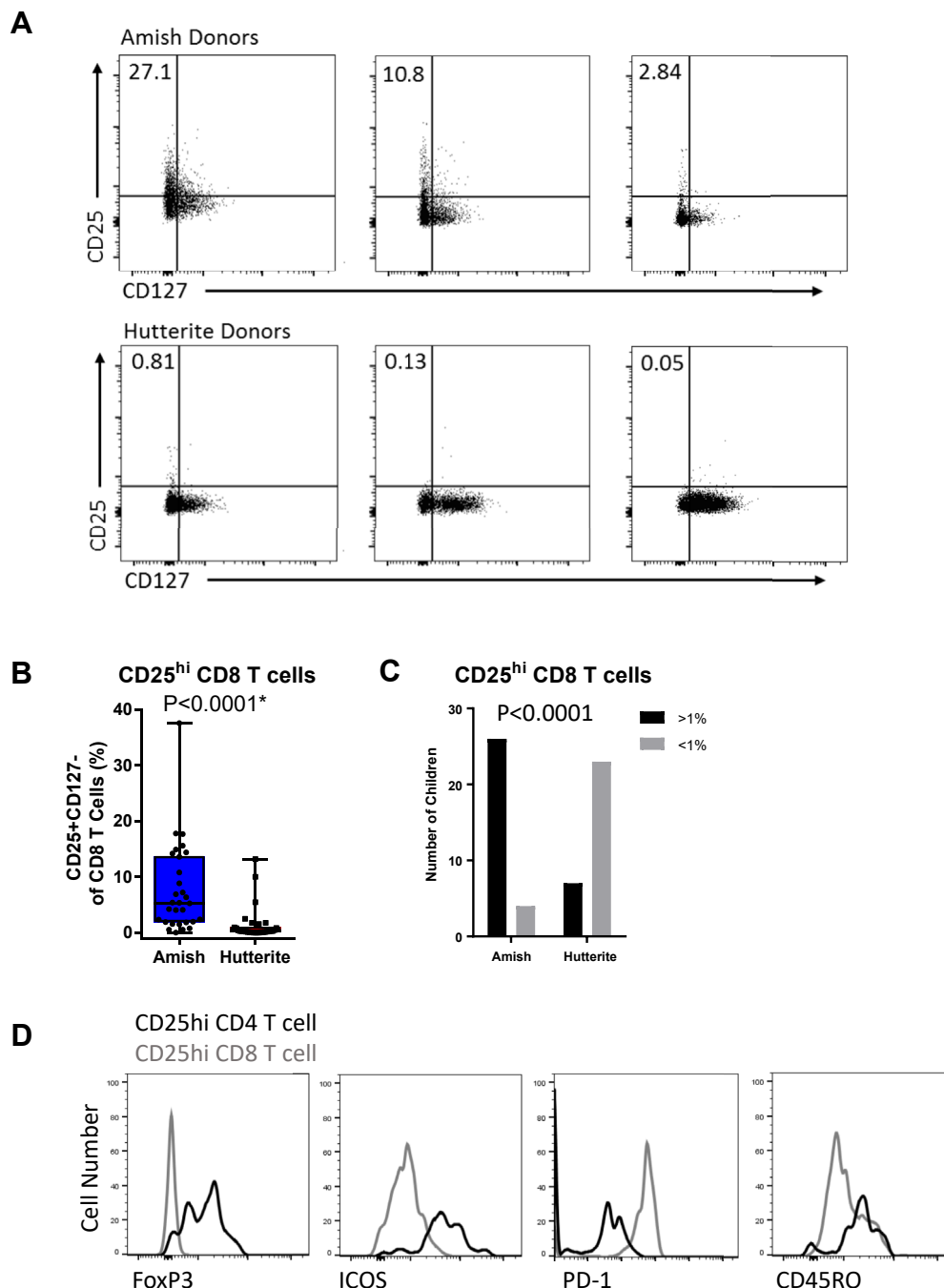


FIG 5. Amish children have a unique population of CD8 T cells that express high levels of CD25. **A**, Example flow plots showing expression of CD25 and CD127 on CD3⁺CD8⁺ T cells in 3 Amish and 3 Hutterite donors. **B**, Percentage of CD25^{hi}CD127^{lo} CD8 T cells in each group with *P* values from the nonparametric Mann-Whitney test. Asterisks indicates a significant difference after multiple testing correction. **C**, Proportion of Amish or Hutterite children who had less than 1% (gray bars) or greater than 1% (black bars) CD8 T cells exhibiting the CD25^{hi}CD127^{lo} phenotype. *P* values were from the Fisher exact test. **D**, Expression of FoxP3, ICOS, PD-1, and CD45RO was compared between CD25^{hi}CD127^{lo} CD8 T cells (gray line) and CD25^{hi}CD127^{lo} CD4 T cells (black line) from the same Amish donor.

and Hutterite children after T-cell stimulation (Fig 6, D).²⁶ Yet in both Amish and Hutterite children, IFN- γ levels were correlated with the percentage of CD28^{null} CD8 T cells ($r_s = 0.573$, $P = .005$ and $r_s = 0.410$, $P = .047$ in Amish and Hutterite children, respectively; Fig 6, E).

Previously, we found that gene expression of a module of coexpressed innate immune genes was greater in the PBLs of Amish compared with Hutterite children.² To understand the relationship between these innate immune genes and allergic sensitization, we correlated serum IgE levels to 3 genes in the innate

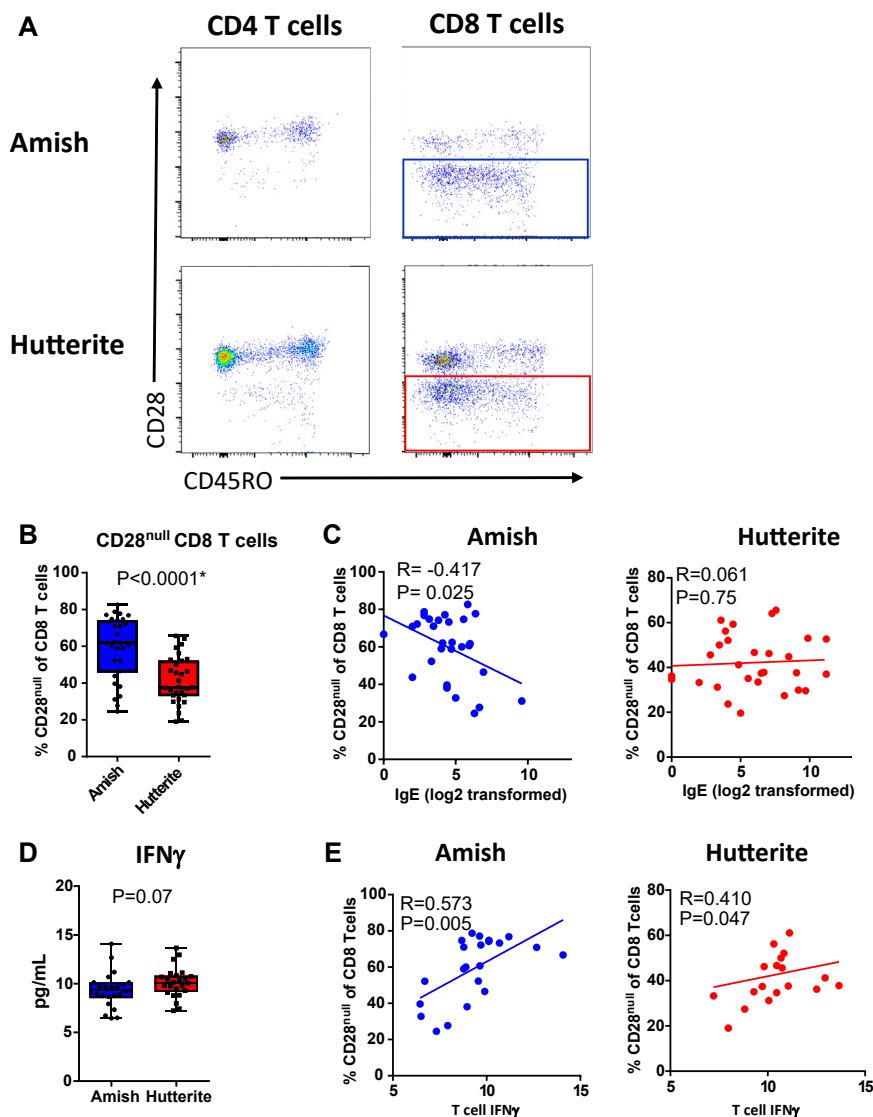


FIG 6. CD8 T cells from Amish children have low levels of the costimulatory molecule CD28 and correlate with increased IFN- γ and low IgE levels. **A**, Example flow plots of the expression of CD28 and CD4 and CD8 T cells in Amish and Hutterite children. **B**, Proportion of CD28^{null} CD8 T cells of total CD8 T cells with *P* values from the nonparametric Mann-Whitney test. Asterisks indicates a significant difference after multiple testing correction. **C**, Correlation of CD28^{null} CD8 T cells and serum IgE levels, as measured by means of ELISA. **D**, IFN- γ levels were measured in supernatants of whole blood from 21 of the 30 Hutterite children and 22 of the 30 Amish children stimulated with anti-CD3 and anti-CD28 antibodies for 30 hours. The children who were omitted have a low-affinity allele in Fc γ RIIA that reduces binding of the anti-CD3 and anti-CD28 antibodies.²⁶ **E**, Correlation of IFN- γ and IgE levels in Amish and Hutterite children. The *R* value is the Spearman rho correlation coefficient and associated *P* value.

network identified from the module (*IRF7*, *TNF*, and *TNFAIP3*) that have significantly increased expression in Amish children (see Fig E7 in this article's Online Repository at www.jacionline.org).² *TNF* and *TNFAIP3* exhibited a positive correlation with the frequency of CD28^{null} CD8 T cells, and all 3 genes exhibited a negative correlation with serum IgE levels in Amish children (Fig 7). Of the 3 genes tested, *TNF* expression exhibited the strongest correlation with CD28^{null} CD8 T-cell counts ($r_s = 0.592$, $P = .001$) and IgE levels ($r_s = -0.521$, $P = .006$). No significant correlations were found in Hutterite children. The Hutterite children with asthma were not driving these findings because all comparisons were examined with the 6 asthmatic

children excluded (see Tables E2 and E3 in this article's Online Repository at www.jacionline.org). A summary of our observations on immune activation in Amish children based on our previous and current studies is shown in Fig 8.²

DISCUSSION

The Amish farm environment shapes the innate immune response in children,² and we hypothesized that this would lead to altered adaptive T-cell responses. Here we show that the Amish children had a lower proportion of T cells, including CD4, CD8, and DN T-cell subsets, compared with Hutterite children.

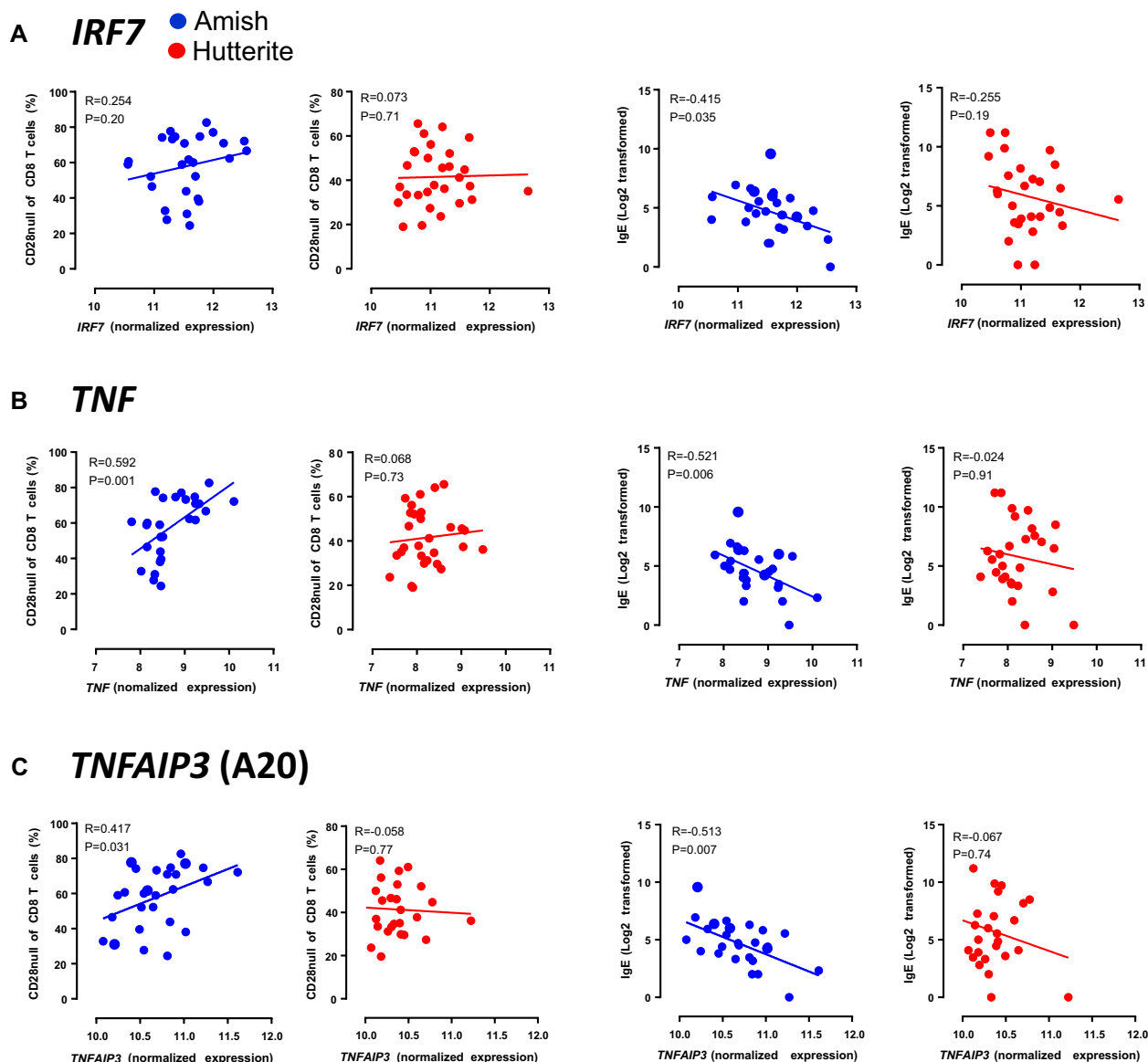


FIG 7. High expression of innate immune genes correlates with increased numbers of CD28^{null} CD8 T cells and low IgE levels in Amish children. The association between normalized expression of an *IRF7* (A), *TNF* (B), and *TNFAIP3* (C) with CD28^{null} CD8 T-cell counts and IgE levels is shown. Gene expression in Amish and Hutterite PBL samples was assessed by means of microarray after culture in medium for 30 hours. The *R* value is the Spearman rho correlation coefficient and associated *P* value.

All 3 T-cell subsets in the Amish children expressed less CD28, a costimulatory molecule critical for effector T-cell function. ICOS levels were lower on conventional CD4 T cells in Amish compared with Hutterite children, suggesting less activation of CD4 T cells. Yet ICOS was expressed at a much greater level on Treg cells in the Amish children. These data indicate that the Amish children have specific activation of Treg cells that might contribute to their reduced levels of IgE. High ICOS expression on Treg cells from Amish children was correlated with expression of the inhibitory receptor ILT5, but not ILT3, on monocytes. Finally, Amish children had increased percentages of CD28^{null} CD8 T cells, and these cells were correlated with increased IFN- γ production and a reduction in circulating

IgE levels in Amish children. Interestingly, the percentage of CD28^{null} CD8 T cells also correlated with genes within the innate gene network that was upregulated in Amish blood. Thus our findings suggest a model in which environmental exposures in the Amish community drive expression of an *IRF7*/*TNF* pathway, promote unique T-cell phenotypes that produce IFN- γ , and inhibit IgE.

We were surprised to find that healthy Amish children between 6 and 14 years of age already have as much as 80% CD28^{null} CD8 T cells, a phenotype generally associated with aging and chronic diseases, especially chronic viral infections.^{23-25,27} In our earlier study of the innate response in Amish children, we reported that *IRF7* was a key transcription

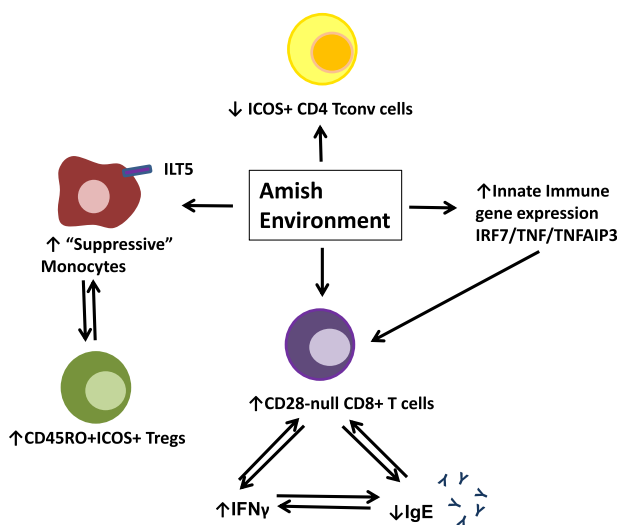


FIG 8. Effects of Amish environment on circulating immune cells: potential mechanisms for reduced asthma risk. Our studies in Amish and Hutterite children support a model in which microbial exposures in the Amish drive neutrophil emigration from bone marrow and induce suppressive monocytes (previously published in the *New England Journal of Medicine*).² These monocytes are associated with activated Treg cells that might be inhibiting effector T cells or antigen-presenting cells. The Amish environment also leads to unique CD8 T-cell phenotypes, including CD25^{hi}CD127^{lo} CD8 T cells with an unknown function. Increased numbers of CD28^{null} CD8 T cells were associated with increased IFN- γ and decreased IgE levels. These cells might be directly or indirectly suppressing T_H2 responses to reduce atopy and asthma risk. *Tconv*, Conventional CD4 T cells.

factor upregulated in PBLs from Amish compared with Hutterite children.² IRF7 is well known as a viral response gene that initiates type 1 interferon production. Although we do not know the past or ongoing viral exposures in Amish children, the positive correlation of CD28^{null} CD8 T cells with IFN- γ release suggests that viral exposures could be contributing to this phenotype. Furthermore, our observation that greater IFN- γ levels were associated with lower IgE levels suggests that this phenotype might be a marker of protection against atopy, although it is unclear whether CD28^{null} CD8 T cells directly contribute to this protection. The majority of Amish children also had CD8 T cells expressing CD25, whereas almost none of the Hutterite children had these cells. Although the function of these cells is not known, they express markers consistent with an exhausted type of CD8 T cells.^{28,29}

Monocytes from Amish children had low expression of HLA-DR,² which has been associated with repeated endotoxin exposure³⁰ and chronic infections^{31,32} in other studies. These HLA-DR-low monocytes expressed high levels of 2 inhibitory receptors, ILT3 and ILT5 (Fig 3).² ILT3 is notable in that it can drive the differentiation of both Treg cells and CD8 suppressor cells that can inhibit inflammation.^{15,16,33,34} Therefore it was surprising that ILT3 levels were negatively correlated with CD45RO⁺ICOS⁺ Treg cell counts in Amish children. ILT5 is a related immunoreceptor tyrosine-based inhibition motif-containing family member that is upregulated in tolerogenic dendritic cells.³⁵ The function of ILT5 and its relationship to T-cell differentiation is currently unknown,¹⁵ but our data suggest that ILT5 might be important in induction of Treg cells in

the Amish environment. Multiple studies have linked increased numbers of Treg cells with protection from asthma and have shown that children exposed to farm environments, particularly to consumption of raw milk, have increased numbers of Treg cells.^{36,37} Together, these data support the hypothesis that increased levels of tolerogenic monocytes drive Treg cell activation in the Amish children.

Our study has a number of limitations. First, differences in cell phenotypes between Amish and Hutterite children might be due to an epiphenomenon. For instance, we have insufficient data to link our findings to either microbial and environmental exposures or the presence or absence of atopy.

Second, although children with high total IgE levels in our study were more likely to also have additional markers of atopic disease, including increased eosinophil counts and positive allergen-specific IgE results, we cannot rule out the possibility that these findings reflect exposure to unknown parasitic infections in these children.

Finally, our study is limited by the overall small sample size and the relatively few atopic children, especially among the Amish. Future studies with larger sample sizes will yield additional insight into the effect of different environments on cell phenotypes associated with allergic sensitization.

Age and sex had little to no effect on our T-cell phenotypes, but IFN- γ levels became significantly different in Amish and Hutterite children when these covariates were removed. This suggests that immune system skewing can change with age or sex and is consistent with known differences in age-dependent allergy and asthma prevalence in male and female subjects.³⁸ Although 6 of the 30 Hutterite children were asthmatic, those subjects did not drive the differences in cell phenotypes we observed between the Amish and Hutterite children. Even when asthmatic patients were excluded, Hutterite children had increased numbers of ICOS⁺ conventional CD4 T cells, decreased ILT receptor expression on monocytes, fewer CD45RO⁺ICOS⁺ Treg cells, and fewer CD28^{null} CD8 T cells compared with Amish children. We do not know whether these cell phenotypes are associated with asthma risk or allergic sensitization. Instead, our data suggest that the Hutterite environment is associated with the development of distinct cell phenotypes that are related to greater IgE production.

Overall, our assessment of T-cell subsets in Amish children supports a model in which sustained microbial exposures lead to chronic antigen stimulation. Exposure to this milieu in turn results in changes to the innate immune system² and is correlated with changes to the adaptive immune system characterized by low expression of T-cell costimulatory molecules. Thus the Amish farm environment broadly regulates both innate and adaptive immunity. Understanding the mechanisms driving the protective effects of the Amish farm environment on both innate and adaptive immunity can generate robust avenues of study for asthma and allergic disease prevention.

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Key messages

- Environmental exposures in children can drive unique T-cell phenotypes that are associated with circulating IgE levels.
- Regulatory T cells and CD8 T cells from Amish children display a phenotype characteristic of chronic activation, whereas conventional CD4 T cells display a less activated phenotype.

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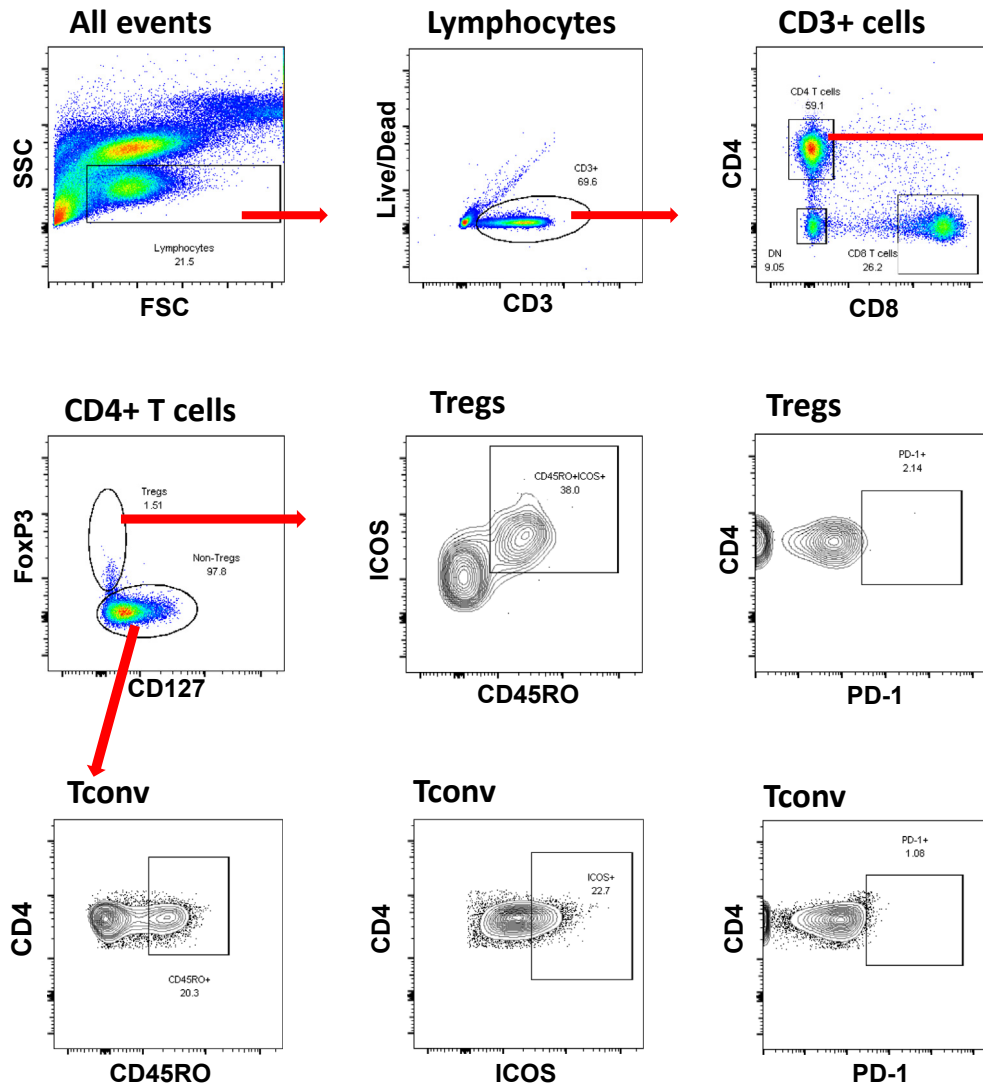


FIG E1. Gating strategy to examine CD4⁺ Treg and conventional CD4⁺ T (*Tconv*) cell phenotypes in Amish and Hutterite children. Thawed PBLs were stained with antibodies against CD3, CD4, CD8, CD45RO, ICOS, and PD-1. Cells were fixed, permeabilized, and stained for FoxP3. CD4⁺ Treg cells were gated as lymphocyte sized (CD3⁺CD4⁺CD8⁻FoxP3⁺CD127⁻). Treg cells were also analyzed for the frequency of CD45RO⁺ICOS⁺ and PD-1⁺ cells. Conventional CD4 T (*Tconv*) cells were gated as lymphocyte sized (CD3⁺CD4⁺CD8⁻FoxP3⁻CD127^{+/+}). Conventional CD4 T cells were additionally examined for the frequency of cells expressing CD45RO, ICOS, or PD-1. *FSC*, Forward scatter; *SSC*, side scatter.

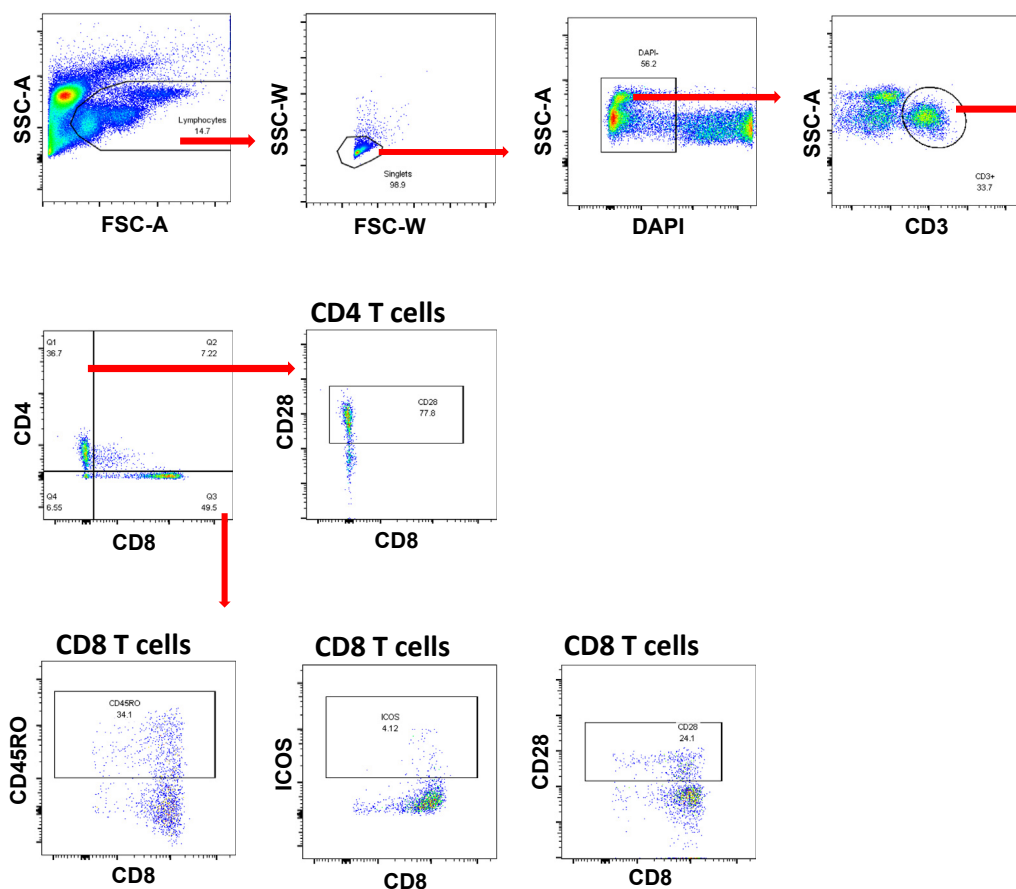


FIG E2. Gating strategy to examine CD28 expression on CD4 T cells and activation markers on CD8 T cells in Amish and Hutterite children. Thawed PBLs were stained with antibodies against CD3, CD4, CD8, CD45RO, ICOS, and CD28. 4'-6-Diamidino-2-phenylindole dihydrochloride was added directly before acquiring events to distinguish live and dead cells. CD4 T cells were gated as shown and examined for expression of CD28. CD8 T cells were examined for expression of CD45RO, ICOS, and CD28. *FSC-A*, Forward scatter area; *FSC-W*, forward scatter width; *SSC-A*, side scatter area; *SSC-W*, side scatter width.

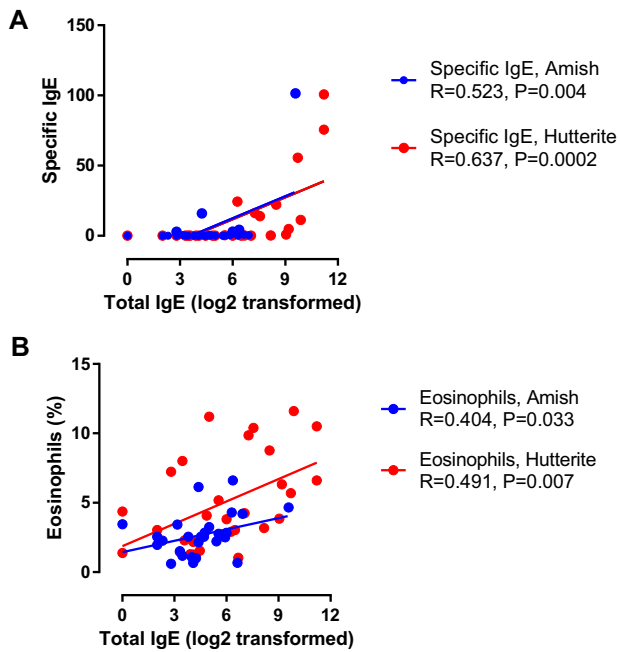


FIG E3. Allergen-specific IgE levels and eosinophil counts strongly correlate with total IgE levels in Amish and Hutterite children. **A**, Correlation of total IgE to allergen-specific IgE. Specific IgE is the sum of IgE levels measured for the following allergens: *Dermatophagoides pteronyssinus*, cat dander, German cockroach, *Alternaria alternata*, mixed grass, and mixed trees. *Open symbols* indicate atopic children, as defined by any specific IgE levels of greater than 0.7 kU_A. **B**, Correlation of total IgE levels to frequencies of blood eosinophils measured by using flow cytometry as Siglec-8⁺CD66b⁺. The *R* value is the Spearman rho correlation coefficient and associated *P* value.

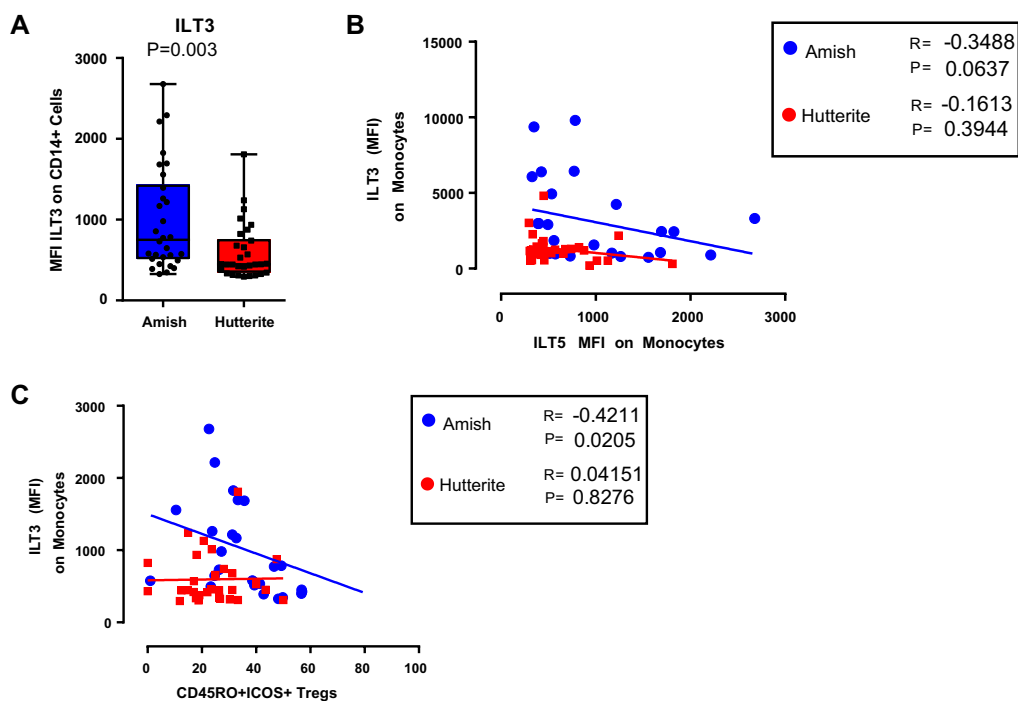


FIG E4. Relationship between ILT3 and ILT5 levels on circulating monocytes. **A**, Expression of ILT3 (mean fluorescence intensity [MFI]) on CD14⁺CD66b⁺ cells in Amish and Hutterite children. *P* values were from the Mann-Whitney nonparametric test. **B**, Correlation of ILT3 and ILT5 expression on monocytes in Amish (red dots) and Hutterite (blue dots) children. **C**, Correlation of ILT3 levels on monocytes with the proportion of activated CD45RO⁺ICOS⁺ Treg cells. *R* values are from the Spearman rho correlation coefficient with associated *P* values.

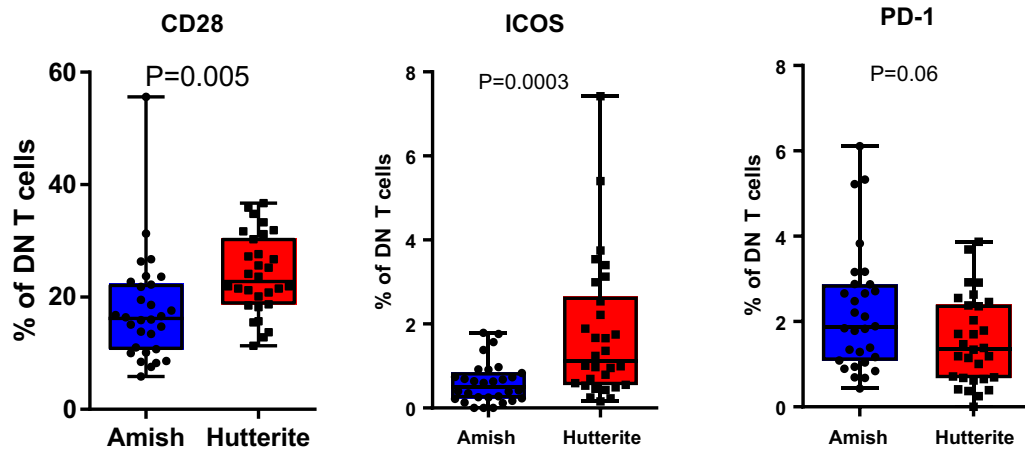


FIG E5. Expression of activation markers on CD4 and CD8 DN T cells. Levels of the costimulatory markers CD28 and ICOS and the coinhibitory molecule PD-1 were measured on DN T cells in Amish and Hutterite children by using flow cytometry. *P* values were from the Mann-Whitney nonparametric test.

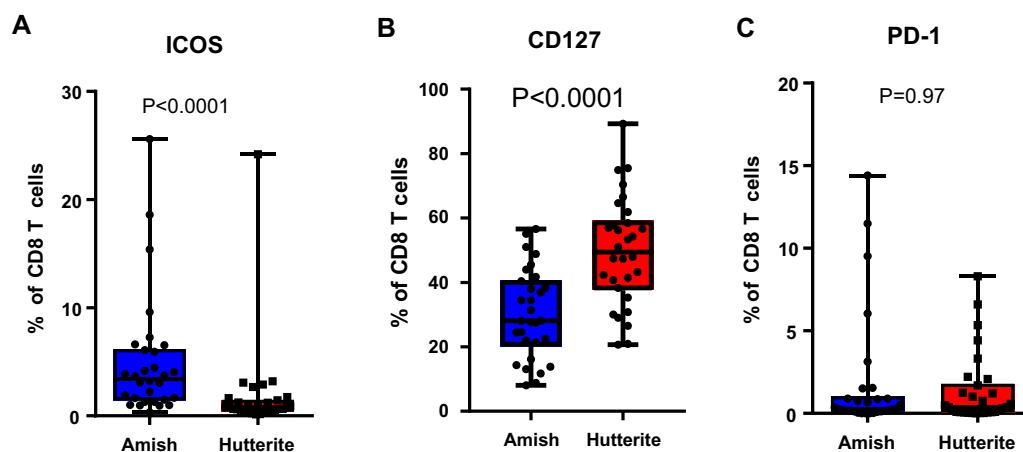


FIG E6. Expression of activation markers on CD8 T cells. Frequency of CD3⁺CD8⁺ T cells expressing the markers ICOS (**A**), CD127 (**B**), and PD-1 (**C**) were measured in Amish and Hutterite children using flow cytometry. *P* values were from the Mann-Whitney test.

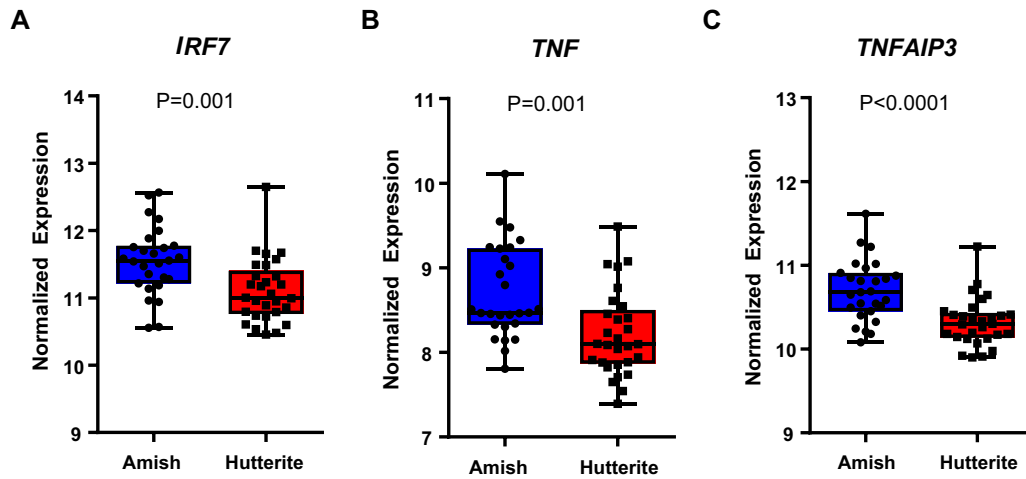


FIG E7. Innate gene expression. Expression of *IRF7* (A), *TNF* (B), and *TNFAIP3* (C) was measured by using a microarray in PBLs isolated from Amish and Hutterite children. *P* values were from the unpaired Student *t* test.

TABLE E1. Demographic and clinical characteristics of the study populations

	Amish (n = 30)	Hutterite (n = 30)
Median age (y [range])	11 (8-14)	12 (7-14)
No. of female subjects	10	10
No. of sibships	15	14
No. of children with asthma	0	6
No. of positive specific IgE results (>0.7 kU _A /L)	5	9
No. of positive specific IgE results (>3.5 kU _A /L)	2	9
Median total serum IgE level (1st-3rd quartile [kU/L])	21 (10-57)	64 (15-288)

TABLE E2. Group comparison *P* values with asthmatic patients excluded or age and sex regressed out*

Figure	Description	Mann-Whitney test, all children	Mann-Whitney test, no asthmatic children	Age-sex regression,† likelihood ratio test
1, A	T cells (% of cells)	.0044	.0063	.06
1, B, left	CD4 T cells (% of CD3)	.2169	.4807	.8
1, B, middle	CD8 T cells (% of CD3)	.5793	.8124	1
1, B, right	DN T cells (% of CD3)	.2284	.6737	.5
1, C, left	CD45RO (% of CD4)	.3998	.377	.8
1, C, middle	CD45RO (% of CD8)	.949	.9189	1
1, C, right	CD45RO (% of DN)	.9611	.9965	.9
2, A	Treg cells (% of CD4)	.1493	.2969	.7
2, C	CD45RO ⁺ ICOS ⁺ (% of Treg cells)	.0009	.001	.01
2, D	PD-1 (MFI on Treg cells)	.0001	.0003	.01
3, A	ILT5 on monocytes	<.0001	<.0001	2.00E-04
3, B	ILT5 on neutrophils	.6865	.8292	.8
4, A	CD28 (% of CD4 Tconv cells)	.0466	.0311	.09
4, B	CD127 (% of CD4 Tconv cells)	.0039	.0029	.001
4, C	ICOS (% of CD4 Tconv cells)	<.0001	<.0001	9.00E-06
4, D	PD-1 (% of CD4 Tconv cells)	.0157	.0512	.4
5, B	CD25 ^{hi} CD8 T cells (% of CD8)	<.0001	<.0001	1.00E-04
6, B	CD28 ^{null} CD8 T cells	<.0001	<.0001	1.00E-04
6, D	IFN-γ	.066	.1032	.01

Figure	Description	Fisher exact test, all children	Fisher exact test, no asthmatic children	
5, C	CD25 ^{hi} CD8 T cells (no. of children with >1%)	<.0001	<.0001	NA

MFI, Mean fluorescence intensity; NA, not available; Tconv, conventional CD4 T.

*The Hutterite group contained 6 asthmatic patients, and the Amish group contained no asthmatic patients. The Bonferroni-corrected *P* value for the Mann-Whitney test is less than .0025 (shown in boldface).†Conditional logistic regression was performed in R software by using a likelihood ratio test with associated *P* values. Significant differences are indicated by a *P* value of less than .05 (boldface values).

TABLE E3. Correlations with asthmatic samples excluded*

Figure	Description	Spearman r, all children	P value, all children	Spearman r, no asthmatic children	P value, no asthmatic children
3, C	ILT5 vs CD45RO ⁺ ICOS ⁺ Treg cells (Amish)	0.4696	.0088	NA	NA
3, D	ILT5 vs CD45RO ⁺ ICOS ⁺ Treg cells (Hutterite)	−0.2646	.1576	−0.4046	.0499
4, E, left	ICOS vs IgE (Amish)	0.089	.653	NA	NA
4, E, right	ICOS vs IgE (Hutterite)	0.373	.046	0.4602	.0271
6, C, left	CD28 ^{null} versus IgE, Amish	−0.417	.025	NA	NA
6, C, right	CD28 ^{null} versus IgE, Hutterite	0.061	.753	−0.0890	.6865
6, E, left	CD28 ^{null} versus IFN-γ, Amish	0.573	.005	NA	NA
6, E, right	CD28 ^{null} versus IFN-γ, Hutterite	0.410	.047	0.4364	.0480
7, A, left	CD28 ^{null} versus IRF7, Amish	0.254	.201	NA	NA
7, A, left middle	CD28 ^{null} versus IRF7, Hutterite	0.073	.707	0.167	.4355
7, A, right middle	IgE versus IRF7, Amish	−0.415	.035	NA	NA
7, A, right	IgE versus IRF7, Hutterite	−0.255	.191	−0.1903	.3962
7, B, left	CD28 ^{null} versus TNF, Amish	0.592	.001	NA	NA
7, B, left middle	CD28 ^{null} versus TNF, Hutterite	0.068	.726	0.0741	.7368
7, B, right middle	IgE versus TNF, Amish	−0.521	.0063	NA	NA
7, B, right	IgE versus TNF, Hutterite	−0.024	.905	0.0672	.7663
7, C, left	CD28 ^{null} versus TNFAIP3, Amish	0.417	.031	NA	NA
7, C, left middle	CD28 ^{null} versus TNFAIP3, Hutterite	−0.058	.767	0.0278	.8973
7, C, right middle	IgE versus TNFAIP3, Amish	−0.513	.007	NA	NA
7, C, right	IgE versus TNFAIP3, Hutterite	−0.067	.736	−0.0079	.9721

Significant differences are shown in boldface.

NA, Not available.

*The Hutterite group contained 6 asthmatic patients, and the Amish group contained no asthmatic patients.