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Immunodeficiency and Autoimmune Enterocolopathy Linked to NFAT5 Haploinsufficiency

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The link between autoimmune diseases and primary immunodeficiency syndromes has been increasingly appreciated. Immunologic evaluation of a young man with autoimmune enterocolopathy and unexplained infections revealed evidence of immunodeficiency, including IgG subclass deficiency, impaired Ag-induced lymphocyte proliferation, reduced cytokine production by CD8+ T lymphocytes, and decreased numbers of NK cells. Genetic evaluation identified haploinsufficiency of NFAT5, a transcription factor regulating immune cell function and cellular adaptation to hyperosmotic stress, as a possible cause of this syndrome. Inhibition or deletion of NFAT5 in normal human and murine cells recapitulated several of the immune deficits identified in the patient. These results provide evidence of a primary immunodeficiency disorder associated with organ-specific autoimmunity linked to NFAT5 deficiency. The Journal of Immunology, 2015, 194: 2551–2560.

It has been increasingly appreciated that patients with primary immunodeficiency syndromes exhibit an increased susceptibility to infections, as well as paradoxical manifestations of autoimmunity (1, 2). Patients with well-recognized disorders, such as common variable immunodeficiency, are susceptible to bacte-

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Abbreviations used in this article: AIE, autoimmune enteropathy; AZA, azathioprine; CAVX, calcineurin; CD, Crohn’s disease; DN, dominant negative; IBD, inflammatory bowel disease; PI, propidium iodide; sIgA, short hairpin RNA; SNP, single nucleotide polymorphism; UC, ulcerative colitis; UPD, uniparental disomy.

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NFAT5 function exhibit impaired proliferation and survival (16, 17). Importantly, lymphoid tissues were shown to be hypomolar compared with blood, suggesting that the ability of lymphocytes, via induction of NFAT5 and related pathways, to adapt to osmotic stress may be important in the initiation of immune responses (18). However, NFAT5 deficiency has not been reported to be associated with human disease.

In this article, we describe a patient with a diagnosis of AIE who presented with symptoms of autoimmunity. Immunologic evaluation demonstrated defects in innate and adaptive immunity, whereas genetic testing revealed de novo haploinsufficiency of NFAT5. We confirmed that the patient had significantly impaired induction of NFAT5 mRNA and protein in response to osmotic stress. Using both dominant-negative (DN) and RNA interference approaches in human and murine lymphocytes, we demonstrate that reduced NFAT5 activity disrupted the ability of T cells to produce TNF-α and to survive in hyperosmolar conditions. Analysis of colonic tissue from patients with active inflammatory bowel disease (IBD), another immune-mediated disease, revealed reduced NFAT5 expression at the mRNA level. Together, these results suggest that NFAT5 may play an important role in immune responses and that NFAT5 deficiency may be linked to human autoimmunity.

Materials and Methods

Study participants

The study protocols with informed consent were approved by the Institutional Review Board/Human Research Protections Program at the University of California, San Diego. Written informed consent was obtained from participants.

Human PBMC isolation and cell culture

PBMCs were isolated from whole human blood on a Ficoll-Paque PLUS (GE Healthcare) gradient. Cell numbers were quantitated using an Accuri C6 Flow Cytometer (BD Biosciences). PBMCs were cultured in AIM V medium (Life Technologies) with human IL-2 (PeproTech) and activated with anti-human CD3 (clone HIT3a) and CD28 (clone CD28.2) Abs (eBioscience). Jurkat cells were grown in RPMI 1640 media (Mediatech) with 10% FBS, 2 mM L-glutamine (Sigma-Aldrich), 50 μM 2-ME (Life Technologies), and penicillin and streptomycin (Sigma-Aldrich). To evaluate lymphocyte function, PBMCs were cultured with anti-CD3 and anti-CD28 Abs in AIM V medium for 24 h; 0.25 mOsm/kg brefeldin A (Sigma-Aldrich) were added during the final 6 h of culture prior to fixation and staining for intracellular cytokines and survival in hypertonic conditions (IBD), another immune-mediated disease, revealed reduced NFAT5 expression at the mRNA level. Together, these results suggest that NFAT5 may play an important role in immune responses and that NFAT5 deficiency may be linked to human autoimmunity.

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Survival in hyperosmotic conditions

Jurkat cells or human PBMCs were cultured in conventional media (280 mOsm/kg, “isotonic”) or media adjusted to 420 mOsm/kg (“hypertonic”) with sterile 4 M sodium chloride (Life Technologies). PBMCs also were evaluated with sterile 4 M sodium chloride (Life Technologies). PBMCs were isolated and activated as described above. Cells were cultured in isotonic or hypertonic conditions for 5 d and then assessed for viability by flow cytometry. The percentage of live lymphocytes was determined by forward scatter and side scatter properties (Supplemental Fig. 2). Viability in hypertonic conditions was expressed as the percentage of cell death due to hypertonicity: the difference between the percentage of live cells in isotonic media and the percentage of live cells in hypertonic media, normalized to the percentage of live cells in isotonic media (% live lymphocytes in isotonic culture − % live lymphocytes in hypertonic culture / % live lymphocytes in isotonic culture). As a secondary, confirmatory method to evaluate cell survival, cells were stained with anti–annexin V and propidium iodide (PI; eBioscience). The percentage of cell death was presented as the difference in annexin V+PI+ cells in hypertonic versus isotonic conditions. Each experiment was repeated at least twice, with ≥3 biological replicates each in Jurkat cells and ≥10 biological replicates in each human PBMCs.

Mice

All animal work was done in accordance with Institutional Animal Care and Use Guidelines of the University of California, San Diego. Mice heterozygous and null for Nfat5 were described previously (9, 19). Nfat5+/- mice were maintained in an isogenic 129/Sm background and were crossed to obtain Nfat5−/− and control Nfat5+/- littermates.

Generation of bone marrow chimeras

Donor mice were treated with 5-fluorouracil; after 4 d, bone marrow cells were harvested and cultured with IL-3, stem cell factor, and IL-6 (PeproTech) overnight and then retrovirally transduced with control or DN NFAT5 constructs daily for 3 d. lethally irradiated (1000 rad) Rag1−/−deficient mice were injected i.v. with transduced bone marrow cells. Blood from recipient mice was obtained for FACS analysis after 8 wk.

Single nucleotide polymorphism arrays and sequencing

The samples analyzed consisted of four cases affected with autoimmune enterocolopathy and seven unaffected controls. HumanCoreExome SNP Arrays were performed. We used the GeneChip module (1.9.4) with Illumina’s GenomeStudio V2011.1. Data were normalized using default parameters, and LogR Ratios and B Allele Frequencies were exported for further analysis in R. Because the platform is designed to detect additional rare variants (exome) that may interfere with our copy number analysis and measurement of heterozygosity, we used only probes measuring common single nucleotide polymorphism (SNPs) corresponding to Illumina’s HumanCore BeadChip (287,064/526,811 probes). An interval of 139 kb was sequenced using 16 long-range PCR amplifications (Supplemental Table II) in 11 individuals. The sequencing generated between 245 × 10^6 and 1594 × 10^6 reads/sample, of which >96% mapped to the genome. The targeted sequencing identified all variants in the interval; therefore, it was used to more accurately measure the rate of heterozygosity. The F statistic was measured using the 451 SNPs genotyped by sequencing and using those present in dbSNP (20).

DNA methylation analysis

DNA was bisulfite modified using the EZ DNA Methylation-Gold Kit (Zymo Research). Methylation of NFAT5 promoter regions was quantified by bisulfite pyrosequencing (PSQ HS 96A pyrosequencing system; QIAGEN), as described previously (21). Methylation levels of CpG sites were analyzed, and mean methylation values for NFAT5 promoter regions were determined. The following primers were used: NFAT5-methylation forward, 5'-GATTGAAAAGGAAGATTATT-3'; NFAT5-methylation reverse, 5'-AATACTACTATTTCCACACATTTGTA-3'; and sequencing primer, 5'-TTTAACAGAATTTTTAAGGTTT-3'.

Plasmid constructs and retroviral transduction

The DN effect of the IDD5 domain of NFAT5 was reported previously (14). GFP or IDD5-GFP was first cloned into the vector pENTR11 (Life Technologies) and subsequently transferred to the KMV-DV retroviral vector, yielding control (KMV-DV-GFP) and DN NFAT5 (KMV-DV-DD5-DD5-GFP) constructs. A short hairpin RNA (shRNA) to silence NFAT5 gene expression was generated using previously described methods (22). The following oligonucleotides (ValueGene), including restriction sites, were used: shLac transfector nontargeting control (5'-TGGTCTGTGA-CAGTGAGCGCCTGGCTAAGTCTTTAGAAAGACCGACGAGTTAATACAGAGATCAGGGCTTGCTCTAGCTCGGA-3') and shNFAT5 (5'-TGGTCTGTGA-CAGTGAGCGCCTGGCTAAGTCTTTAGAAAGACCGACGAGTTAATACAGAGATCAGGGCTTGCTCTAGCTCGGA-3') and shNFAT5 (5'-TGGTCTGTGA-CAGTGAGCGCCTGGCTAAGTCTTTAGAAAGACCGACGAGTTAATACAGAGATCAGGGCTTGCTCTAGCTCGGA-3'). The constructs were digested with EcoRI and XhoI restriction enzymes (New England Biolabs) to generate sticky ends and cloned into the MSCV-LTRmriR30-PIG vector (Thermo Scientific) to generate control shRNA and NFAT5 shRNA plasmids. Viral packaging in transfected 293T cells was performed as previously described (23). To transduce cells with retrovirus containing the constructs, PBMCs were isolated and activated as described above for 2 d. Jurkat cells or PBMCs were resuspended in retroviral supernatants with polybrene at 0.8 μg/ml (American Bioanalytical) and centrifuged at 2500 rpm at room temperature for 90 min. Cells were subsequently cultured for 2 d prior to assessment of cytokine production or survival in hypertonic conditions. Cells expressing constructs were detected on the basis of GFP expression.

Flow cytometry

Cells were stained with the following fluorochrome-conjugated Abs: anti-human CD4 (clone RPA-T4), anti-human CD68 (clone SK1), anti-human CD16 (clone 426), anti-human CD56 (clone HCD56), anti-human TNP-α (clone MAb11), anti-mouse CD4 (clone GK1.5), anti-mouse CD8a (clone 53-6.7), anti-mouse CD49b (clone DX5), anti-mouse CD122 (clone TM-b1), and anti-mouse TNF-α (clone MP6-XT22; all from BioLegend). For intracellular
RNA extraction from cells and colonic biopsies

Total RNA was extracted from PBMCs or Jurkat cells using TRIzol (Life Technologies). One microliter of GlycoBlue (Life Technologies) was added before RNA precipitation with isopropanol. RNA was washed with 70% ethanol and resuspended in nuclease-free water. Equal amounts of total RNA were reverse transcribed to generate cDNA using MultiScribe Reverse Transcriptase and random primers (Applied Biosystems). To extract RNA from colonic biopsies, each sample was immediately placed into RNA STAT-60 (Tel-Test) and then placed on ice. The samples were then loaded onto RNeasy Lipid Tissue Mini columns (Qiagen), according to the manufacturer’s protocol. RNA was eluted in 40 μl TE buffer. RNA was quantified using RiboGreen reagent (Life Technologies).

Measurement of mRNA levels

Quantitative real-time PCR was performed in triplicate using 30 ng cDNA and SsoAdvanced SYBR Green Supermix on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Data analysis was done using Bio-Rad CFX Manager 2.1. The relative amount of NFAT5 mRNA was determined on the basis of the threshold cycle for each PCR product (Cq). Gene expression in PBMCs was normalized with ACTB levels. Gene expression in colonic biopsies was normalized using an average of calnexin (CANX) and RPLP0. ACTB expression was shown to vary in inflamed gastrointestinal mucosa; in contrast, expression of RPLP0 and CANX was shown to be relatively stable in gastrointestinal tissue, even in the setting of inflammation (24, 25). The following primers were used to detect their expression (24, 25): forward, 5'–CAGGTTGGATT–3'; reverse, 3'–CTCCTTAATGTCACGCACGAT–3'; CANX forward, 5'–TGGTGCTGATGGGCAAGAAC–3'; reverse, 5'–AGTGGACATTGAAGG–3'; RPLP0 forward, 5'-TGTTGCTGATGGGCAAGAAC-3'; and actin, 5'–GGAACTCTGT–3'.

Immunoblotting

Proteins were extracted with lysis buffer (50 mM HEPES [pH 7.4], 80 mM NaCl, 5 mM MgCl2, 10 mM EDTA [pH 8], 5 mM sodium pyrophosphate, 1% Triton X-100) and protease inhibitor cocktail (Sigma-Aldrich). Fifty micrograms of protein was resolved on a 4–20% SDS polyacrylamide gel (Invitrogen), transferred onto nitrocellulose membranes (Bio-Rad), and analyzed by immunoblotting using anti-NFAT5 (Thermo Scientific), anti-NFAT1 (Thermo Scientific), and anti–β-actin (Sigma-Aldrich) primary Abs, followed by anti-mouse IRDye 800, and anti-rabbit DyLight 680–conjugated secondary Abs (Rockland).

Statistical analysis

Statistical analysis of the majority of experimental data was performed using a paired Student t test. The Mann–Whitney U test was used to compare expression of NFAT5 in colonic tissue. A p value < 0.05 was considered significant.

Results

Clinical presentation and evaluation

A 19-y-old man born to nonconsanguinous parents of northern European descent was affected with numerous sinopulmonary infections during the first few years of life. At 7 y of age, he began to experience frequent episodes of abdominal pain, intermittent fevers, and nonbloody diarrhea associated with an eczematous rash on his extremities. He was given a presumptive diagnosis of IBD and treated over the next several years with courses of mesalamine derivatives and various immunosuppressives, including corticosteroids, azathioprine, and 6-mercaptopurine, without clear sustained benefit. Endoscopic evaluation of his upper and lower gastrointestinal tracts were remarkable only for mild nodularity in the terminal ileum (Fig. 1A) and rectum (Fig. 1B). H&E staining of biopsy specimens from the duodenum (Fig. 1C) and colon (Fig. 1D) revealed intraepithelial lymphocytes, an absence of goblet cells, and abundant apoptotic enterocytes, consistent with AIE. This diagnosis was confirmed by the presence of anti-goblet cell Abs, and an additional laboratory evaluation was negative for anti-enterocyte Abs and did not otherwise reveal evidence of a known primary immunodeficiency or systemic autoimmune disorder (Table I).

FIGURE 1. Endoscopic and histologic features of gastrointestinal tissue from the proband patient diagnosed with autoimmune enterocolopathy. Upper endoscopy and colonoscopy were performed on the patient; images from the terminal ileum (A) and rectum (B) are shown. H&E staining of biopsy specimens from the duodenum (C) and colon (D) (original magnification ×20). Arrows indicate apoptotic enterocytes.
Given the patient’s history of unexplained infections, an extensive immunologic evaluation was performed to rule out a known primary immunodeficiency syndrome or systemic autoimmune disease (Supplemental Table I). Diagnostic tests excluded severe combined immunodeficiency, immune dysregulation polyendocrinopathy enteropathy X-linked, common variable immunodeficiency, chronic granulomatous disease, systemic lupus erythematosus, and celiac disease, among other disorders. Normal absolute numbers and percentages of naive and memory B and T lymphocytes, as well as regulatory T cells, were observed. Levels of serum Igs (IgG, IgA, and IgM) and Abs to childhood vaccinations were within normal limits.

**Immunologic evaluation reveals immune deficiency**

More comprehensive immunologic testing revealed deficits in various components of innate and adaptive immunity (Table I). Although the numbers of mature B cell subsets (naive, memory, class-switched) in the blood were within normal limits, abnormalities in certain B cell subsets were observed. These included a decrease in the number of Ag-experienced B cell populations, including CD21+ T2 B cells, transitional B cells, and CD38+IgM− plasmablasts (26, 27), along with a corresponding increase in the number of IgM+ naive B cells and CD21− T1 B cells. Although total serum IgG was normal, the patient exhibited a selective IgG subclass deficiency, with a reduction in IgG2 and a complete absence of IgG4 that persisted over time (Supplemental Table II).

Although lymphocyte proliferation in response to mitogens was normal, Ag-specific proliferation in response to Candida albicans or tetanus toxoid was absent (Table I). Moreover, CD8+, but not CD4+, T lymphocytes appeared to be functionally impaired, with a reduced ability to degranulate and to produce the proinflammatory cytokines IFN-γ and TNF-α. Finally, innate immunity was impaired, with a reduction in the absolute number and percentage of CD56+CD16+ NK cells. Together, this constellation of immune deficits involving disparate components of both innate and adaptive immunity suggested the presence of a previously unrecognized primary immunodeficiency disorder.

At the time of the immunologic evaluation, the patient was taking azathioprine (AZA) at a low, subtherapeutic dose that is unlikely to have biologic effects. The therapeutic efficacy of AZA...
is most closely associated with the 6-thioguanine nucleotide metabolite levels in the blood. The patient’s 6-thioguanine nucleotide level was 169 pmol/10^8 RBCs, a concentration that is well below the established therapeutic range associated with a higher likelihood of clinical response: 230–400 pmol/10^8 RBCs. This range was determined by clinical studies that identified metabolite concentrations associated with therapeutic responses (28–30). Nonetheless, although the patient’s AZA dose was subtherapeutic, we cannot entirely exclude the possibility that the drug may have influenced the results of the immunologic evaluation.

**Genetic evaluation reveals hemizygous loss of NFAT5**

To explore a genetic cause for these immunologic abnormalities, we looked for potential copy number variation using an oligo-SNP array. This analysis revealed a 559-kb deletion at 16q22.1 (69,580,702-70,139,542) in one chromosome that contained eight genes: NFAT5, MIR1538, NQO1, NOB1, WWP2, MIR140, CLEC18A, and PDXDC2 (Fig. 2A). We tested both parents, neither of whom harbored a similar deletion, indicating that the deletion in the proband was de novo. None of the deleted genes has been associated with human disease; with the exception of NFAT5, little is known about the function of the genes in this region.

NFAT5 is a transcription factor and a member of the family of Rel-like domain-containing factors, which includes NF-κB and the calcineurin-dependent NFATc proteins (12, 14). NFAT5 was demonstrated to play a critical role in regulating responses to extracellular hypertonicity in a variety of cell types, in particular, renal cells and cells of the innate and adaptive immune systems (9, 12–15). Moreover, NFAT5 was shown to regulate expression of a number of cytokines, growth factors, and surface receptors in lymphocytes and macrophages in a tonicity-independent manner (9, 12, 14). Finally, recent work implicated NFAT5 in the induction of pathogenic Th17 lymphocytes in a murine model of autoimmunity (31, 32), raising the intriguing possibility that abnormalities in components of the osmoadaptation pathway might result in human disease.

To confirm that haploinsufficiency of NFAT5 resulted in reduced expression in the patient’s cells, PBMCs isolated from the patient

![FIGURE 2](files/fig2.png)

**FIGURE 2.** NFAT5 deficiency associated with autoimmune enterocolitis. (A) Copy number status of NFAT5. The log ratio (upper panel) and B allele frequency (lower panel) of the Infinium HumanCore BeadChip array located ∼10 Mb around the NFAT5 gene (asterisk). Expression of NFAT5 protein (B) and mRNA (C) from PBMCs isolated from the proband and a healthy control measured after stimulation for 3 d with anti-CD3 and anti-CD28 Abs, followed by culture in hypertonic media for 1 d.

![FIGURE 3](files/fig3.png)

**FIGURE 3.** Copy number status of NFAT5. (A–C) The log ratio (upper panels) and B allele frequency (lower panels) of the Infinium HumanCore BeadChip array located ∼10 Mb around the NFAT5 gene (asterisk) are displayed for three individual patients with autoimmune enterocolitis.
and an unrelated healthy control were subjected to hypertonic culture conditions. Levels of NFAT5 protein and mRNA were assessed by immunoblotting and real-time quantitative PCR. We observed a 6-fold reduction in NFAT5 protein (Fig. 2B) and a 5.3-fold reduction in NFAT5 mRNA levels (Fig. 2C) in the patient’s cells compared with control cells.

We investigated NFAT5’s status in three additional patients with AIE using copy number SNP arrays and targeted resequencing. Although the arrays did not demonstrate NFAT5 deletion (Fig. 3), NFAT5 sequencing revealed a deficit in heterozygous variants, suggesting a loss of heterozygosity, or uniparental disomy (UPD), in all of the patients. All four AIE patients had an F statistic, the expected heterozygosity within a population, >0.8, whereas 0 of 7 of the unaffected controls had an F statistic that surpassed this threshold (Fig. 4). In addition, we considered an additional population control and examined 78 white individuals from the 1000 Genomes Project. We measured the F statistic using the 451 SNPs for which the genotypes were available. Only 10/78 of the population controls had such a high F ($p \approx 2.6 \times 10^{-17}$). UPD occurs when both homologs of a chromosomal segment are inherited from only one parent. UPD can cause disease if an individual inherits two abnormal copies of a gene from the parent of origin, resulting in an altered level of gene expression (33, 34). Aberrant hypermethylation of the NFAT5 promoter was not detected in the proband nor in the additional patients with AIE (Supplemental Fig. 1). Nonetheless, AIE patients exhibited a significantly higher incidence of UPD than did our measured controls and the population controls, raising the possibility that inheritance of NFAT5 abnormalities from one parent might influence disease penetrance.

**NFAT5 deficiency is associated with immunologic deficits in T lymphocyte function**

To provide a mechanistic link between NFAT5 deficiency in the proband and the abnormalities observed in his immune system, we tested whether inhibition of NFAT5 function in immune cells would recapitulate the patient’s deficits. Because prior studies demonstrated that NFAT5 deficiency in proliferating murine T lymphocytes impairs their survival capacity in hypertonic conditions (16, 18), we first tested the viability of the patient’s PBMCs and observed reduced survival in hypertonic culture conditions (Fig. 5A) relative to control cells.

To provide evidence that this effect might be due to NFAT5 deficiency, we used a DN NFAT5 construct, which was shown to interfere with dimerization, thereby reducing NFAT5 transcriptional activity by ∼75% (14). Using a retroviral approach, we expressed the DN NFAT5 or control construct in Jurkat cells, a human T lymphocyte cell line, and observed that proliferating cells expressing the DN NFAT5 construct exhibited a survival defect in hypertonic conditions (Fig. 5B) similar to that displayed by the patient’s PBMCs. As a second, confirmatory method to measure cell viability, we stained the cells for annexin V and PI, with similar results (Fig. 5D).

As a confirmatory approach, we generated an shRNA construct that reduced NFAT5 expression by ∼50% (Supplemental Fig. 2). Jurkat cells were transduced with retrovirus-expressing control shRNA or shRNA targeting the NFAT5 gene and subsequently cultured in isotonic or hypertonic media. We observed that proliferating cells with reduced expression of NFAT5 exhibited decreased viability in hypertonic conditions (Fig. 5C, 5E, Supplemental Fig. 2).

To determine whether NFAT5 deficiency might be responsible for decreased production of TNF-α by the patient’s CD8+ T cells (Fig. 5F, Table I), we expressed the control or DN NFAT5 constructs in PBMCs isolated from healthy controls. CD8+ T cells expressing the DN NFAT5 construct exhibited reduced production of TNF-α (Fig. 5G), analogous to the defect observed in the patient’s CD8+ T cells. To provide further evidence that NFAT5 regulates T lymphocyte cytokine production, we generated bone marrow chimeras in which CD8+ T cells have diminished NFAT5 function. Bone marrow cells from wild-type mice were transduced with control or DN NFAT5 constructs and adoptively transferred into lethally irradiated Rag1-deficient mice. After reconstitution, CD8+ T cells from the blood were analyzed for intracellular cytokine production. We observed reduced TNF-α production in CD8+ T cells expressing the DN NFAT5 construct compared with those cells expressing the control construct (Fig. 5H). Taken together, these human and murine data are consistent with prior evidence that TNF-α is a direct target of NFAT5 (9, 14) and suggest that NFAT5 deficiency may underlie the impaired functional capabilities of the proband’s T lymphocytes.

**NFAT5 deficiency may be associated with a reduction in NK cells**

We next investigated whether NFAT5 deficiency might be related to the finding of reduced NK cells in the patient (Fig. 6A, Table I). Because a reduction in the number of NK cells had not been reported previously in NFAT5-deficient mice, we analyzed NK cells from wild-type, NFAT5-heterozygous, and NFAT5-null mice. The percentages and absolute numbers of CD122+CD49b+ NK cells were reduced in a dose-dependent manner, with a modest reduction observed in NFAT5-heterozygous mice and a more pronounced reduction in NFAT5-null mice (Fig. 6B). These results suggested that NFAT5 deficiency might contribute to the development and/or survival of NK cells, although the underlying mechanism remains unknown and it remains possible that other factors may be involved.

**NFAT5 expression is reduced in patients with IBD**

To determine whether the link between reduced NFAT5 expression and autoimmunity might be generalizable to other immune-mediated diseases, we examined NFAT5 mRNA expression in intestinal tissue biopsies from patients with IBD. Crohn’s disease (CD) and ulcerative colitis (UC), which together make up IBD, are believed to result from an aberrant immune response to commensal gut microbes, leading to chronic intestinal inflammation. Compared with healthy controls, we observed that NFAT5 mRNA expression was significantly reduced in patients with active UC and CD (Fig. 7), raising the possibility that NFAT5 and other components of the osmoreadaptation pathway may be dysregulated in IBD.
FIGURE 5. Dysregulated T lymphocyte responses linked to NFAT5 deficiency. (A) Survival capacity of proliferating PBMCs isolated from the proband and 13 healthy controls. PBMCs were activated with anti-CD3 and anti-CD28 Abs and cultured in isotonic (280 mOsm/kg) or hypertonic (420 mOsm/kg) media; viability was quantified using flow cytometry based on forward and side scatter properties. Survival is expressed as the percentage cell death in hypertonic media normalized to isotonic media (% live lymphocytes in isotonic culture - % live lymphocytes in hypertonic culture / % live lymphocytes in isotonic culture). Jurkat cells were retrovirally transduced with control or DN NFAT5 constructs (B and D) or control or NFAT5 shRNA constructs (C and E). Cells were rested for 2 d and then cultured in either isotonic or hypertonic media for an additional 5 d. Cells expressing constructs were detected on the basis of GFP positivity. Cell survival was calculated as in (A). Cells were stained with annexin V and PI to quantify apoptotic and (Figure legend continues)
**FIGURE 6.** Decreased NK cell frequency in the absence of NFAT5. (A) NK cell frequencies in the proband and 25 healthy controls, assessed as the percentage of CD16^+CD56^+ events gated on lymphocytes. (B) NK cell frequencies in the spleen of wild-type (Wt/Wt), NFAT5-heterozygous (Wt/Nfat5^+^), and NFAT5-null (Nfat5^-/-Nfat5^-) mice were assessed as the percentage of CD49b^-CD122^- events gated on lymphocytes (left panel). The total NK cell numbers in each mouse genotype were quantified (right panel). Error bars represent SEM. *p < 0.05, **p < 0.001.

**Discussion**

In the current study, we describe a patient with a previously unrecognized primary immunodeficiency syndrome affecting components of innate and adaptive immunity, predominantly manifesting as autoimmune enterocolitis. Although we cannot completely rule out the contribution of other genes found in the patient’s deleted segment, to our knowledge these studies provide the first evidence linking this syndrome with a deficiency in NFAT5, which has not been associated with disease in mice or humans previously. Several groups showed that an important consequence of NFAT5 deficiency in mice is impaired lymphocyte proliferation and survival in hypertonic culture conditions (16, 18). The critical need for osmoadaptative mechanisms in lymphocytes is underscored by the finding that lymphoid tissues are hyperosmotic relative to blood (18). Thus, because lymphocytes encounter microbes within hypertonic lymphoid tissues, these cells must be capable of osmoadaptation to proliferate and orchestrate antimicrobial functions. Our observation that lymphocytes from the proband exhibited a reduced ability to survive in hypertonic conditions ex vivo suggests that these cells also may be functionally impaired in vivo in the setting of microbial challenges. This impairment may be more pronounced in the gut microenvironment, where rapid shifts in tonicity due to fluid absorption combined with constant microbial stimuli may explain, in part, the predominance of gastrointestinal manifestations in the proband (35).

Functional defects observed within different types of immune cells in the proband support the contention that NFAT5 deficiency may indeed confer a state of immunodeficiency. First, CD8^- T lymphocytes from the patient exhibited reduced degranulation and production of the proinflammatory cytokine TNF-α, consistent with prior evidence that TNF-α is a downstream target of NFAT5 (9, 14). Second, NK cells, innate immune cells with important roles in antiviral and antitumor immunity, were reduced in the proband and in NFAT-deficient mice. These results raise the possibility that NFAT5 influences the development and/or survival of NK cells, although the precise mechanisms will require additional investigation, and other factors are likely to be contributory. Taken together, these findings suggest a role for NFAT5 in innate and adaptive immune responses against microbial pathogens.

The clinical significance and etiology of the findings within the patient’s B lymphocyte compartment remain unclear. Certain developing B cell subsets appeared to be dysregulated in the patient, raising the possibility that BAFF, a regulator of B cell proliferation and differentiation and a known gene target of NFAT5 (36), might be involved. However, the patient’s BAFF levels and BAFF receptor levels were normal, suggesting that the dysregulation of the patient’s B cell subsets may be linked to NFAT5 via an alternate mechanism or unrelated to NFAT5 deficiency altogether. Moreover, the patient exhibited an IgG2 subclass deficiency, which has been associated with an increased susceptibility to sinopulmonary infections, as well as certain autoimmune disorders (37–39). However, the presence of protective titers of Abs to microbes against which he was vaccinated seemed to suggest functional humoral immunity and contrasts with the...
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finding of reduced Ag-specific Abs in immunized NFAT5−
heterozygous mice (18).

The patient exhibited a defective proliferative response against
*C. albicans* and tetanus toxoid but not against mitogens, sug-
gestig an Ag-specific deficiency. The patient had been vaccinated
against tetanus and had likely been exposed to *Candida*, which is
ubiquitously found in the environment, arguing against a lack of
primary exposure to these Ags as the underlying mechanism.
Another possibility is a defect in the ability of the proband to form
memory T cells makes this possibility less likely. Finally, the
patient’s lymphocytes might exhibit a defect in their ability to be
activated by Ag and undergo proliferation. However, the precise
mechanism underlying the patient’s defect in Ag-specific prolif-
eration remains unknown.

Thus, our findings provide the first evidence, to our knowledge,
that genetic abnormalities in NFAT5 might be associated with
AIE. However, we did not find the identical genetic deletion in the
AIE patients that were evaluated, making it unlikely that abnor-
nalities in NFAT5 underlie all cases of AIE. Nonetheless, we
observed that NFAT5 deletion in the proband was associated with
defects in lymphocyte function, and inhibition of NFAT5 in
normal murine and human cells recapitulated those deficits. Toget-
ner with the finding of reduced NFAT5 gene expression in the intesti-
nal mucosa of patients with IBD, these results suggest that
NFAT5 and other components of the osmoadaptation pathway
may play a previously unappreciated role in a variety of human
immune-mediated diseases and highlight the need for additional
studies.

Although the association between primary immunodeficiency and
autoimmunity has long been appreciated, the molecular
mechanisms underlying this association are not understood. It was
suggested that defects in one component of immunity result in
inadequate control of microbes, leading to a persistent and patho-
logic state of activation within a second arm of immunity that
ultimately triggers an autoimmune response (1, 2). Autoimmune
manifestations are often treated with immunosuppressive medi-
cations; however, if the initial triggering event is indeed immu-
nodeficiency, an alternative therapeutic approach might be to
correct the immune component that is defective. Taken together,
our findings suggest that patients presenting with unexplained
infections and/or autoimmune manifestations should be consid-
ered for a comprehensive genetic and immunologic evaluation that
may reveal new insights into the mechanisms underlying their
conditions.

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Disclosures

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