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NFAT5 Regulates T Lymphocyte Homeostasis and CD24-Dependent T Cell Expansion under Pathologic Hypernatremia

Rosa Berga-Bolaños, Katherine Drews-Elger, Jose Aramburu, and Cristina López-Rodríguez

Immune cells rely on the transcription factor NFAT5 to adapt to hypertonic stress. The hypertonicity-dependent role of NFAT5 in T cells in vivo remains unclear because mouse models of NFAT5 deficiency have produced substantially different T cell phenotypes. In this study, we analyzed the T cell compartment in NFAT5-null and T cell-specific NFAT5 knockout mice. We found that NFAT5-null mice had constitutive, pronounced hypernatremia and suffered a severe immunodeficiency, with T cell lymphopenia, altered CD8 naive/memory homeostasis, and inability to reject allogeneic tumors. By contrast, T cell-specific NFAT5 knockout mice had normal plasma tonicity, rejected allogeneic tumors, and exhibited only a mild, low-penetration memory bias in CD8 cells. Notably, when T cells from these mice were cultured ex vivo in hypernatremic media, they exhibited features found in NFAT5-null mice, with pronounced naive/memory imbalance and impaired homeostatic survival in response to IL-7, as well as a severe inhibition of their mitogen-induced proliferation. By analyzing surface receptors whose expression might be affected in NFAT5-deficient cells, we identified CD24 as a novel NFAT5 target induced by hypertonicity both in vitro and in vivo, and required to sustain T cell expansion under osmotic stress. NFAT5 bound to the CD24 promoter in response to hypertonicity facilitated the local derepression of chromatin and enhanced the expression of CD24 mRNA and protein. Altogether, our results indicate that the systemic hypernatremia of NFAT5-null mice is a major contributor to their immunodeficiency, and highlight the role of NFAT5 and CD24 in the homeostasis of T cells under osmotic stress in vivo. The Journal of Immunology, 2010, 185: 6624–6635.

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The NFAT5, also known as tonicity enhancer binding protein (TonEBP), is a transcription factor required for the adaptation of mammalian cells to osmotic stress (27, 28). NFAT5 induces the expression of enzymes and transporters that increase the intracellular concentration of compatible organic osmolytes, thus allowing cells to function under prolonged hypertonic stress conditions (27–30). Mammals maintain a constant plasma tonicity in a narrow range around 300 milliosmoles per kilogram (mOsm/kg), whereas certain anatomical niches, such as the renal medulla (at >1200 mOsm/kg) and the matrix between intervertebral discs (420–450 mOsm/kg) are naturally hypertonic. However, dehydration and osmoregulatory disorders can cause plasma hypernatremia and systemic hypertonicity that affect the entire organism (31–38). Previous work by us and others had shown that NFAT5 is essential for cells of the renal medulla (39), but can also be activated in leukocytes (T cells, B cells, macrophages) by hypertonicity levels in the range recorded in plasma and tissues of patients and animal models of hypernatremia (360–430 mOsm/kg) (40–44). Under these tonicity conditions, NFAT5 is critical for the cell cycle progression and proliferation of T cells (42, 45). The analysis of mouse models hemizygous for Nfat5 (Nfat5+/−) or its regulator, Brx, had revealed common defects between them, such as weaker Ag-specific Ab responses and reduced splenocyte numbers (43, 45). In addition, Nfat5 hemizygous mice presented thymic hypocellularity of both CD4 and CD8 cells (45). An initial interpretation attributed these defects to the mildly hypertonic milieu of lymphoid organs (~330 mOsm/kg) (45). However, our characterization of conditional knockout mice that lacked NFAT5 only in T cells showed that these mice had normal numbers and proportions of thymocyte and splenocyte populations (42), suggesting that the natural tonicity of lymphoid organs was not overtly deleterious for T cells in vivo. In view of these differences, we decided to analyze in greater detail the T cell compartment in NFAT5-null and T cell-specific conditional knockout mice.

In this paper, we show that NFAT5-null mice suffer severe T cell lymphopenia, impaired ability to reject allogeneic tumors, and exhibit pathological hypernatremia in plasma with hypertonicity values of 360–411 mOsm/kg. These mice also had significantly greater proportions of central and effector memory CD8 T cells, and reduced numbers of naive cells, which together with their persistent lymphopenia suggested an ongoing homeostatic response that was, however, insufficient to compensate for the T cell depletion. By contrast, T cell-specific NFAT5 knockout mice (Nfat5fl/fl, CD4-Cre) had isotonic plasma, normal T cell numbers with only a mild bias toward memory in CD8 cells, and were competent to reject allogeneic tumors; and 3) the NFAT5-deficient mice showed that these mice had normal proportions of naive and central memory CD8 T cells, and were not overtly impaired in their ability to reject tumors.

Materials and Methods

Mice

Mice used in this study have been previously described (39, 42). Because NFAT5-null mice in a mixed 129/sv-C57BL/6 background had a severe mortality rate from late embryonic development to early perinatal stages (39, 45), we bred them for more than 10 generations to a pure 129/sv background and observed that the rate of survival of NFAT5-null mice (Nfat5−/−) increased, with more than 50% of the expected mendelian ratio of Nfat5−/− mice reaching adulthood (not shown). In contrast, we did not obtain any NFAT5-null mice in a pure C57BL/6 background (not shown). Nfat5−/− mice were maintained in a syngeneic 129/sv background, and were crossed to produce Nfat5−/− mice and control Nfat5+/+ littermates. Conditional mice that lack NFAT5 in T cells, Nfat5ΔTH, CD4-Cre or Nfat5ΔBV mice, were maintained on a pure C57BL/6 background. All mice were analyzed between 6 to 9 wk of age. Mice were bred and housed in specific pathogen-free conditions, and animal handling was performed according to institutional guidelines approved by the ethical committee (Parc de Recerca Biomèdica de Barcelona Animal Care and Use Committee).

Tumor growth assay

J558L mouse plasmacytoma cells (BALB/c background) were cultured in DMEM supplemented with 10% (v/v) FBS, 2 mM l-glutamine, and antibiotics (46). Cells were harvested, washed three times in PBS, and resuspended at a density of 5 × 10⁶ cells/200 μl and injected s.c. in the abdominal skin (shaved) of 6- to 8-wk-old C57BL/6, 129/sv, or syngeneic BALB/c mice of the indicated genotypes. Mice that did not develop tumors by 15 d were defined as "tumor free." Mice were sacrificed 15 d after implantation or when tumors exceeded 1.5 cm in diameter.

Hypertonic stress

The osmolality of the culture medium was measured in a VAPRO 5520 vapor pressure osmometer (Wescor, Logan, UT) using the VetScan Comprehensive Diagnostic Profile reagent rotor (Comprehensive Metabolic Panel, reference 400-0028; Abaxis Medical Diagnostics, Union City, CA) combined with the use of the VetScan Chemistry Analyzer (VS2; Abaxis Medical Diagnostics), following manufacturer’s instructions.

Analysis of plasma metabolic parameters

Quantitative determination of blood urea nitrogen (BUN), amylase (AMY), sodium, or calcium concentrations in heparinized whole blood was done using the VetScan Comprehensive Diagnostic Profile reagent rotor (Comprehensive Metabolic Panel, reference 400-0028; Abaxis Medical Diagnostics, Union City, CA) combined with the use of the VetScan Chemistry Analyzer (VS2; Abaxis Medical Diagnostics), following manufacturer’s instructions.

Splenocytes and thymocytes were obtained from 6- to 9-wk-old Nfat5−/− and littermate Nfat5+/+ 129/sv, or Nfat5−/− CD4-Cre and littermate Nfat5ΔTH, C57BL/6 mice. The genotype of each mice was confirmed when experiments were performed. Thymus and spleen were disected, and splenocytes were depleted of erythrocytes by hypotonic lysis using 1 × BD Pharm Lyse (Cat. 555899; BD Biosciences, San Jose, CA). Proliferating T cells were obtained by activating splenocytes (2.5 × 10⁶ cells/ml) with 2.5 μg/ml Con A (Cat. C-2010; Sigma-Aldrich, St. Louis, MO) plus 25 ng/ml recombinant human IL-2 (Proleukin; Chiron [formerly Eurocetus]; Emory, Atlanta, GA) in culture medium (DMEM; Life Technologies, Rockville, MD) supplemented with 10% FBS (Cat. 10270-106; Life Technologies), non-essential amino acids (Life Technologies), 2 mM l-glutamine (Life Technologies), 50 μM β-mercaptoethanol (Life Technologies), 1 mM sodium...
pyrurate (Life Technologies), and antibiotics penicillin and streptomycin (Life Technologies). Splenocyte cultures, grown under isometric or hypotonic conditions, were stained for flow cytometry analysis or were cultured under stress conditions for 3 or 6 d with either Con A plus IL-2 or with 10 ng/ml recombinant mouse IL-7 (Cat. 2340072; ImmunoTools, Friesoythe, Germany) for 24 h in complete medium supplemented with IL-2. Finally, they were adjusted to 2 × 10^6 cells/ml and cultured under isometric or hypotonic conditions, as indicated in the figure legends.

Abs

The rabbit anti-NFAT5 polyclonal Abs recognizing its DNA binding domain, the C-terminal and the N-terminal regions of NFAT5, were previously described (42, 47). Goat anti-pyrurate kinase (AB1235) was purchased from Chemicon International (Temecula, CA), HRP-labeled rabbit anti-IgG (Cat. NA934-V) was from Amersham Biosciences (Uppsala, Sweden), and HRP-, labeled anti-goat IgG (Cat. P010.60) was from DAKO Denmark A/S (Glostrup, Denmark). Hamster anti-mouse CD3 (Cat. 553058) and hamster anti-mouse CD28 (Cat. 553295) were from BD Biosciences, and goat anti-hamster IgG was from MP Biosciences (Solon, OH; Cat. 55397). PE-labeled Abs (anti-IgGk, κ (Cat. 553989), anti-CD3 (Cat. 553049), anti-CD4 (Cat. 553049), anti-CD8 (Cat. 553033), anti-CD24 (clone M16/9, Cat. 553526), anti-CD28 (Cat. 553297), anti-CD2L (Cat. 555151), anti-CD69 (Cat. 552537), anti-TCRβ (Cat. 553754), and anti-IL-7Re (Cat. 552543), FITC-labeled Abs (anti-CD4 (Cat. 553047), anti-CD8 (Cat. 553031), anti-B220 (Cat. 553088), anti-CD25 (Cat. 553072), and anti-CD44 (Cat. 551133), PerCP-labeled anti-CD4 (Cat. 553052), and allophycocyanin-labeled anti-CD8 (Cat. 553035) were from BD Biosciences. The 20C9 hybridoma clone producing an anti-CD24 blocking Ab (48) was kindly provided by Dr. Yang Liu (University of Michigan, Ann Arbor, MI). The hybridoma was grown under conditions that enhance Ab accumulation in the culture media (49), and the supernatant was used in a 1:10 dilution to block CD24 in T cells cultured in hypotonic conditions. For some experiments, the 20C9 hybridoma supernatant was concentrated by amm0-nium sulfate precipitation (49), dia lyzed against PBS, and titrated (as in Fig. 5D and Supple mental Fig. 5A) to determine its optimal anti-CD24 blocking dilution.

Flow cytometry

Two × 10^6 cells were blocked for 20 min in 1× PBS containing 3% FBS, 0.1% sodium azide, and anti-FcγR Ab (1 μg/10^6 cells; Cat. 553142; BD Biosciences). Cells were then incubated with surface marker-specific Abs in the same solution (1 μg/10^6 cells) and analyzed by FACS Calibur or LSR II flow cytometers (BD Biosciences) and FlowJo (Tree Star, Ashland, OR) software. For monitoring proliferation, cells were labeled with 5 μM Vybrant carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) (Ref. V12883; Invitrogen) in PBS at room temperature for 7 min; then an equal volume of FBS was added and cells were subsequently incubated for 5 min. Unincorporated CFDA-SE was removed by washing cells twice with complete medium.

Adaptive transfer assays

Proliferating T cells from a pool of Nfat5^{g−/−}, Cd4−/− or control Nfat5^{+/−}, Cd4−/− mice (2–4 mice per group) were obtained as described earlier by activating spleenocytes with 2.5 μg/ml Con A plus IL-2 in 2–3 complete medium for 24 h. Cells were next depleted of residual B cells, labeled with 5 μM CFDA-SE, and incubated (10 × 10^6 cells/200 μl) into the peritoneum of Nfat5^{g−/−} or control Nfat5^{+/−} syngeneic host mice (2–3 mice per group). After 5 or 6 d, blood samples were taken from the tail vein to measure plasma osmolality, mice were sacrificed to analyze the induction of CD24, and the proliferation rate of transferred CD4+ and CD8+ cells was obtained from the spleen of host mice. For the adaptive transfer assays blocking CD24, a pool of T cells from control Nfat5^{+/−} and Cd4−/− mice was cultured for 3 d with 1 or 2.5 μg/ml Con A plus IL-2 in hypotonic conditions (350 mM NaCl). Cells were then depleted of residual B cells, labeled with 5 μM CFDA-SE, incubated with a saturating dilution of concentrated 20C9 Ab, and then inoculated into the peritoneum of Nfat5^{g−/−} or control Nfat5^{+/−} mice (2–3 mice per group). Transferred cells were isolated 48 h later from the spleen and analyzed for their proliferation and persistence of the 20C9 blockade.

Protein sample preparation and Western blot analysis

Cells were lysed (30 min at 4˚C) in 50 mM HEPES (pH 7.4), 80 mM NaCl, 5 mM MgCl2, 10 mM EDTA, 1% Triton X-100, 5 mM sodium pyrophosphate, 20 μM β-glycerophosphate, and protease inhibitors PMSF, leupeptin (Cat. L2884; Sigma-Aldrich), aproatin (Cat. 236624; Roche, Indianapolis, IN), and pepstatin A (Cat. P5318; Sigma-Aldrich). Lysates were cleared by centrifugation (18,000 × g, 10 min, 4˚C), and the protein concentration in the supernatants was determined using the BCA Protein Assay (Cat. 23227; Pierce, Rockford, IL). Equal amounts of protein from each sample were separated in SDS-polyacrylamide gels under reducing conditions, transferred to nitrocellulose membranes (Protran 10401396; Whatman, Dassel, Germany), and detected with specific primary Abs followed by HRP-labeled secondary Abs and ECL (Supersignal West Pico Chemiluminescent Sub- strate; Pierce). Pyruvate kinase was used as protein loading control.

Measurement of mRNA levels

Total RNA was isolated using the RNeasy system (Cat. 74104; Qiagen, Valencia, CA) following manufacturer’s instructions and quantified in a NanoDrop (ND-1000) spectrophotometer. Two micrograms total RNA were reverse-transcribed to cDNA using SuperScript III RT (Life Technologies) and random primers (Invitrogen). For real-time quantitative PCR, LightCycler 480 SYBR Green I Master Mix (Cat. 11608521; Roche) and a LightCycler 480 system (Roche) were used following the instructions provided by the manufacturer. Samples were normalized to L32 mRNA levels using the LightCycler 480 SWL 5.1 software. Primer sequences for the PCR reactions were: 5′-ATT GTC GAC CAG CAA CTT GC-3′; 5′-ATT CGC CCA AAA GGG AAC TGG AG-3′; reverse: 5′-GAA AGC CTG GCT GTG TTC TG-3′; CD24—forward: 5′-CAG ATC CCT ACT TAC CA-3′; reverse: 5′-ACG GTG CAA CAG ATG TTG GG-3′.

Chromatin immunoprecipitation

T-cell blasts grown for 96 h and stimulated during 6 or 12 h with 500 mOsm/kg (40 × 10^6 cells in 20 ml) were fixed with 0.75% formaldehyde for 10 min at room temperature. Formaldehyde was then quenched with 3 ml of 2.5 M glycine for 5 min. After washing the cells twice with cold PBS, and once with cold PBS with 1 mM PMSF, cells were lysed using 1 ml lysis buffer (50 mM HEPES, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 5 μg/ml leupeptin/aproatin, 1 μg/ml pepstatin A, 10 mM NaF, 10 mM sodium orthovanadate, and 0.1% β-glycerophosphate) for 30 min on ice. Lysates were sonicated (Bioruptor; Diagenode, Liège, Belgium) for 10 cycles of 30 s to obtain DNA fragments between 500 and 1000 bp, and centrifuged to remove insoluble debris. Supernatants were collected and 3% of each sample was saved to be used as a measure of chromatin input for normalization. The rest of the sample was diluted 10 times in dilution buffer (20 mM Tris-HCl, pH 8, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 1 mM PMSF, 5 μg/ml leupeptin/aproatin, 1 μg/ml pepstatin A, 10 mM NaF, 10 mM sodium orthovanadate, and 0.1% β-glycerophosphate) for immunoprecipitation. Samples were preclariﬁed with protein A-Sepharose beads (Cat. 17-0780-01; Amersham) that were previously blocked with salmon sperm DNA (Cat. 11 467 140 001; Roche) and 1× BSA (B9001S; NEB, Beverly, MA) overnight at 4˚C. After removing the preclariﬁed beads, 20 μl preimmune serum, or a mixture of two rabbit polyclonal NFAT5-speciﬁc Abs that recognize its N-terminal and DNA binding domains (47) (10 μg each) were added to the lysed and cross-linked, incubated samples overnight at 65˚C with 6 μg/ml RNase (Cat. 11 119 915 001; Roche). DNA was puriﬁed using the Qiagen PCR puriﬁcation system (Cat. 281049) and then subjected to real-time quantitative PCR. Immunoprecipitated DNA from each sample was normalized to its respective chromatin input. Previous sequences for the PCR reactions were: C2β promoter—forward: 5′-CAG ATC TTC ACT TAC CAG AC-3′, reverse: 5′-GAG AGA CAG GAG GTG TTC TG-3′; NFAT5—forward: 5′-CAG CCA AAA GGG AAC TGG AG-3′; reverse: 5′-GAG ATC CCT ACT TAC CA-3′.
Statistical analysis

Statistical significance of the experimental data was determined with paired Student t test.

Results

NFAT5-null mice have lymphopenia restricted to their T cell compartment

Although mice hemizygous for Nfat5 (Nfat5+/−) suffer lymphopenia (45), we recently reported that a mouse model lacking NFAT5 only in T lymphocytes (Nfat5fl/fl, CD4-Cre) had normal numbers of T cells (42). We therefore considered that non-T cell-autonomous effects might underlie the differences between both mouse models. To address this possibility, we analyzed different parameters indicative of T cell dysfunction in NFAT5-null mice (39) and conditional, T cell-specific Nfat5fl/fl, CD4-Cre mice. Whereas NFAT5-null mice had a pronounced reduction in size and cellularity of their spleen (Fig. 1A), Nfat5fl/fl, CD4-Cre mice had normal numbers of splenocytes and thymocytes despite lacking NFAT5 in these populations (42) (Supplemental Fig. 1A–C). This reduction was more severe than that reported for Nfat5 hemizygous mice (45) but was nonetheless proportional to the decreased body size (39) (Fig. 1A). Therefore, when the spleen cellularity of NFAT5-null mice was normalized to body weight and compared with that of wild-type littermates, we observed a specific reduction in the relative number of T lymphocytes, but not in B cells (Fig. 1B). Thymocyte numbers were also reduced with respect to body size in these mice, similar to what was described for Nfat5 hemizygous mice (45) (Fig. 1A). T cell lymphopenia in NFAT5-null mice affected both CD4 and CD8 cells, but was more severe in the latter (Fig. 1B). Similar results were obtained for the distribution of T and B cells in peripheral blood of NFAT5-null or control littermates (not shown). These observations indicated that NFAT5-null mice had a severe T cell lymphopenia.

Impaired allogeneic tumor rejection in NFAT5-null mice

In view of the pronounced T cell lymphopenia of NFAT5-null mice, we analyzed their ability to mount an in vivo immune response that are primarily dependent on T cells. We used a model of allogeneic tumor rejection, highly dependent on CD8 lymphocytes (49). In this assay, JS88L plasmacytoma cells (BALB/c background, H-2d) develop tumors when inoculated s.c. in syngeneic mice but are rejected by immunocompetent mice of either C57BL/6 or 129/sv background (both H-2d). As shown in Fig. 2, 70% (9/13 mice) of the NFAT5-null mice (129/sv) developed a tumor 14 d after the inoculation, whereas none of the wild-type littermate mice did (0/13). Interestingly, mice that lacked NFAT5 only in their T cells (Nfat5fl/fl, CD4-Cre, C57BL/6) were competent to reject the tumor (Fig. 1C). As a positive control, 100% of the syngeneic BALB/c mice (5/5) tested in parallel developed a tumor (Fig. 1C). The inability of NFAT5-null mice to reject allogeneic cells indicated a substantial T cell immunosuppression. However, this did not seem to be due just to the loss of NFAT5 in T cells, because Nfat5fl/fl, CD4-Cre mice were not immunosuppressed.

T cells from NFAT5-null mice display characteristics of impaired homeostasis

The lymphopenia observed in NFAT5-null mice could be associated with the reduced thymic output, but might also involve defects in the homeostasis of T lymphocytes in periphery. We analyzed whether mature T cells in NFAT5-null mice had altered representation of naive, central memory and effector memory populations. We observed that the pool of CD8 cells from NFAT5-null mice was markedly skewed toward central memory (CD44hi, CD62Llo) and effector memory (CD44hi, CD62Llo) phenotypes, with a concomitant reduction in the proportion of naive cells (CD44lo, CD62Lhi; Fig. 1D). With regard to CD4+ lymphocytes, their naive/memory proportions were less affected, although 56% of the mice analyzed (5/9) had a moderate increase (<1.5-fold) in their proportion of effector memory CD4+ cells, and 33% (3/9) had fewer naive CD4+ cells (<20% less than in wild-type mice; Fig. 1D). The increased proportion of memory cells, particularly in the CD8 compartment, suggested that a homeostatic response was being activated in NFAT5-null mice. However, their persistent lymphopenia indicated that this response was insufficient to compensate for the depletion of the T cell pool. The finding that lymphopenia and the imbalance in naive/memory cell proportions were more pronounced in CD8 T cells was also consistent with the inability of NFAT5-null mice to reject allogeneic tumor cells. Given that the TCR complex and the IL-7R are essential to preserve the homeostasis of naive T cells, we analyzed their expression in T cells from NFAT5-null mice. The levels of surface TCRβ, CD3, and IL-7Rα in CD4 and CD8 T cells from NFAT5-null mice were only slightly lower than in lymphocytes from Nfat5fl/fl littermates (Supplemental Fig. 2), indicating that Nfat5+/− T cells did not have a substantial defect in the expression of these receptors. The analysis of Nfat5fl/fl, CD4-Cre mice showed a mild bias toward central and effector memory in CD8 cells in some of the animals: five of nine mice analyzed had their proportions of central memory CD8 cells increased 1.5 times, on average, with respect to wild-type mice, and four of nine exhibited a 1.4-fold increase in the proportion of effector memory CD8 cells (Supplemental Fig. 1D). Nonetheless, the frequency and magnitude of this bias were substantially lower than in NFAT5-null mice. This result, together with the normal numbers and function of T cells in Nfat5fl/fl, CD4-Cre mice (Fig. 1C and Supplemental Fig. 1), indicated that the severe T cell phenotype of NFAT5-null mice could not be simply attributed to the lack of NFAT5 in mature T cells.

Hyperosmolality and hypernatremia in plasma of NFAT5-null mice

Given that mice lacking Nfat5 have severe atrophy of their renal medulla (39), we considered that this condition might alter the concentration of plasma biomarkers such as urea, sodium, calcium, and AMY, which are informative to assess renal dysfunction. We found that NFAT5-null mice had abnormally high concentrations of sodium, urea (BUN), and AMY in their plasma, but had no significant differences in the plasma levels of calcium when compared with control mice (Fig. 2A). This analysis revealed that NFAT5-null mice displayed markers of renal damage in their blood (BUN and AMY), and importantly, they suffered from pathologic hypernatremia. Consistent with this, sera from NFAT5-null mice were markedly hypertonic, with osmolality values of 364–411 mOsm/kg (Fig. 2B). Sera from heterozygous mice were moderately hypertonic with an average value of 336 mOsm/kg (Fig. 2B). By contrast, sera from Nfat5+/− littermate mice, or mice deficient in NFAT5 only in T cells (Nfat5fl/fl, CD4-Cre), were in the range of normotonic values (average of 317 mOsm/kg; Fig. 2B). These results showed that blood cells in NFAT5-null mice were chronically exposed to pathologic hypertonicity that, as we had previously shown, would require NFAT5 for appropriate T cell adaptation (41, 42). Therefore, certain defects observed in mature T lymphocytes of NFAT5-null mice could be because of their continuous exposure to hypertonic stress in vivo.

NFAT5-dependent and -independent effects of hypernatremia on T cell survival, expansion, and proportions of naive and memory cells

To analyze the specific effect of pathologic hypernatremia on the survival, expansion, and homeostatic responses of NFAT5-deficient T cells, we used in vitro cultures of splenocytes from Nfat5fl/fl, CD4-Cre...
mice. In these mice, only T cells lacked NFAT5; furthermore, they had not been exposed to any prior osmotic stress in vivo. Cells from Nfat5fl/fl, CD4-Cre or control mice were cultured in the presence of IL-7 (conditions of homeostatic survival) or stimulated with mitogens (Con A and IL-2) to activate their expansion, either in isotonic conditions and impair the expansion capacity of wild-type lymphocytes or in hypernatremic media with tonicity levels (380 or 420 mOsm/kg) in the range found in the plasma of hypernatremia caused a severe depletion of NFAT5-null mice. Comparison of this result with the in vivo phenotype (Supplemental Fig. 3A) strongly suggested that Nfat5fl/fl, CD4-Cre cells, indicating that accumulation of central memory cells caused by osmostress was independent of NFAT5 and T cell expansion.

The results showed that after 6 days of culture with IL-7, hypernatremia caused a severe depletion of Nfat5fl/fl, CD4-Cre T cells, but only moderate lymphopenia in wild-type ones (Fig. 3). With regard to the expansion of T cells induced by mitogenic stimulation, the proliferation of wild-type CD4 cells was minimally affected by hypertonicity (380 and 420 mOsm/kg), whereas CD8 cells proliferated at a progressively slower rate as tonicity increased (Fig. 3). By contrast, hypertonic stress stalled the expansion of both CD4 and CD8 Nfat5fl/fl, CD4-Cre cells.

Hypertonic stress also caused a moderate accumulation of central and effector memory CD4 T cells in IL-7 cultures of wild-type cells, whereas in Nfat5fl/fl, CD4-Cre lymphocytes, which expressed normal levels of the IL-7Rα (Supplemental Fig. 3A), it induced a much greater accumulation of effector memory cells, both CD4 and CD8, and a significant reduction of the naive pool in both subsets (Fig. 4A). These effects were evident at greater tonicity levels (420 mOsm/kg), and already noticeable after 3 d of culture (Fig. 4A and Supplemental Fig. 3B). Regarding the naive/memory proportions in mitogen-stimulated cultures, essentially all T cells differentiated to a mixture of central and effector memory after 6 d of stimulation in isotonic conditions, and hypertonic stress skewed their differentiation toward central memory in both CD4 and CD8 cells (Fig. 4B and Supplemental Fig. 3C). In this case, the proportions of central and effector memory cells in isotonic and hypertonic conditions were similar between wild-type and Nfat5fl/fl, CD4-Cre cells, indicating that accumulation of central memory cells caused by osmostress was independent of NFAT5 and T cell expansion.

These ex vivo assays showed that pathologic hypertonicity could cause a certain degree of lymphopenia under homeostatic survival conditions and impair the expansion capacity of wild-type lymphocytes, and that both effects were exacerbated in NFAT5-deficient cells. Comparison of this result with the in vivo phenotype of NFAT5-null and Nfat5fl/fl, CD4-Cre mice strongly suggested that the lymphopenia of the former was due to the effect of systemic
Plasma levels of sodium (Na⁺), BUN, calcium (Ca²⁺), and AMY. White and black circles represent individual Nfat5⁺/+ and Nfat5⁻/⁻ samples, respectively. Where shown, mean values for each cluster of samples are represented by a horizontal line. Values above the measuring range of the instrument (>180 mmol/l for Na⁺; >180 mg/dl for BUN) are indicated.

A. Osmolality (mOsm/kg) of sera from Nfat5⁺/+, Nfat5⁻/⁻, Nfat5⁻/⁻, Nfat5⁻/⁻, Nfat5⁻/⁻, CD4-Cre, and Nfat5⁻/⁻, CD4-Cre mice. Each circle corresponds to one mouse, and average values are represented as a horizontal line.

B. Concentration (mM) of Na⁺, Ca²⁺, and AMY (U/L) in sera from wild-type (Nfat5⁺/+), CD4-Cre (Nfat5⁻/⁺), and Nfat5⁻/⁻ mice.

FIGURE 3. Effect of hypernatremia on the IL-7–dependent survival and mitogen-induced expansion of wild-type and NFAT5-deficient T cells. Numbers of CD4⁺ and CD8⁺ cells cultured under isotonic (300 mOsm/kg) or hypertonic (380 or 420 mOsm/kg) conditions with IL-7 (left graphs) or Con A plus IL-2 (right graphs) were calculated at day 0 and after 3 and 6 d of culture. Values shown are relative to the number of Nfat5⁺/+ control, CD4-Cre cells at day 0 (arbitrary value of 1) and correspond to the mean ± SEM; n = 4. *p < 0.05; **p < 0.01.
the number of wild-type T cells to a similar extent as the reduction observed in NFAT5-deficient cells (from *Nfat5*<sup>fl/fl</sup>, CD4-Cre mice) exposed to hypernatremia alone (Fig. 5C) and also inhibited the proliferation of T cells cultured in hypertonic conditions (Fig. 5D). Therefore, CD24 contributed to sustain the expansion of mature T lymphocytes under conditions of persistent hypertonicity.

We then asked whether the specific lack of NFAT5 in T cells impaired the expression of CD24 and proliferation in response to hypernatremia in vivo. Mitogen-activated T cells from *Nfat5*<sup>fl/fl</sup>, CD4-Cre or *Nfat5*<sup>wt/wt</sup>, CD4-Cre control mice were labeled with CFDA-SE and adoptively transferred into hypertonic (*Nfat5*<sup>2<sup>−/−</sup></sup>) or control isotonic (*Nfat5*<sup>+/+</sup>) mice. Three or 5 d later, CD4 and CD8 transferred T cells were isolated from the spleen of host mice and analyzed for their expression of CD24 and proliferation rate. We observed that NFAT5 was required to sustain CD24 expression (Fig. 6A) and T cell proliferation (Fig. 6B) in vivo in hypernatremic hosts.

We next sought to analyze whether blockade of CD24 affected the proliferation of T cells in hypernatremic hosts. We first observed that binding of 20C9 blocking Ab to CD24 effectively competed the binding of Ab M1/69 to CD24 in a dose-dependent manner (Supplemental Fig. 5A), which allowed us to monitor the efficacy and persistence of the blockade of CD24 by the 20C9 Ab. Because CD24 is expressed in vivo in different cell types, and it is also involved in regulating diverse immune and nonimmune functions (24), we tried to block CD24 specifically in T cells before transferring them into hypernatremic hosts. For this, wild-type T cells were cultured with Con A plus IL-2 in hypertonic medium (380 mOsm/kg) for 3 d to induce CD24 (as shown in Fig. 5B). Cells were then labeled with CFDA-SE and incubated with blocking concentrations of 20C9. The efficiency of the blockade of CD24 with the 20C9 Ab was confirmed before transferring the cells (as in Supplemental Fig. 5A). 20C9-treated or untreated control T cells were transferred into hypertonic (*Nfat5*<sup>2<sup>−/−</sup></sup>) or isotonic (*Nfat5*<sup>+/+</sup>) hosts, and their proliferation and CD24 expression and blockade were monitored 48 h later. This experiment showed that blockade of CD24 in T cells before adoptive transfer caused an impairment of their proliferation in some of the hosts, although the overall effect was relatively mild (Supplemental Fig. 5B). We also observed that, despite the initial blockade with 20C9, T cells transferred into hypertonic mice were able to rein-duce CD24 (Supplemental Fig. 5C). These results suggest that the rapid turnover of CD24 in vivo might have precluded an inhibitory effect of the 20C9 Ab. Another interpretation could be that once T cells had induced CD24 in vitro after several days in hypertonic media, they might be less sensitive to the inhibitory effect of the Ab.

**FIGURE 4.** Effect of hypernatremia on the relative amount of naive and memory cells in wild-type and NFAT5-deficient lymphocytes. Representative dot plots and proportions of naive, central memory, and effector memory CD4<sup>+</sup> and CD8<sup>+</sup> cells from *Nfat5*<sup>wt/wt</sup>, CD4-Cre and *Nfat5*<sup>fl/fl</sup>, CD4-Cre mice after 6 d of culture in isotonic (300 mOsm/kg) or hypertonic (380 and 420 mOsm/kg) media with IL-7 (A) or Con A plus IL-2 (B). Graphics represent the proportion of each respective subset in hypertonic conditions relative to isotonic medium (value of 1). Values correspond to the mean ± SEM; *n* = 4. *p* < 0.05; **p** < 0.01.
NFAT5 binds to the Cd24 promoter in response to hypernatremia and facilitates a transcriptionally permissive chromatin configuration

We analyzed whether the poor induction of CD24 in NFAT5-null T cells was due to defective accumulation of its mRNA in response to hypernatremia. *Nfat5*fl/fl, CD4-Cre T cells and wild-type controls were either induced to proliferate (stimulated with Con A plus IL-2 or anti-CD3 plus anti-CD28 Abs) in moderately hypertonic medium (380 mOsm/kg) for 3 d (Fig. 7A) or allowed to proliferate first in isotonic medium during 3 d and then subjected to acute hypertonic shock (500 mOsm/kg) for 2 to 12 h (Fig. 7B). Both types of treatments induced the accumulation of the mRNA of Cd24 in T cells in a NFAT5-dependent manner. Because the Cd24 promoter region (−2300 to +525) contains nine elements that fit the consensus binding site for NFAT5 (T/A/CGGAAA) (47, 51), we tested whether this factor could be recruited to this promoter in response to hypernatremia. Con A plus IL-2–stimulated T cells from control mice were subjected to acute hypernatremia for 6 or 12 h, and then used for chromatin immunoprecipitation experiments to monitor the in vivo occupancy of the Cd24 promoter by NFAT5. As shown in Fig. 7C, binding of NFAT5 to the 5' regulatory region of Cd24 was detectable in isotonic conditions and was strongly increased in a hypertonicity-dependent manner, indicating that Cd24 is a direct target of NFAT5. Next, we explored whether NFAT5 regulated the chromatin configuration of the Cd24 promoter during the response of T cells to hypernatremia. We monitored two histone modifications, trimethyl histone H3K4 (lysine 4 of histone H3) and trimethyl histone H3K27 (lysine 27 of histone H3), that associate with active or repressed chromatin, respectively (52). Our results showed that the regulatory region of Cd24 was constitutively marked by the trimethylation of histone H3K4 regardless of tonicity and NFAT5 (Fig. 7D). In contrast,
Our analysis of the T cell compartment of NFAT5-null and T cell-specific knockout mice shows that the former had a pronounced immunodeficiency associated with a severe hypernatremia, whereas mice that lacked NFAT5 only in T cells had the same plasma osmolality as wild-type mice and largely normal T cell parameters. We have identified NFAT5-sensitive effects of pathologic osmostress in T cell homeostatic survival, naive/memory balance, and expansion capacity. We also show that CD24, a relevant regulator of T cell homeostatic survival, naive/memory balance, and expansion, is induced in mature T cells in an NFAT5-dependent manner in response to pathologic hypertonicity, and required to sustain T cell expansion under osmotic stress.

Our finding that the plasma of NFAT5-null mice was severely hypernatremic, with toxicity levels in the range 360–411 mOsm/kg recorded in human patients and mouse models of hypernatremic syndromes (31–38), strongly suggests that this hyperosmotic disorder could cause the T cell immunodeficiency of NFAT5-null mice. Although the physiologic toxicity of the thymus and spleen in normal mice was described as mildly hypertonic (~330 mOsm/kg), previous works had found that a toxicity of 340 mOsm/kg did not activate NFAT5 in T cells (41) nor impaired the proliferation of NFAT5-deficient lymphocytes (45). Moreover, we and others had shown that hypertonicity levels >360 mOsm/kg can activate NFAT5 in mature T cells, induce the expression of genes required for osmoreadaptation (41, 42), and inhibit the proliferative capacity of NFAT5-deficient T cells (42, 45). Finally, our results showed that NFAT5-deficient T cells transferred into wild-type hosts proliferated normally, indicating that the physiologic toxicity of lymphoid organs would not be deleterious for T cells lacking NFAT5, and that the toxicity threshold required to activate this factor or affect T cell function in vivo must be higher. Regarding the pronounced systemic hypertonicity of NFAT5-null mice, we must also consider that this defect might have masked other osmostress-independent functions of NFAT5. Such possibility should be kept in mind when interpreting results obtained with NFAT5-null mice, either on immune function or in other systems. Nonetheless, the similarity between the effects of hypertonicity ex vivo on NFAT5+/+ CD4-Cre T cells and the T cell phenotype of NFAT5-null mice suggests that pathologic hypertonicity plays an important role in the immune dysfunction of these mice.

We found that T cell survival and maintenance of naive/memory balance in response to homeostatic cues, such as IL-7, as well as mitogen-mediated proliferation, were substantially impaired by pathologic osmostress in NFAT5-deficient lymphocytes. Considering that maintenance of the circulating T cell pool requires homeostatic survival and proliferation mechanisms, our experiments support the interpretation that the pathologic in vivo hypernatremia of NFAT5-null mice could be a major determinant of their lymphopenia, and possibly contribute to the depletion of naive T cells and bias toward memory. This lymphopenia would be worsened by a reduced output of naive cells from the thymus, which combined with the inability of NFAT5-deficient T lymphocytes to proliferate in a hypertonic milieu would prevent an effective lymphopenia-induced T cell expansion. Many studies on T cell homeostasis do not allow a clear-cut distinction between long-term survival and proliferation to replenish cell numbers. Part of the problem occurs because both processes are linked because lymphopenia-induced proliferation causes naive T cells to slowly acquire the phenotypic and functional characteristics of memory cells (9, 10). In this regard, because NFAT5-deficient T cells proliferate poorly under osmotic stress, it appears more likely that their bias toward memory is due to a reduced survival of naive cells rather than to proliferation-coupled differentiation to memory.

The skewing toward memory and loss of naive CD8 cells in NFAT5-null mice could contribute to their inability to reject allogeneic tumors, a process that requires a fully functional CD8 compartment. In addition, T cells from NFAT5-null mice have a lower surface expression of TCRβ and CD3 than wild-type mice. Because the lifespan of CD8 cells, particularly the naive ones, is greatly reduced when the expression of the TCR is extinguished (53, 54), it might be possible that a reduced TCR level could exacerbate their homeostasis defects in vivo. However, in the ex vivo assays with NFAT5+/+ CD4-Cre lymphocytes, both CD4 and CD8 cells were similarly biased toward memory by hypertonicity; but in vivo in NFAT5-null mice, CD4 cells were less skewed toward memory than CD8 lymphocytes. Several variables could contribute to this difference, for instance, the effect of the in vivo microenvironment in NFAT5-null mice with respect to our culture conditions, or a different sensitivity of CD4 cells of NFAT5-null mice to a prolonged exposure to hypertonicity in vivo.

The homeostasis defects observed in NFAT5-deficient T cells, either in vivo in NFAT5-null mice or ex vivo under hypertonicity, are highly reminiscent of parameters observed in aged mice and humans (15, 55–57). This might reflect the deleterious conditions occurring...
in an organism that suffers from systemic and persistent hyponatremia and, at the same time, lacks NFAT5, a major player in the adaptation and survival to this stress. In this regard, because hypertonicity can induce DNA damage-like responses (42) and oxidative stress (30), it is tempting to speculate that these or other molecular processes that occur in aged cells might also be present in T cells from NFAT5-deficient mice.

Although more than one mechanism could underlie the dependency of T cells on NFAT5 to expand in hypertonic conditions, we focused on analyzing receptors linked to lymphopenia-induced proliferation. We have identified CD24 as an NFAT5-regulated target both in vitro and in vivo. Mature T cells express low levels of CD24, but until now it was unknown that they could induce a robust, sustained expression of this molecule in response to stress signals. Recruitment of NFAT5 to the Cd24 promoter inhibited the local hypermethylation of H3K27, indicating that NFAT5 was required to prevent the repression of this locus in response to stress. NFAT5 could either act by reducing the recruitment of the PRC2 complex, which catalyzes the trimethylation of H3K27 (58, 59), or alternatively, facilitating the recruitment of JmjC-domain–containing demethylases that are specialized in abrogating this epigenetic mark (60).

Our results support a role for CD24 in sustaining the proliferative capacity of wild-type T cells under osmotic stress. Because CD24 is a key player of lymphopenia-induced T cell proliferation (22), its induction in response to hyponatremia suggests that it could facilitate the optimal expansion of T cells under persistent hypertonic conditions, a function that may well be of critical relevance when the pool of T cells is reduced. Interestingly, induction of CD24 by osmotic stress required stimulation with mitogens or via TCR, and was not observed in cells just maintained with IL-7, suggesting a specific role for CD24 induction in T cell expansion. A previous study had also shown that CD24 was required for the acquisition of a memory phenotype in proliferating lymphocytes, particularly CD8 cells (22). However, NFAT5-null mice had a greater proportion of memory CD8 cells than wild-type mice, and memory CD8 and CD4 accumulated in cultures of NFAT5-deficient lymphocytes exposed to osmotic stress. Notably, NFAT5-deficient lymphocytes expressed relatively normal levels of CD24 in isotonic conditions and could even induce it to some extent in response to hypertonicity, although much less potently than wild-type cells. It is possible that this level of CD24 expression could suffice to support the differentiation toward memory, or that the functions of CD24 during isotonic conditions and hypertonic stress might not overlap entirely. Expression of
CD24 might endow T cells with survival advantages during the response to hypertonicity. In this regard, CD24 has a mucin-like structure (61), and mucins have been recently shown to function as sensors for osmotic stress in yeast (62). Although currently it is unknown whether mammalian mucins work as osmosensors, it is conceivable that signaling via CD24 could facilitate the cellular adaptation to hypertonicity. In another scenario, Liu’s group has recently identified a novel role for CD24 in dendritic cells, in which it attenuates inflammatory signals delivered by tissue damage (63). Because persistent hypernatremia can injure cells (64–66), it is tempting to speculate that CD24 might have a similar protective role in T cells exposed to hypertonic stress.

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Disclosures

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