

Spontaneous Organ-Specific Th2-Mediated Autoimmunity in TCR Transgenic Mice

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CD4⁺ T cells that lead to autoimmune gastritis (AIG) in BALB/c mice are either Th1 or Th2 cells. To test whether the phenotype of disease is related to the particular TCR expressed by the pathogenic cell, we have generated several lines of TCR transgenic mice using receptors cloned from pathogenic Th1 or Th2 cells. We previously described spontaneous inflammatory AIG in A23 mice, caused by the transgenic expression of the TCR from a Th1 clone, TXA23. In this study we describe the generation of A51 mouse lines, transgenic for the TCR of a CD4⁺ self-reactive Th2 clone, TXA51. A proportion of A51 mice spontaneously develop AIG by 10 wk of age, with a disease characterized by eosinophilic infiltration of the gastric mucosa and Th2 differentiation of transgenic T cells in the gastric lymph node. The Th2 phenotype of this autoimmune response seems to be related to a low availability of MHC class II-self peptide complexes. This *in vivo* model of spontaneous Th2-mediated, organ-specific autoimmunity provides a unique example in which the clonotypic TCR conveys the Th2 disease phenotype. *The Journal of Immunology*, 2004, 172: 2917–2924.

CD4⁺ T lymphocytes differentiate into two distinct classes, designated Th1 and Th2, characterized by the spectrum of cytokines they secrete (1–3). The Th1 subset, commonly referred to as proinflammatory, produces IFN- γ , whereas Th2 cells secrete IL-4, IL-5, IL-10, and IL-13. In a balanced immune response, Th1 and Th2 functions are counter-regulatory, but some infections tend to elicit predominantly either Th1 or Th2 cytokine-producing cells. Although the causes of autoimmune diseases are complex and multifactorial, one generally accepted scheme is that immune dysregulation, secondary to an infectious process or excessive exposure to an inciting or cross reactive Ag, promotes a Th1 response that leads to progressive inflammation and autoimmunity.

The balance of Th1 and Th2 cytokines in normal responses, the preponderance of Th1 cells in autoimmunity, and examples of Th2 cells exerting a counterinflammatory response suggest that Th1 cells may be effectors in autoimmune diseases, whereas Th2 cells might be suppressive. Indeed, some models of organ-specific autoimmunity, such as insulin-dependent diabetes and experimental allergic encephalomyelitis (EAE),⁴ can be prevented or suppressed by the induction of Th2 cell responses (4, 5). For example, in the NOD mouse, administration of β cell autoantigens (insulin, glutamic acid decarboxylase, or IA-2) protects from diabetes through the induction of Th2

cytokines (6), an effect not observed in IL-4^{o/o} mice (7). In contrast, autoantigen-specific Th2 T cell clones, when transferred to T cell-deficient mice, lead to the development of disease in both insulin-dependent diabetes and EAE models (8, 9). Other examples of organ-specific autoimmune disease can be induced by the elimination of regulatory CD4⁺CD25⁺ T cells by thymectomy on day 3 of life (d3Tx) (10). Autoimmune gastritis (AIG) is commonly observed in d3Tx BALB/c mice and resembles the human disease pernicious anemia, in that the effector T cells and autoantibodies recognize the α and β subunits of the gastric parietal cell H/K-ATPase (11, 12). AIG seems to be primarily Th1-mediated, but autoantigen-specific Th2 cells are found in the gastric mucosa (13–15).

Although the factors that determine the differentiation of Ag-specific CD4⁺ T cells into Th1 or Th2 populations have been extensively studied *in vitro* (16, 17), little is known about the regulation of autoantigen responses *in vivo*. We previously reported the isolation of two pathogenic I-A^d-restricted CD4⁺ T cell clones that recognize distinct epitopes of the H/K-ATPase α -chain (18). TXA23, a Th1 cell, induces, on transfer to *nu/nu* mice, a severe gastritis characterized by a lymphocytic infiltrate and autoantibody production. In contrast, clone TXA51, a Th2 cell, produces IL-4 and IL-10, and on transfer to *nu/nu* mice leads to a severe gastritis characterized by a mixed infiltrate composed of polymorphonuclear leukocytes and eosinophils (18). These cell transfer experiments suggested that the expressed TCR controlled the phenotype of the resulting disease. Like the TXA23 cell transfer model, a transgenic (Tg) mouse line, A23, expressing the TCR of the TXA23 clone, develops a spontaneous and severe autoimmune gastritis in which CD4⁺ T cells produce Th1 lymphokines in the gastric lymph node (GLN) (19). In this study we describe a TCR Tg line generated from the TCR derived from the Th2 T cell clone, TXA51. These Tg mice offer a unique model of Th2 autoimmune disease, and provide evidence consistent with an avidity-based Th2 phenotype of T cell-derived autoimmunity.

Materials and Methods

Mice, Abs, and reagents

BALB/c wild-type, *nu/nu*, *SCID*, and *RAG2^{o/o}* were purchased from National Cancer Institute animal facility (Frederick, MD) and housed under SPF conditions.

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⁴ Abbreviations used in this paper: EAE, experimental autoimmune encephalitis; AIG, autoimmune gastritis; BM, bone marrow; DC, dendritic cell; DP, double positive; d3Tx, day 3 thymectomy; GLN, gastric lymph node; PCAb, parietal cell Ab; PIT, H/K-ATPase $\alpha_{630-641}$; PLL, H/K-ATPase $\alpha_{889-900}$; PLN, peripheral lymph node; SP, single positive; Tg, transgenic.

H/K-ATPase was obtained by purification of microsomes from rabbit stomach (11). Synthetic peptides were provided by Dr. J. Lukszo (Peptide Synthesis and Analysis Unit, National Institute of Allergy and Infectious Diseases, Bethesda, MD) and were >95% pure. The peptides were H/K-ATPase $\alpha_{630-641}$ (PITAKAIAASVG (PIT)) and H/K-ATPase $\alpha_{889-900}$ (PLLCLVGLRPQWE (PLL)). Stock solutions at 2 mg/ml were made in PBS/5% DMSO. Abs used in flow cytometry and specific for CD4 (GK1.5), V β 4 (KT4), CD8 (53-6.7), CD25 (7D4 and PC61), CD69 (H1.2F3), and Fc Block (CD16/CD32; 2.4G2), were purchased from BD PharMingen (San Diego, CA). Abs used for T cell cultures specific for IL-4, IL-12, and IFN- γ were provided by W. E. Paul (National Institute of Allergy and Infectious Diseases, National Institutes of Health), as well as recombinant murine cytokines, IL-4, IL-12, and IL-2.

DNA constructs and generation of A51 Tg mice

Total RNA was extracted from 5×10^6 TxA51 cells using an RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. cDNA was transcribed from 1 μ g of total RNA using oligo(dT) primer, first-strand cDNA synthesis reagents, and Superscript II reverse transcriptase (Life Technologies, Gaithersburg, MD).

The V α segment of TxA51 TCR was identified using a linker-facilitated PCR as previously described (20). Briefly, TCR α -chain cDNA synthesis was primed with α complementary primer (5'-TTTGGGCCCATTGATTTGGGAGTCAA-3'). A double-stranded linker was then ligated to the V-region end of the cDNA. TCR α cDNA was then amplified using a single-stranded primer from the synthetic linker and a nested α primer (5'-caagcgccgcacaggcaggggtctgt-3'). PCR products were ligated to TA vector pCR2.1 (Invitrogen, Carlsbad, CA) for transformation of DH5 α and were sequenced using a single-stranded primer from the synthetic linker. The sequence of a single V α segment (AV17S3) was identified. Full-length TCR α -chain cDNA, beginning with the initiation ATG codon of the leader and ending with the termination codon at the 3' end of α , was then amplified from total TxA51 cDNA by PCR using a specific AV17S3 primer (5'-CCGAATTCATGCTGATTCTAAGCCTGTTGGAGC-3') and a reverse α primer (5'-TCCTACGTATTCAACTGGACCACAGCCTCAGCGTCATGAG-3'), incorporating *Eco*RI and *Sna*BI sites, respectively. Insert DNA was sequenced and subcloned into the *Eco*RI and *Sna*BI sites of a CD2-based expression vector, provided by Dr. D. Kioussis (National Institute of Medical Research, London, U.K.). Prokaryotic sequences were removed by *Not*I/*Sal*I digestion and gel purification before injection into (C57BL/6xBALB/c) F_1 blastocysts. TxA51 stained with anti-V β 4 Ab, but did not react with any of the available anti-V α Abs.

Full-length TCR β cDNA, beginning with the leader exon and ending with the stop codon at the 3' end of C β 2, was amplified by PCR from TxA51 total cDNA using a BV4S1-specific forward primer (5'-ACGCGTCGACCCAGCCATGGGCTCCATTTTCTCAGTTGCTGTG-3') and a reverse TCR β C2 primer (5'-GGTCAGTGGCCTGGTGCTGATGGCCATGCTCAAGAAAAAATTCTGATAACCCGGGGGA-3'). The amplified fragment containing a 5' *Sal*I site and a 3' *Bam*HI site was ligated to pCR2.1 and sequenced. An internal *Sal*I site was removed by directed mutagenesis using the QuickChange (Stratagene, La Jolla, CA) site-directed mutagenesis kit following the manufacturer's instructions. The mutated TCR β cDNA was then excised by *Sal*I/*Bam*HI digestion and ligated to the corresponding sites of pHSE3', a Tg vector that exploits an H-2K b promoter and an Ig enhancer (provided by Dr. H. Pircher, University of Freiburg, Freiburg, Germany). Prokaryotic sequences were removed by *Xho*I digestion and gel purification before blastocyst injection. Transgenic generation and animal husbandry were conducted at the National Institute of Allergy and Infectious Diseases transgenic and knockout facility (Frederick Cancer Research and Development Center, Frederick, MD) under specific pathogen-free conditions. Because of the difficulty in generating transgenic animals in homozygous BALB/c, (BALB/c $\times$ C57BL/6) F_1 blastocysts were injected with purified TCR α and β gene constructs. When weaned, A51 Tg TCR mice were identified by staining of PBL with anti-V β 4 and anti-CD4 Abs and flow cytometry. Three A51 lines were generated from three founder animals and are referred to as A51 line-1, line-2, and line-3. Only lines-1 and -2 have been studied extensively, and data primarily from line-2 are presented in this paper.

Sequence analysis indicated that the TCR α cDNA derived from AV17S3 and AJ7 and its CDR3 region included 12 codons with no N insertions (Fig. 1). The TCR β cDNA was derived from BV4S1, BD2.1, and BJ2.3 (Fig. 1), and its CDR3 region contained six N insertions and comprised 15 codons. The presence of N insertions reflects TdT activity in the thymus before day 3 of life, consistent with previous observations (19, 21). The mice used in these studies were from backcross generations N2 to N7 and are referred to as lines A51-line-1, -line-2, and -line-3. N3 animals from line-2 were also backcrossed to BALB/c-RAG2 $^{o/o}$ mice. The cell

clone from which the TCR AV and BV genes were cloned is referred to as TxA51, whereas the Tg mouse lines are designated A51. Real-time PCR analysis of A51 genomic DNA from line-1 and line-2 indicated that although both lines had the same relative amount of TCRB DNA per cell, line-2 had ~5-fold the number of copies of TCRA. Relative mRNA expression of TCRA transcripts in line-2 was ~2-fold that in line-1, and TCRB was ~0.6 that in line-1.

Real-time PCR analysis

Genomic DNA from 10^8 PBMC isolated from mice of each A51 line was extracted using a DNeasy Tissue Kit (Qiagen). Total RNA from 10^7 positively selected CD4 $^+$ T cells (mouse CD4 Dynabeads M450; Dynal Biotech, Lake Success, NY) was isolated with an RNeasy MiniKit (Qiagen). cDNA was transcribed using 1 μ g of total RNA, random hexamers, first-strand cDNA synthesis reagents, and Superscript II reverse transcriptase (Life Technologies). Real-time PCR amplification of CDR3 junctional regions of α and β Tg cDNA or genomic DNA was performed using either 20 ng of cDNA or 100 ng of genomic DNA, 300 nM AV17S1, and AJ7 primers (A51 α F: 5'-CCGCCACACAGATTGAGGA-3'; A51 α R: 5'-GGTAACACCACCACCTGGGT-3') or BV4 and BJ primers (A51 β F: 5'-CCTGAGCCAAAATACAGCGTT-3'; A51 β R: 5'-GTGCCAGCAGCCAAGGACT-3') in the presence of 200 nM clonotypic TaqMan MGB probes (A51CDR3 α : 5'-6FAM-AGAGGGCTACAGCAACAA-MGBNFQ-3'; A51CDR3 β : 5'-6FAM-CATCCCGACCCCCCA-MGBNFQ-3') with the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Quantitation of α sequences was performed with specific α primers (CaF: 5'-CTGCTGTTCACCGACTTTGA-3'; CaR: 5'-TCATGGTTTTTCGGCACATT-3') in the presence of Syber Green (Applied Biosystems). Amplification of GAPDH sequences was performed with Rodent GAPDH Control reagents (Applied Biosystems). Amplification of known quantities of TxA51 TCR α and β cDNA sequences⁵ inserted into CD2 cassette and pHSE3' expression vectors as well as of known quantities of mouse GAPDH cDNA (BioSource International, Camarillo, CA) were used for quantitation of TCR α and β cDNA. Expression of GATA-3 was analyzed in naive and memory/activated CD4 T cells of peripheral lymph node (PLN) or GLN. After total RNA extraction and cDNA transcription, real-time PCR amplification of GATA-3 and 18S was performed using GATA-3-specific primers (GATA3-F: 5'-CAGAACCGGCCCTTATCA-3'; GATA3-R: 5'-CATTAGCGTTCCTCTCCAGA-3') and probe (5'-6FAM-CGAAGGCTGTGGCA-MGBNFQ-3'), and 18S Pre-Developed TaqMan assay reagents (Applied Biosystems).

Analysis of AIG

Offspring from A51 Tg lines were bled for sera and analyzed for the presence of parietal cell Ab (PCAb) by immunofluorescence between 10 and 15 wk, as previously described (11). For histological analysis of Tg mice and BALB/c *nu/nu* mice, animals were sacrificed at 10–25 wk of age, and stomachs were fixed in 4% paraformaldehyde. Sections were prepared and stained with H&E or Giemsa.

Pathology was evaluated on at least four separate sections of each stomach, based on three histological parameters: inflammation, destruction of parietal and chief cells, and eosinophilia. Each parameter was determined on blinded samples using the following scores, on scales of 1–4. Inflammation was graded as previously described (22): grade 1, normal gastric mucosa that contains a few lymphocytes scattered throughout the submucosa; grade 2, small aggregates containing three or four layers of cells in the mucosa or sparse infiltrates of cells in the submucosa covering 5% of the section; grade 3, frequent and larger infiltrates extending into the mucosa; and grade 4, infiltrates spanning half to the entire width of the mucosa. Eosinophilia was graded as: grade 1, no eosinophils or a few solitary eosinophils restricted to the submucosa; grade 2, small aggregates of more than five eosinophils located in the submucosa; grade 3, larger aggregates and/or the presence of eosinophils within the mucosa; and grade 4, frequent and large aggregates within the submucosa and the gastric glands. Parietal and chief cell destruction were graded depending on the area and extent of destruction: grade 1, integrity of the mucosa; grade 2, parietal and/or chief cell destruction covering <20% of the section; grade 3, parietal and/or chief cell destruction covering 20–50% of the section; and grade 4, parietal and chief cell destruction covering >50% of the section with hyperplasia of mucous and epithelial cells. The sum of the three evaluations was then divided by 3, giving a final score that ranged from <2 (normal), ≥ 2 and ≤ 3 (discrete to mild gastritis), to >3 (severe gastritis).

⁵ The sequences of the TxA51 α - and β -chains have been deposited in GenBank and are available under accession numbers AY324400 and AY324401, respectively.

FIGURE 1. Structures of junctional regions of TXA51 TCR α - and β -chains. V-, D-, and J-gene assignments were made using IMGT/V-QUEST sequence alignment and comparison software of IMGT, the international ImMunoGeneTics database (<http://imgt.cines.fr> (45)). The nomenclature of Arden et al. (46) is used in this study. AV17S3 corresponds to IMGT TRAV16D/DV11*01, and BV4S1 corresponds to IMGT TRBV2*01. Junctions were assigned according to the complementarity-determining residues and framework residues definitions provided by IMGT. Underlined nucleotides represent N insertions.

	V α 17.3					J α 7											
	C	A	M	R	E	G	Y	S	N	N	R	L	T	L	G	K	G
TXA51A	TGT	GCT	ATG	AGA	GAG	GGC	TAC	AGC	AAC	AAC	AGA	CTT	ACT	TTG	GGG	AAG	GGA
AV17S3	TGT	GCT	ATG	AGA	GAG	GG.	...	...	...	...	...	...	...	...	...	...	...
AJ7	...	...	...	...	..G	GAC	TAC	AGC	AAC	AAC	AGA	CTT	ACT	TTG	GGG	AAG	GGA

	V β 4					D β 2.1				J β 2.3											
	C	A	S	S	Q	G	L	G	G	R	D	A	E	T	L	Y	F	G	S	G	
TXA51B	TGT	GCC	AGC	AGC	CAA	GGA	CTG	GGG	<u>GGT</u>	<u>CGG</u>	<u>GAT</u>	GCA	GAA	ACG	CTG	TAT	TTT	GGC	TCA	GGA	
BV4S1	TGT	GCC	AGC	AGC	CAA	G..	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
DB2.1	...	...	...	...	..G	GGA	CTG	GGG	GGG	C..	...	...	...	...	...	...	...	...	...	...	
BJB2.3	...	...	...	...	...	...	...	...	...	...	...	AGT	GCA	GAA	ACG	CTG	TAT	TTT	GGC	TCA	GGA

Flow cytometry

Lymphoid tissues were harvested from A51 mice and non-Tg littermates, processed into single-cell suspensions, and multistained after lysis of RBC with ACK buffer (Biofluids; BioSource International, Camarilla, CA) with various combinations of anti-V β 4, anti-CD4, anti-CD8, anti-CD69, and anti-CD25 Abs in the presence of Fc Block. For intracytoplasmic staining, cells were first fixed and permeabilized with Cytofix/Cytoperm buffer (BD PharMingen), then stained with anti-CD4, anti-V β 4, anti-IL-4, and anti-IFN- γ in Perm-Wash buffer (BD PharMingen) in the presence of Fc Block. After two washes, cells were analyzed by flow cytometry on a FACSCalibur (BD Biosciences, San Jose, CA). Analyses were performed with CellQuest software (BD Biosciences).

Cell purification

Naive and memory/activated CD4 T cells were positively selected from whole-cell suspensions of PLN or GLN with CD4⁺, CD62L^{high}, and CD44^{low} columns following the manufacturer's instructions (R&D Systems, Minneapolis, MN). A purity of 97% for each subpopulation was consistently achieved. Splenic CD11c⁺ dendritic cells (DC) were isolated from BALB/c suspensions of whole splenocytes using the VARIOMACS technique and microbeads coated with Ab against CD11c (Miltenyi Biotec, Auburn, CA), as previously described (23). BALB/c CD11c⁺ bone marrow (BM) DCs were generated by culture of progenitors for 7 days in GM-CSF (5 ng/ml) and IL-4 (10 ng/ml), as previously described (24).

T cell proliferation assays

PLN cells from 15- to 25-wk-old A51 Tg mice were harvested, resuspended in complete medium (RPMI 1640/10% FCS), and then plated in triplicate in 96-well plates (2×10^5 cells/well) in the presence of graded concentrations of peptides. Cells were cultured for 72 h, and [³H]thymidine (10 μ Ci) was added to the culture for the final 6 h. Cultures were then harvested and counted in a beta spectrometer.

T cell differentiation assays

PLN or GLN cells from 15- to 20-wk-old A51 mice were harvested. CD8⁺ T cells were depleted with anti-mouse CD8 M450 Dynabeads (Dyna Biotech, Lake Success, NY) following the manufacturer's instructions. Unbiased culture conditions (null) used cells (0.5×10^6 /ml) cultured in 48-well plates in complete medium for 3 days in the presence of PLL peptide (30 μ M) and anti-IL-4 (10 ng/ml), anti-IL-12 (10 ng/ml), and anti-IFN- γ (10 ng/ml) Abs. In these conditions, de novo differentiation of naive T cells present in the culture is inhibited. Culture conditions promoting Th1 differentiation used IL-12 (10 ng/ml) plus anti-IL-4 (10 ng/ml), and Th2 conditions were established with IL-4 (10^4 U/ml) plus anti-IL-12 (10 ng/ml) and anti-IFN- γ (10 ng/ml). Three days later, supernatants were removed from the cultures and replaced by fresh medium containing IL-2 (50 U/ml). On day 6, PMA (5 ng/ml; Sigma-Aldrich, St. Louis, MO), ionomycin (500 ng/ml; Sigma-Aldrich), and monensin (GolgiStop; BD PharMingen, San Diego, CA) were added, and 6 h later, cells were harvested for cytoplasmic staining of IL-4, IFN- γ , V β 4, and CD4.

IgE ELISA

Serial dilutions of control and A51 mouse serum and of an IgE standard (clone 27-74; BD PharMingen) were incubated overnight at 4°C on plates coated with anti-mouse IgE (clone R35-72; BD PharMingen). Captured IgE was then detected with 2 μ g/ml biotinylated anti-mouse IgE (clone R35-92; BD PharMingen), followed by a 1/1000 dilution of streptavidin-HRP (BD PharMingen). Tetramethylbenzidine substrate was added, and

the reaction was allowed to develop at room temperature for 20–30 min and was read spectrophotometrically at 650 nm. An IgE standard curve was established and used for calculation of serum IgE levels, expressed in nanograms per milliliter.

Transfer of AIG

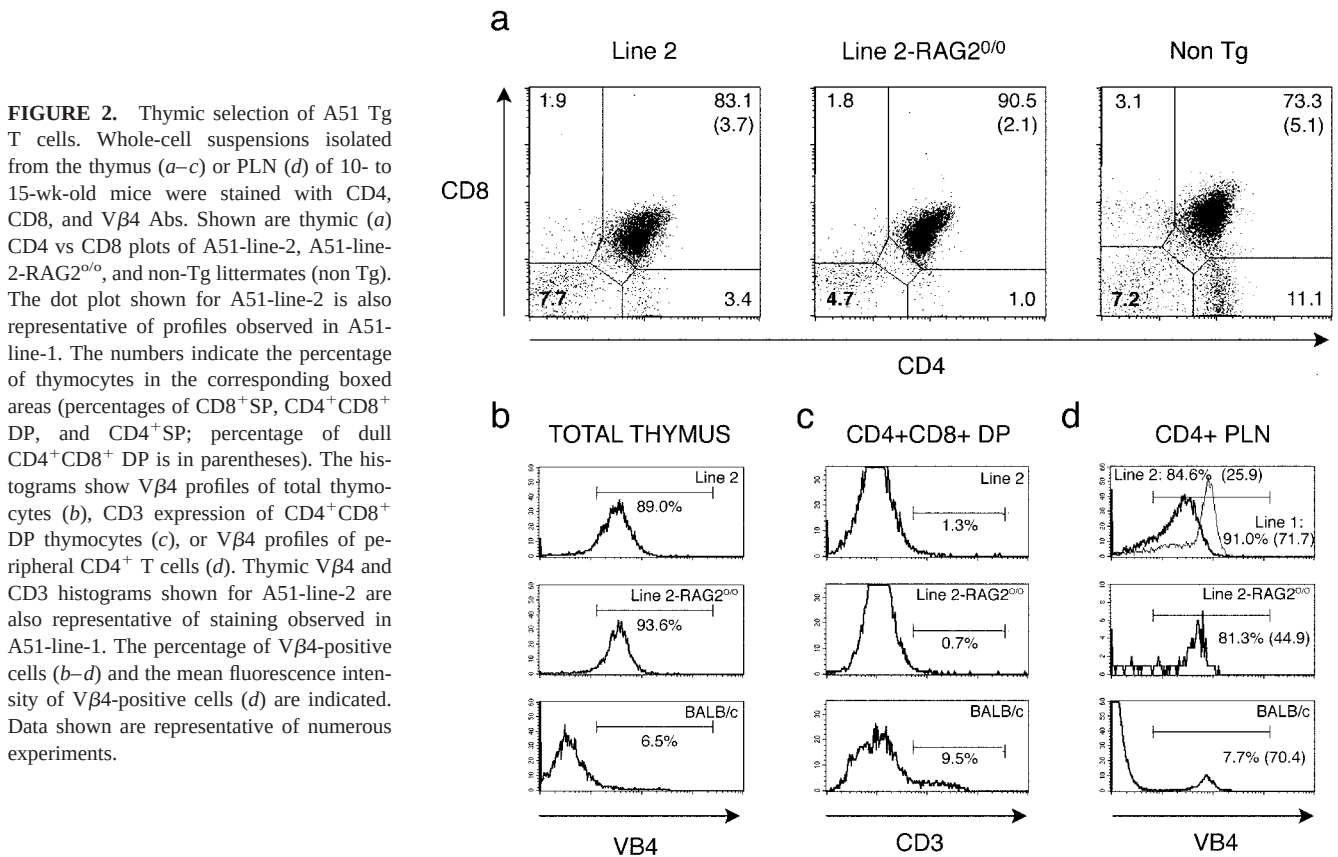
Thymuses were harvested from 15- to 20-wk-old A51 Tg mice and processed into single-cell suspensions. Following removal of RBC by ACK lysis, cells were resuspended in PBS at a density of 1.6×10^6 to 5×10^7 cells/ml. Cells in a final volume of 200 μ l were injected i.v. into 12-wk-old BALB/c *nu/nu* or BALB/c mice. Animals were analyzed for AIG by detection of anti-PCAb in sera 7 wk after cell transfer, and stomachs were analyzed for pathology as described above.

Results

Generation of A51 Tg mice and development of Tg T cells

To understand the relationship between TCR structure and T cell differentiation, and the role of Th2 cells in the pathogenesis of AIG, we generated Tg mouse lines expressing the TXA51 TCR. Founder animals expressed TXA51 β -chain on >80% of CD4⁺ PBLs and also expressed α -chain mRNA. Real-time PCR analysis revealed that α and β transgenes were transmitted to the germline of A51 mice at different relative copy numbers in A51 line-1 and line-2 (see *Materials and Methods*). With respect to cell surface protein expression, V β 4 staining of CD4⁺ T cells was consistently higher in line-1 than in line-2 (Fig. 2d), presumably related to the complexities of the balance of mRNA expression, protein expression, assembly of α - and β -chains, and down-regulation of TCR following engagement of ligand. Both lines developed a disease of similar Th2 phenotype.

The size of the thymus and the number of cells recovered were equivalent in A51 mice and control littermates, indicating grossly normal thymic development. Analysis of CD4, CD8, and V β 4 expression of A51 thymocytes (Fig. 2, a–c) revealed that >85% ($86.4 \pm 3.3\%$) of thymocytes expressed the TCR β transgene (Fig. 2b and data not shown). The proportion of CD4⁺CD8⁺ double-positive (DP) cells in the Tg lines was higher than that in littermate controls ($77.5 \pm 5.4\%$ in littermate controls compared with $89.2 \pm 2.3\%$ in line-1 ($p < 0.0001$), $86.8 \pm 2.9\%$ in line-2 ($p = 0.0005$), and $94.1 \pm 0.4\%$ in line-2-RAG2^{o/o} ($p = 0.044$)). Lower percentages of CD8⁺ single-positive (SP) thymocytes were found ($3.55 \pm 1.7\%$ in littermate controls vs $2.1 \pm 0.5\%$ in line-1 ($p = 0.0071$), $1.35 \pm 0.5\%$ in line-2 ($p = 0.0003$), and $1.13 \pm 0.2\%$ in line-2-RAG2^{o/o} ($p = 0.044$)). Unexpectedly, CD4⁺ SP cells represented a lower proportion of the cells in A51 mice ($3.5 \pm 0.9\%$ in line-2 ($p < 0.0001$) and $3.3 \pm 1.2\%$ in line-2 ($p < 0.0001$) vs $9.5 \pm 2.2\%$ in littermate controls; Fig. 2a). In line-2-RAG2^{o/o} mice, the frequency of CD4⁺ SP was even lower ($0.9 \pm 0.1\%$; $p = 0.036$). Thus, the ability of thymocytes to mature from DP to SP (CD4⁺ and CD8⁺) was less efficient in both A51 and A51-RAG2^{o/o} mice.



Normally, DP thymocytes undergoing positive selection up-regulate TCR surface expression as they differentiate into CD4⁺ or CD8⁺ SP cells (25). The proportion of DP thymocytes with increased levels of TCR (CD3^{high}) was decreased in A51 and A51-RAG2^{0/0} mice compared with littermate controls ($2.1 \pm 0.9\%$ in A51 line-1 and line-2 and $0.8 \pm 0.5\%$ in line-2-RAG2^{0/0} vs $7.0 \pm 2.4\%$ in littermate controls; Fig. 2c). The proportion of dull CD4^{low}CD8^{low} DP cells, representing later transitional stages between DP and SP thymocytes (26), was slightly decreased in A51 lines and in line-2-RAG2^{0/0} (Fig. 2a) compared with littermate controls ($2.5 \pm 1.3\%$ in line-1 ($p = 0.001$), $2.1 \pm 1.1\%$ in line-2 ($p = 0.0005$), and $1.8 \pm 0.7\%$ in line-2-RAG2^{0/0} ($p = 0.044$) vs $4.9 \pm 1.1\%$ in littermate controls). Overall, the equivalent proportion of CD4⁺CD8⁺ double-negative cells ($8.6 \pm 3.5\%$ in littermate controls, $5.1 \pm 1.8\%$ in line 1, $8.0 \pm 2.3\%$ in line-2, and $4.1 \pm 0.8\%$ in line-2-RAG2^{0/0}), the increased proportion of DP cells, and the lower percentages of CD3^{high} DP and dull DP suggested that positive selection in A51 and A51-RAG2^{0/0} was somewhat inefficient. However, it is possible that in addition to inefficient positive selection of A51 thymocytes, some degree of deletion of Tg A51 thymocytes may also be responsible for the decreased numbers of mature CD4⁺ SP thymocytes.

Despite low numbers of CD4 SP thymocytes, mature CD4⁺ T cells populated peripheral lymphoid tissue effectively ($39.9 \pm 5.6\%$ in line-1 and $39.02 \pm 4.4\%$ in line-2 vs $45.8 \pm 3.3\%$ in littermate controls). Mature CD8 T cells were also found in similar proportions in A51 mice ($19.3 \pm 3.7\%$ in line-1 and $24.2 \pm 1.4\%$ in line-2) and in non-Tg littermates ($18.4 \pm 1.8\%$). In line-2-RAG2^{0/0}, CD4 and CD8 T cells represented, respectively, 10.2 ± 2.1 and $1.3 \pm 0.7\%$ of PLN cells. In A51 and A51-RAG2^{0/0} lines, peripheral TCR V β 4 transgene expression was similar to that seen in the thymus ($93.7 \pm 2.0\%$ of CD4 SP cells; Fig. 2d). The level

of expression of TCR V β 4 chain varied between the two A51 lines and was higher in line-1 than in line-2 (Fig. 2d).

A51 mice spontaneously develop AIG

The TXA51 clone induces AIG in T cell-deficient animals upon adoptive transfer (18). Transgenic expression of the TXA51 TCR in H-2^d T cells also caused AIG; histological analysis of 85 10- to 25-wk-old H-2^d A51 animals showed that 58.8% (50 of 85) of them had signs of gastritis. The incidence of AIG in line-2 was significantly higher than that in line-1 (80.0 vs 52.2%; $p = 0.0375$). An inflammatory infiltrate of the submucosa or extending into the mucosa was associated in varying degrees with the loss of parietal and chief cells and replacement with mucus-producing cells (Fig. 3). The infiltrate was mixed, composed of lymphocytes and a variable proportion of polymorphonuclear cells identified mostly as eosinophils (Fig. 3c). The development of AIG in A51 mice was spontaneous, with the earliest histological signs seen at 10 wk of age. The severity of AIG in A51 mice was evaluated histologically (see *Materials and Methods*). Forty percent (31 of 85) of A51 animals had mild gastritis, and 18.8% (16 of 85) had a more severe form. Severe cases were more common in line-2 (65 vs 4.6% in line-1; $p < 0.0001$).

The presence of anti-parietal cell Abs (PCAb) was tested by immunofluorescence; 39.5% of A51 animals had high titers of PCAb (data not shown). The incidence of PCAb was significantly higher in line-2 than in line-1 (60.3 vs 27.8%; $p < 0.0001$). A strong correlation between the presence of PCAb and AIG was found, especially for severe forms of AIG (data not shown). In line-2-RAG2^{0/0} mice, despite fewer CD4⁺ mature T cells in the periphery than in A51 lines, all animals (15 of 15) showed gross indications of gastritis (enlarged gastric lymph node), and those

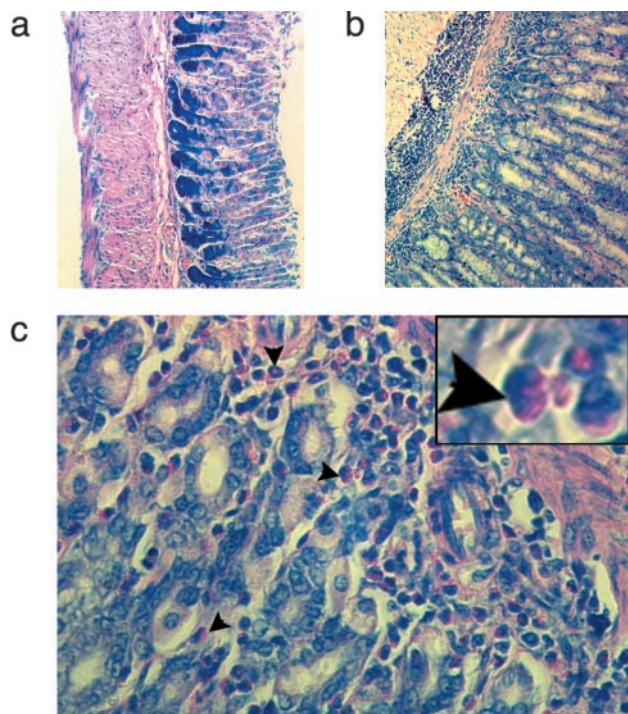


FIGURE 3. AIG in A51 Tg mice. Paraffin-embedded sections of stomach from a non-Tg animal (*a*) and a 12-wk-old A51 animal (*b*) were stained with Giemsa (magnification, $\times 20$). A section from the same A51 animal observed at higher magnification is shown in *c*. Infiltrating eosinophils are indicated by arrows. A blow-up of a cluster of eosinophils is shown in the inset.

examined microscopically (three of three) showed advanced histological features of AIG with a similar eosinophil/polymorphonuclear phenotype (data not shown).

A51 Tg T cells transfer disease to immunodeficient animals

To determine whether A51 Tg T cells mediate AIG, we analyzed the ability of naive cells, such as thymocytes, to transfer AIG to both immunodeficient and immunocompetent mice (see *Materials and Methods*). Four groups of four BALB/c *nu/nu* mice received i.v. 3.7×10^5 , 1.11×10^6 , 3.3×10^6 , or 10×10^6 thymocytes from A51 line-2 animals. Two groups of four BALB/c mice also received either 3.3×10^6 or 10×10^6 thymocytes from the same A51 line-2 animals. Analysis of both PCAb and gastric pathology 7 wk after transfer revealed that although 50% (four of eight) recipient BALB/c mice produced PCAb, none of these showed definite signs of gastritis. The histological score was 0.7 ± 0 in the group that had received 10×10^6 thymocytes and 1.5 ± 0.2 in the group that had been given 3.3×10^6 cells (mean of both groups, 1.1 ± 0.5). By contrast, in the four groups of *nu/nu* recipients, all mice (16 of 16) developed gastritis and 87.5% (14 of 16) of them were positive for PCAb in the serum (data not shown). In all but one of the *nu/nu* recipients, the induced gastritis was very severe; the histological score was 4 ± 0 in the group that had received 1.1×10^6 cells, 3.8 ± 0.2 in the group receiving 3.3×10^6 cells, 3.5 ± 0.2 in the third group (1.1×10^6 cells), and 3.8 ± 0.5 in the fourth group (0.3×10^6 cells transferred). The overall score for all four groups was 3.8 ± 0.3 . The single animal with milder gastritis (score of 3) belonged to the group that had received 0.3×10^6 cells. The histological phenotype of the gastritis induced by transfer of Tg cells to *nu/nu* animals was similar to that of A51 mice or *nu/nu* recipients that had received the TXA51 clone. Large infiltrates containing eosinophils were observed in all animals. It has

been reported that transfer of relatively large numbers (3×10^7) of non-Tg thymocytes from adult BALB/c *nu⁺/nu⁻* animals to *nu/nu* recipients may lead to AIG in a proportion of recipient animals (27, 28), but these recipients always have the inflammatory histological phenotype of lymphocytes without the eosinophils also observed in d3Tx mice or A23 Tg mice. Thus, Tg A51 thymocytes mediate a gastritis in which both the incidence and the histological phenotype are clearly different from those of the gastritis induced by non-Tg cells. In addition, A51 T cells from PLN and GLN, which contain T cells naturally primed to the gastric Ags, could induce a histologically similar AIG after transfer to *nu/nu* recipients. Taken together, these results indicate that A51 T cells mediate a Th2-type AIG in A51 mice.

A51 Tg T cells differentiate into Th2 cells in vivo

To confirm the peptide specificity of the Tg lines, we assayed the proliferation of PLN A51 T cells in response to the cognate peptide recognized by TXA51, PLL($\alpha_{889-901}$). A51 T cells specifically proliferated in response to PLL, but not to the control PIT($\alpha_{630-641}$) (Fig. 4*a*). The expression of the early activation marker, CD69, was substantially increased in the GLN of A51 animals (50.6% of CD4⁺, V β 4⁺ T cells in A51 compared with 15% in non-Tg littermates; data not shown), but not in other peripheral sites, indicating that the priming of naive A51 CD4 T cells occurs in vivo in the GLN that drains the target organ. The cellular phenotype (characterized by the presence of eosinophils in the gastric mucosa) of the spontaneous gastritis observed in A51 mice mimicked the disease induced by transfer of the TXA51 clone to *nu/nu* animals, suggesting that A51 Tg CD4⁺ T cells undergo Th2 differentiation in vivo.

We examined the pattern of cytokine secretion of activated cells in the GLN. A51 T cells isolated from either PLN or GLN were stimulated in vitro with the PLL peptide in the presence of anti-IL-4, anti-IFN- γ , and anti-IL-12 neutralizing Abs. Under these null conditions, differentiation of naive cells is inhibited, and cytokine production is induced only in cells that have already been primed and have undergone Th1 or Th2 differentiation in vivo. Such previously primed A51 cells produced IL-4, but no IFN- γ (Fig. 4*b*). IL-4-producing cells represented 10% of PLN cultures and 20% of GLN cultures.

As the transcription factor GATA-3 is preferentially expressed in Th2 cells (29), we quantified its expression by real-time PCR in purified A51 T cells. GATA-3 transcription was enhanced in A51 CD62L^{low} CD44^{high} activated/memory CD4⁺ T cells compared with A51 CD62L^{high} CD44^{low} naive CD4⁺ T cells, at levels comparable to those observed in Th2 polarized A51 cells (Fig. 4*c*). Another indication of the in vivo Th2 phenotype of A51 T cells was that serum levels of total IgE in A51 mice were increased compared with controls, with a number of animals registering IgE levels >1000 ng/ml, almost 1000-fold the level in controls animals (Fig. 4*d*). These data show that A51 CD4⁺ T cells are primed in vivo in the GLN, where they undergo Th2 differentiation.

Low density of I-A^d-PLL complexes favors Th2 differentiation of A51 T cells

Numerous factors influence the Th1 or Th2 differentiation of naive T cells upon their interaction with cognate MHC class II/peptide expressed on APCs. The strength of the signal delivered through the TCR, depending on the overall avidity of the TCR for the MHC class II/peptide complex, is one critical factor (16, 30, 31). Low avidity can result from 1) low intrinsic affinity of the TCR for the MHC/peptide complex; 2) low affinity of the peptide for the MHC class II molecule, resulting in low surface density of MHC class II/peptide ligand; 3) inefficient generation of antigenic peptide via

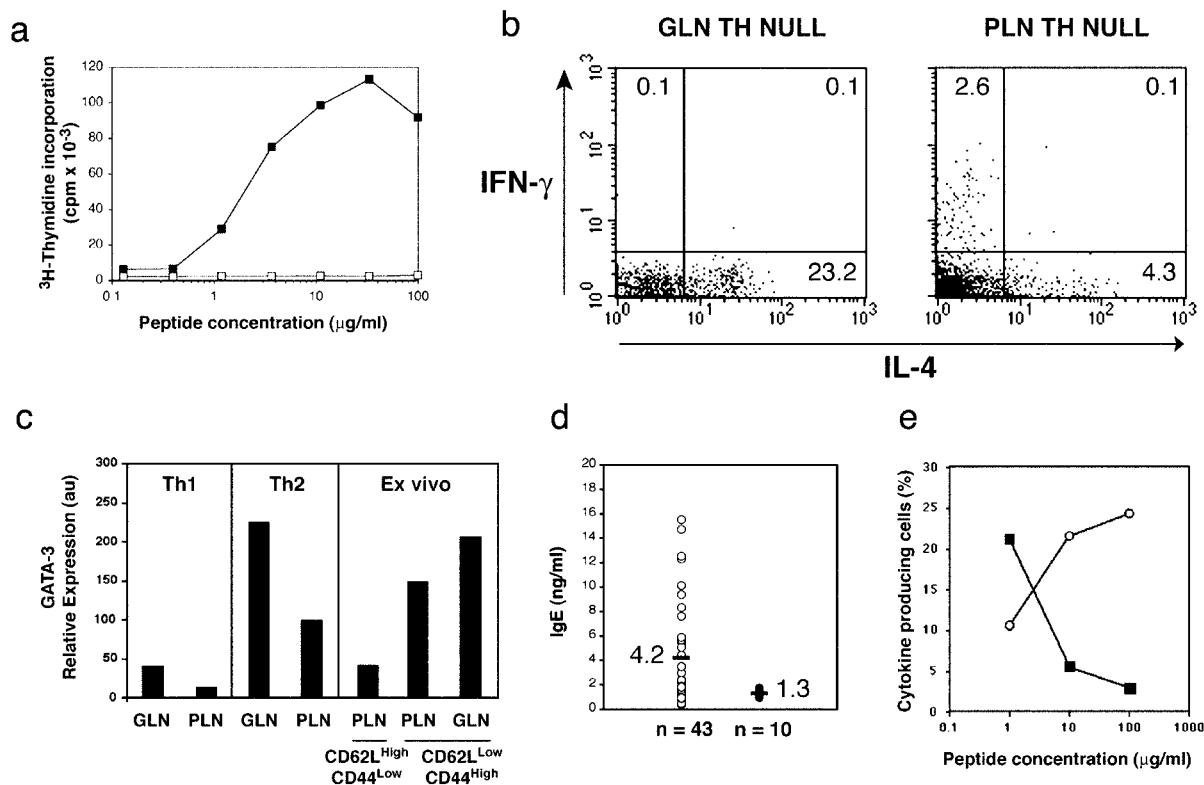


FIGURE 4. A51 Tg T cells undergo Th2 differentiation in vivo. *a*, Cells isolated from PLN of A51 animals (line-2) were cultured in the presence of graded concentration of PIT (□) or PLL peptide (■) and assayed 72 h later for proliferation. Triplicate determinations always varied by <10%. Non-Tg animals do not proliferate to either Ag in a primary response. *b*, Whole suspensions isolated from the GLN or PLN of 10- to 20-wk-old A51 mice were cultured in the presence of 30 μM PLL peptide, under null conditions, as described in *Materials and Methods*. After 6 days, cells were stained for cytoplasmic IFN-γ and IL-4. IL-4 vs IFN-γ dot plots are shown for PLN and GLN cultures gated on CD4⁺Vβ4⁺ cells. The percentages of cells in each quadrant are indicated. The data presented in this study are representative of multiple experiments performed with T cells from A51-line-1 and -line-2 mice. *c*, GATA-3 expression was quantified by real-time PCR in A51 subpopulations of T cells isolated from PLN or GLN of A51 mice: CD4 naive T cells (CD62L^{high}-CD44^{low}) and CD4 T cells with an activated/memory phenotype (CD62L^{low}-CD44^{high}) (right). Control cultures of PLN or GLN cells in the presence of 30 μM PLL peptide and under Th1 or Th2 differentiating conditions were also analyzed for GATA-3 expression (left and middle). *d*, Total IgE serum levels were quantified by ELISA in A51 mice (○) and control littermates (●). *e*, Purified A51 CD4 naive T cells were cultured under neutral conditions in the presence of the indicated concentrations of PLL peptide and BALB/c splenic DCs (T:APC ratio = 5:1). After 6 days, cells were restimulated and stained for intracytoplasmic IL-4 (■) and IFN-γ (□). The percentages of CD4⁺Vβ4⁺ cells secreting either IL-4 or IFN-γ are shown. The results presented in this study are representative of at least three independent experiments.

Ag processing pathways, resulting in low surface density of MHC-II/peptide ligand; 4) low level expression of TCR; or 5) some combination of these mechanisms. To understand the parameters driving the Th2 differentiation of A51 T cells in vivo, we simulated in vitro the priming of naive A51 CD4⁺ cells with graded concentrations of the PLL peptide pulsed onto BALB/c splenic DC (Fig. 4e). At low doses of peptide, a greater proportion of cells (21.4%) were induced to produce IL-4, whereas at high doses of peptide, IL-4 production decreased, and IFN-γ production correspondingly increased to 24.4%. These results suggest that the low level of presentation of the PLL epitope in vivo after processing of the native Ag by APCs could be responsible for the Th2 phenotype of the autoimmune response in A51 mice.

To evaluate the relative efficiency of the generation of I-A^d-PIT and I-A^d-PLL complexes resulting from the processing of the H/K-ATPase and the endogenous MHC class II presentation pathways in BM DCs, we used the original T cell clones, TXA23 and TXA51. These clones were stimulated with BALB/c DCs pulsed with the whole protein Ag. As shown in Fig. 5, TXA23 responded more vigorously than TXA51 to gastric microsomes as a source of the H/K-ATPase. This result suggests that the TXA51 epitope, PLL, may be generated less efficiently than the TXA23 epitope, PIT, or that the PLL peptide has a lower affinity for I-A^d than PIT,

leading to a lower density of I-A^d/PLL complexes at the membrane. The observations that 1) naive Th2 cells from the Tg animals can be directed to differentiate into Th1 cells by increasing doses of antigenic peptide presented on professional APCs (splenic

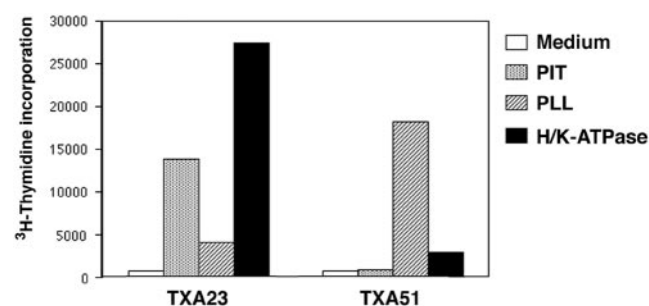


FIGURE 5. H/K-ATPase-pulsed DCs poorly activate TXA51 T cell clone. BALB/c BM DCs were pulsed with optimal concentrations of PIT (30 μM), PLL (30 μM), or H/K-ATPase (5 μg/ml). TXA23 and TXA51 T cell clones were cultured in the presence of pulsed DC and assayed for proliferation 72 h later. The data shown are representative of three experiments.

DCs); and 2) the endogenous processing of H/K-ATPase for presentation to the TXA51 clone appears relatively inefficient suggest that the observed *in vivo* Th2 phenotype of the A51 Tg T cells is related to an overall low level activation of the T cell.

Discussion

Constitutive expression of the TXA51 TCR in a large proportion of developing and mature T cells results in AIG of indolent phenotype, characterized by late onset of disease, pathological eosinophilia in the gastric mucosa, and elaboration of Th2 cytokines. The A51 transgenic mice represent a unique model for autoimmune disease, and they illustrate that the *in vivo* response to a self-Ag can be Th2 and yet also result in pathological autoimmunity. This model of Th2-mediated organ-specific autoimmune disease provides an important example that challenges the simple view that Th2 responses are necessarily protective, whereas Th1 responses are pathogenic (32). Indeed, all other known TCR-transgenic models for autoimmune diseases suggest that Th1 responses are required for pathogenicity. This Th2 model suggests that there is a narrow window of T cell activation by self Ags, dictated by the TCR itself, in which autoimmune reactivity may coexist with a distinct cytokine phenotype.

The pathogenicity of autoreactive Th2 T cells has been demonstrated previously in cell transfer experiments. Both Th1 and Th2 cells generated from MBP-specific TCR Tg mice transfer EAE to RAG-1^{o/o} mice and to $\alpha\beta$ T cell-deficient mice (9), and Th2 cells induce diabetes in NOD.scid mice (8). In an Ag-specific model, transfer of either Th1 or Th2 T cells to RAG-2^{o/o} animals results in severe wasting disease and colitis (33). In each of these examples, Th2-polarized cells home to the target organ and induce tissue destruction in immunodeficient animals. Importantly, self-reactive Th2 cells only rarely transfer disease to wild-type mice, suggesting that regulatory mechanisms are present in immunocompetent animals that are lacking in immunodeficient mice.

Other Th2-mediated models of autoimmunity are either Ab-mediated (34) or involve gut Ags (35–37). A triggering event such as an inflammatory insult, immunization with a self-Ag or a hapten, or a defect in the generation of CD4⁺CD25⁺ regulatory T cells is required to break tolerance and induce disease. Examples of such Th2-mediated models are the experimental colitis induced by oxazolone in SJL/J mice (35, 38), the spontaneous colitis in TCR α knockout C57BL/6 mice (36, 37), and the HgCl₂-induced autoimmune glomerulonephritis in rats (34). By contrast, in the A51 model presented in this study, the transgenic expression of an H/K-ATPase-reactive TCR on a BALB/c background is sufficient to induce AIG spontaneously. This suggests that the usual requirement for Th2 autoimmunity of a decrease in regulatory cell function can be countered by an increase in proportionate representation of pathogenic cells in the TCR-Tg model.

Peripheral autoreactive T cells are presumably kept under control or anergized by regulatory T cells. In both A51 (data not shown) and A23 models (19), CD4⁺CD25⁺ regulatory T cells exist in the thymus and the periphery. These cells are functional, at least in the A23 model, as they are immunosuppressive *in vitro* (19). Nevertheless, regulatory T cells are not sufficient to control autoreactive T cells in the Tg TCR setting, as both A23 and A51 mice develop AIG. This loss of tolerance may be related to the presence of large numbers of H/K-ATPase-reactive T cells that overwhelm the available CD4⁺CD25⁺ regulatory cells or to the delayed development of such regulatory cells consequent to the transgenic expression of a TCR α -chain (39).

The T cell clone used as the donor of the TCR for this Tg model of AIG was derived from an animal that developed AIG after d3Tx (18). Thus, it represents a cell that was positively selected in the

thymus and escaped negative selection there, but is normally kept under control by organ-specific regulatory cells. One might have expected that such cells would undergo positive selection efficiently. However, in A51 mice, fewer thymocytes than normal are positively selected, suggesting a low avidity of the TXA51 TCR for the selecting ligand in the thymus. By contrast, in the A23 model, there is strong skewing toward CD4 differentiation with increased numbers of CD4⁺ SP thymocytes (19), suggesting higher avidity of the TXA23 TCR for its selecting ligand. These differences in thymic selection suggest either that the intrinsic affinities of the two different TCR for their ligands differ, or that their selecting ligands, as a result of differences in processing and presentation, are differentially available.

DCs are the crucial APCs involved in the priming of naive T cells *in vivo*. A recent study identified CD11c⁺CD8a⁺ DC as the cell subset responsible for the constitutive uptake of H/K-ATPase in the gastric mucosa and its presentation in the draining GLN in BALB/c mice under normal noninflammatory conditions (23). CD11c⁺ DCs in the GLN, but not in other PLNs, constitutively process H/K-ATPase and present H/K-ATPase- α -derived epitopes, such as the PIT epitope. The *in vivo* generation and presentation of the PLL epitope by DCs in the GLN were not tested directly in this study. However, several lines of evidence suggest that the presentation of I-A^d-PLL complexes at the surface of APCs might be less efficient than that of I-A^d-PIT complexes. Firstly, although the original TXA51 clone was generated from d3Tx GLN T cells stimulated with the whole H/K-ATPase, it cannot be restimulated *in vitro* with H/K-ATPase, but only with the PLL peptide, whereas the TXA23 clone generated in the same conditions can be restimulated either with H/K-ATPase or the PIT peptide (data not shown). Secondly, in this study we show that BM CD11c⁺ DCs, when pulsed *in vitro* with the H/K-ATPase, induce strong proliferation of the TXA23 clone, but weak proliferation of the TXA51 T cell clone, which, by contrast, can be efficiently activated with PLL-pulsed DCs. This relative inefficiency of I-A^d-PLL presentation could be related to inefficient generation of the PLL sequence upon processing of the H/K-ATPase and/or lower affinity of the PLL peptide for I-A^d, leading to a short half-life of I-A^d/PLL complexes at the cell surface. One consequence of a low availability of I-A^d-PLL complexes might be competition among naive A51 Tg T cells for activating ligand in the GLN. This, together with a low input of T cells from the thymus to the periphery, may result in the observed delay in disease onset in A51 mice compared with A23 mice.

Another consequence of a low availability of I-A^d-PLL complexes might be the preferential induction of Th2 differentiation of A51 T cells during priming. Indeed, *in vitro* and in the absence of pathogen-derived products and cytokines, the Th effector phenotype induced by priming with DCs depends essentially on the Ag dose. A high dose of Ag promotes Th1 differentiation, whereas a low dose suppresses Th1 development and allows Th2 differentiation (16, 40–43). In our model we showed similarly that *in vitro*, unfractionated splenic CD11c⁺ DCs drive the Th2 differentiation of naive A51 T cells only at low doses of the PLL peptide, suggesting that a low efficiency of PLL presentation *in vivo* might be critical for the Th2 differentiation of A51 T cells. In this regard, it is interesting to note that another group was able to establish from the GLN of a d3Tx BALB/c mouse a CD4⁺ T cell clone reactive to an epitope of the H/K-ATPase ($\alpha_{891-905}$) that includes most of the residues of the PLL peptide (44). This clone expresses a different TCR than the TXA51 clone and is a Th1 cell. Thus, presentation of the PLL epitope in GLN may lead to either Th1 or Th2 differentiation of autoreactive T cells upon priming, and we suspect that the critical factor determining T cell fate is the intrinsic

affinity of the particular TCR involved. Overall, the Th2 phenotype of the autoimmune response in A51 mice results from several co-existing aspects of this model system: first, the level of endogenous presentation of the initiating autoimmune Ag, PLL, is below a critical level for the induction of a Th1 response; and second, the intrinsic affinity of the A51 TCR is sufficiently low that the level of MHC-II/peptide complexes generated endogenously is only adequate for stimulation of Th2 differentiation.

In summary, the A51 lines represent a new Tg model of spontaneous Th2-mediated, organ-specific, autoimmune disease. In this model a narrow window of avidity of TCR/MHC-self-peptide interaction seems to control the egress of self-reactive T cells from the thymus, the efficient priming of mature T cells in the draining LN of the target organ, and, ultimately, their differentiation into pathogenic Th2 cells.

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