Factors affecting the susceptibility of the mouse pituitary gland to CD8 T-cell-mediated autoimmunity

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SUMMARY
We have previously shown, in a transgenic mouse model, that the pituitary gland is susceptible to CD8 T-cell-mediated autoimmunity, triggered by a cell-specific model autoantigen, resulting in pan-anterior pituitary hypophysitis and dwarfism. In the present study, we now demonstrate that antigen dose, the T-cell precursor frequency, the degree of lymphopenia and the context of target antigen expression, are important parameters determining the time course and extent of the pathological consequences of CD8 T-cell-mediated autoimmunity. Furthermore, our data indicate that the pituitary gland is susceptible to CD8 autoimmunity following an inflammatory insult such as a viral infection. As lymphocytic hypophysitis may be manifest in other autoimmune conditions, and the pituitary gland may be susceptible to T-cell-mediated pathology after immunization with a virus expressing soluble pituitary antigen, it is important to consider that strategies based on vaccination against soluble pituitary gonadotrophins could have other unexpected endocrine consequences.

INTRODUCTION
Thymic clonal deletion is the major mechanism of T-cell tolerance to self-antigens. Recent studies have shown that even several 'tissue-specific' and developmentally regulated proteins are expressed by rare medullary thymic epithelial cells (mTECs)1,2 and it has been proposed that T-cell engagement of self-peptides, expressed by mTECs, may impose a regulatory T-cell phenotype. Despite this additional mechanism of thymic regulation, CD8 T cells with self-antigen specificity do leave the thymus and enter the periphery, with the potential to cause autoimmunity (reviewed in ref. 3). In most situations these T cells will not become autoaggressive, but instead may engage major histocompatibility complex (MHC) antigen yet remain unresponsive,4 be activated and undergo proliferation before dying of activation-induced cell death,5,6 or never encounter their cognate antigen and remain ignorant.7,8

Ignorance may occur because T cells do not migrate to the site of antigen expression, or the antigen may not be presented in the correct context for T-cell recognition. For example, cross-presentation of soluble protein is relatively inefficient,9 and proteins such as hormones, which occur in small quantities in the bloodstream, may not reach sufficiently high concentrations to be cross-presented to CD8 T cells. However, when soluble antigen is present in high quantities, CD8 T-cell tolerance may occur by cross-presentation to T cells by professional antigen-presenting cells (APCs)10 and, in some cases, non-professional APCs.11 Cell-associated (necrotic or apoptotic cells) antigen is efficiently cross-presented to CD8 T cells12 and this is a well-described mechanism of peripheral tolerance to tissue-restricted antigens.12

The role of CD8 T cells in autoimmune diseases is gradually becoming better understood. In mice, it has been shown that CD8 T cells can mediate experimental autoimmune encephalomyelitis (EAE)13,14 and diabetes,15,16 caused by pancreatic β-cell damage. Indeed, endocrine organs with a specialized vasculature into which large quantities of secreted protein are abruptly secreted, may be particularly susceptible to an autoimmune pathology, readily detected by the obvious phenotypic consequences of endocrine deficiency.17 Although reportedly rarer than other autoimmune endocrinopathies, the pituitary gland of humans is susceptible to such attack.18 A putative autoimmune has recently been described,19 but the role of CD8 T cells in lymphocytic hypophysitis is unknown.

We previously developed a model of this condition in transgenic mice and showed that the mouse pituitary gland is susceptible to CD8 T-cell-mediated autoimmunity, triggered by
a cell-specific model autoantigen, resulting in pan-anterior pituitary hypophysitis, multiple hormone deficiencies and dwarfism. In the present study we now demonstrate that antigen dose, the context of expression of the target antigen, the T-cell precursor frequency and the degree of lymphopenia, are important parameters in determining the time course, extent and pathological consequences of CD8 T-cell-mediated autoimmunity. This model also shows that the pituitary gland may be highly susceptible to CD8 autoimmunity following an inflammatory insult, such as a viral infection.

**MATERIALS AND METHODS**

*Mice*

Rag1<sup>−/−</sup> H-2<sup>b</sup> (Rag<sup>−/−</sup>), F5 Rag1<sup>−/−</sup> H-2<sup>b</sup> (Rag<sup>−/−</sup> F5) mice<sup>21</sup> and F5 Rag1<sup>−/−</sup> H-2<sup>b</sup> mice expressing green fluorescent protein (GFP) under control of the CD2 promoter in all T cells (GFP-F5),<sup>22</sup> were bred in the animal facilities of the National Institute for Medical Research (London, UK), in accordance with established guidelines. 48-Rag1<sup>−/−</sup> H-2<sup>b</sup> mice, transgenic for the influenza nucleoprotein A/NT/60/68 (NP), and the double-transgenic F1 intercross with F5, were as described previously<sup>20</sup> and were termed 48-Rag<sup>−/−</sup> and 48-Rag<sup>−/−</sup> F5 throughout this study. These mice expressed the NP epitope fused to the human growth hormone (hGH) signal peptide, which was driven by a 40-kb hGH locus control region (LCR) promoter that restricts expression to the regulated secretory vesicle pathway in pituitary GH cells (somatotrophs).<sup>20</sup> In this study, additional mice were created that had a truncated variant of this construct (see below); these Δ2-Rag<sup>−/−</sup> H-2<sup>b</sup> mice, and the double-transgenic F1 intercross with F5, are termed Δ2-Rag<sup>−/−</sup> and Δ2-Rag<sup>−/−</sup> F5, respectively. Finally, mice transgenic for NP on a polyclonal C57BL/10 background are called 48-B10 and Δ2-B10. Table 1 summarizes the genotypes of the strains used.

**Construction of the Δ2-GH-NP cosmid for generating transgenic animals**

Polymerase chain reaction (PCR) mutagenesis was used to delete the hGH-coding sequence between the first codon of hGH exon 2 and the last four codons of exon 5 (Fig. 1a). The deletion removed virtually all of the hGH signal peptide and was created using the ExSite<sup>®</sup> PCR site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) and the following primers: 5′-

![Image](image.png)

**Figure 1.** Expression of the influenza nucleoprotein A/NT/60/68 (NP) in the pituitaries of Δ2-Rag<sup>−/−</sup> and 48-Rag<sup>−/−</sup> mice. (a) Schematic representation of the NP transgenic constructs used to generate the Δ2-Rag<sup>−/−</sup> and 48-Rag<sup>−/−</sup> lines. The NP gene replaced the sequence between exons 2 and 5 of the human growth hormone (hGH) gene. Note the deletion of the growth hormone (GH) signal sequence (SS) in the Δ2-Rag<sup>−/−</sup> construct. Arrows indicate the binding sites of primers used for genotyping and non-quantitative reverse transcription–polymerase chain reaction (RT–PCR). (b) Non-quantitative RT–PCR of pituitary RNA and PCR of genomic DNA from adult Δ2-Rag<sup>−/−</sup> and 48-Rag<sup>−/−</sup> mice.

AGCTGTGAGCTTCTAGCTGCAGCCGAGGTTG-3′ and 5′-GCCTGGGGAGAAACCAGAGGCAAC-3′ (5′ phosphorylated). The deletion left the splice acceptor region between exon 1 and exon 2 intact and generated a *PvuII* site at the junction between exon 2 and the remainder of exon 5. The NP insert was derived from influenza A/NT/60/68 nucleoprotein (GenBank accession no. J02137). An NP fragment was generated by PCR and was identical to the NP expressed in the previously described ‘48′ line,<sup>20</sup> except for changes to the 5′ end to allow in-frame fusion with the mutated hGH exon 2 (see above). The primers used for the PCR were: sense, 5′-CAGCTGCGCGCTGTGATGG-3′; antisense, 5′-CAGCTGCGAGCTGATGTCGAGGACTGTGATG-3′. The NP fragment was flanked by *PvuII* sites and encoded both a truncated NP protein (amino acids 2, 327–498) and the influenza haemagglutinin (HA) epitope, YPYDVPDYA. NP was subcloned into the neo *PvuII* site in vector pN3/M-ΔGH-NP that contained the mutated hGH sequence flanked by *MluI* sites. The mutated, transgenic sequence was subcloned into the B2K cosmid,<sup>23</sup> containing a 40-kb insert including the hGH LCR, to generate the construct for microinjection. The final product would be predicted to express an HA-tagged NP fusion protein, but with only the first three residues of the hGH signal peptide, and would thus not be expected to enter the regulated secretory pathway.

**Generation of transgenic mice**

Cosmid cosΔGH-NP DNA was linearized by digestion with NotI. The fragment was purified by gel extraction and column elution (Schleicher and Schuell, Dassel, Germany) and adjusted

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<th>Mouse strain</th>
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GFP, green fluorescent protein; MHC, major histocompatibility complex; TCR, T-cell receptor.
to a concentration of 1–5 ng/μl in 0.5 mM EDTA, 1 mM Tris-HCl, pH 7.5. Transgenic mice were generated by pronuclear microinjection of fertilized oocytes of superovulated Rag1−/− H-2b mice, followed by oviductal transfer into pseudopregnant recipients.

Genotyping of transgenic animals
Genomic DNA from tail biopsies was analysed for transgene DNA by a standard PCR using NP-specific primers: forward, 5’-GCTCACTAGCGGAATGCG-3’; and reverse, 5’-AGGCCTCTGGTGTAGTTGTC-3’. The PCR conditions were: 94° for 45 seconds, 52° for 30 seconds, and 72° for 45 seconds, per cycle, for 35 cycles.

Reverse transcription–polymerase chain reaction (RT–PCR)
Total RNA was extracted from whole pituitaries using the TRIZOL® reagent (Life Technologies, Rockville, MD). cDNA was generated with reverse transcriptase and random hexamers (GeneAmp® RNA PCR Core Kit; Perkin Elmer, Shelton, NJ) and used in a standard PCR with the NP-specific primers and conditions specified above. The semiquantitative RT–PCR was carried out using age-matched pituitaries from male and female Δ.2-Rag−/− and 48-Rag−/− mice. cDNA was reverse transcribed from approximately 1 μg of total RNA, using Superscript III (Invitrogen, Carlsbad, CA) and an oligo-dT primer, according to the manufacturer’s instructions. Dilutions of the cDNA were used in a PCR with primers that were specific for GH gene sequences and amplified both endogenous and transgenic cDNA: forward, 5’-AACACTACGGTTCTGGGAGACG-3’; and reverse, 5’-AGGCATCTAATTATTAGTGGACGAA-3’. The PCR conditions were: 94° for 30 seconds, 58° for 30 seconds, 72° for 1 min, per cycle, for 29 cycles (these conditions were previously tested to show that at the dilution range used this number of cycles is still in the linear range of amplification).

In vitro antigen-presentation assay
The relative quantity of transgenic NP in Δ.2-Rag−/−, 48-Rag−/− and Rag−/− (negative control) pituitaries was estimated in an in vitro cross-presentation assay. Pituitaries were homogenized in complete Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 5% fetal calf serum (FCS). F5 lymph node T cells were incubated at 37° for 10 min at a cell density of 10⁶/ml with 1 μM carboxy fluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) in phosphate-buffered saline (PBS) and washed thoroughly. Bone-marrow-derived H-2b SCs were generated in vitro by 6 days of culture in granulocyte–macrophage colony-stimulating factor (GM-CSF)-containing medium, as previously described.²⁴ Pituitary extracts were titrated and incubated with 1 × 10⁷ dendritic cells (DCs) and 5 × 10⁴ CFSE-labelled F5 T cells in complete IMDM, containing 5% FCS, for 5 days at 37°. NP peptide (ASNNMDAM) (1 nM) was used as a positive control. Proliferation of F5 T cells was assessed by pooling cells from triplicate wells and analysis by flow cytometry, as described below.

Virus infection and cell transfers
For virus infections, a total of 10⁶ plaque-forming units (PFU) of influenza virus A/NT/60/68 was injected intravenously (i.v.) in 50 μl of phosphate-buffered saline (PBS). In some experiments, spleen and lymph node cells containing a total of 5 × 10⁶ GFP-F5 CD8 T cells, were co-injected with virus i.v., or transferred alone, into host mice, in a total volume of 0.2 ml. To monitor in vivo proliferation, 10 × 10⁶ F5 T cells were labelled with CFSE, as described previously,²⁵ and injected i.v. into recipient mice in a total volume of 0.2 ml of air-buffered IMDM. Before injection, total spleen and lymph node cells (10⁷/ml) were incubated with 2.5 μM CFSE in PBS at 37° for 10 min. Four days after transfer, T cells were recovered from spleen and lymph nodes and analysed for CFSE by fluorescence-activated cell sorter (FACS) analysis.

FACS analysis
Single-cell suspensions of pituitaries, spleen or lymph nodes were stained by sequential incubations on ice with antibodies specific for cell-surface markers followed by streptavidin-fluorochrome conjugates, when necessary. Non-specific staining via FcR binding was blocked by initial incubation with the anti-FcR clone, 2.4G2. Cell data were acquired on a FACScalibur (Becton-Dickinson Co., San Jose, CA) using CELLQUEST software. Antibodies and secondary reagents are listed as follows: CD8α-APC (53-6:7); CD8α-phycocerythrin (PE) (53-6:7); T-cell receptor (TCR)-β-APC (H57); CD44-biotin (IM7) and streptavidin-Cy7PE (all Becton-Dickinson). NP-epitope (ASNNMDAM)-specific T cells were detected using recombinant soluble dimeric mouse H-2Db-immunoglobulin fusion proteins (DimerX I; Becton-Dickinson). Dimers were reconstituted overnight at 37° in PBS at a concentration of 10 μg/ml in a 160-m excess of peptide. Cells were stained with the H-2Db-immunoglobulin reagent (2.5 μg/ml) for 1 hr on ice followed by incubation with PE-conjugated anti-mouse immunoglobulin G (IgG)1 monoclonal antibody (mAb) A85-1 (Becton-Dickinson). Intracellular interferon-γ (IFN-γ) was detected in T cells following a 4-hr stimulation at 37° in complete IMDM containing phorbol-12,13 dibutyrate (PdBu) (50 ng/ml), ionomycin (50 ng/ml) and brefeldin A (10 μg/ml). Cells were washed, stained for cell-surface markers (as described above) and then fixed in 1% paraformaldehyde for 15 min on ice and permeabilized in 1% Nonidet P-40 (NP-40) for 3 min on ice. Cells were incubated with rat anti-interferon-γ (IFN-γ) or rat IgG1-PE antibody isotype control (Becton-Dickinson). Analysis of FACS data was performed with either CELLQUEST (Becton-Dickinson) or FLOWJO software (Tree Star Inc., San Carlos, CA).

Radioimmunoassays
Mouse GH in pituitary extracts was assayed by radioimmunoassay (RIA),²⁶ using reagents kindly provided by NIDDK (Bethesda, MD). Unless otherwise stated, data are shown as mean ± standard deviation (SD). A two-tailed Student’s t-test was used to estimate P-values, and a P-value of <0.05 was considered significant.

RESULTS
Relative expression of NP by pituitary somatotrophs
To study the role of the context of antigen expression in vivo, we used two distinct lines of transgenic mice that expressed an NP
epitope exclusively in the somatotrophs of the anterior pituitary gland. In ‘48’ strain mice (described above and previously\(^2\)), pituitary somatotrophs produce NP with a signal peptide that directs its packaging to secretory vesicles and secretion in a manner similar to that of GH. The new line reported here as ‘\(\Delta 2\)’, expresses the same NP epitope, but from a truncated product that lacks a signal peptide, so that the protein is retained in the cytoplasm of somatotrophs and does not reach the regulated secretory pathway. Each of these transgenic lines was bred with either Rag\(^{1/-}\) F5 mice, which express a monoclonal population of CD8 T cells specific for influenza A/NT/60/68 NP epitope 366–374, Rag\(^{1/-}\) mice or C57BL/10 (B10) mice. Expression of NP mRNA in both the 48-Rag\(^{1/-}\) and \(\Delta 2\)-Rag\(^{1/-}\) transgenic strains was demonstrated by RT–PCR (Fig. 1b), confirming that mRNA transcripts of the correct size were present in transgenic pituitaries.

NP protein was detected by Western blotting and immunocytochemistry in the 48-Rag\(^{1/-}\) strain only and not in the \(\Delta 2\)-Rag\(^{1/-}\) strain. This indicated that the \(\Delta 2\)-Rag\(^{1/-}\) strain expressed lower amounts of NP protein. Alternatively, faster degradation of NP protein in the cytoplasmic compartment in the \(\Delta 2\)-Rag\(^{1/-}\) strain might compromise its detection with HA antibody.

To determine relative expression levels in a semiquantitative manner, two assays were performed. First, a novel T-cell-based bioassay was devised to directly measure the amount of protein in pituitaries. Pituitary extracts from age- and gender-matched 48-Rag\(^{1/-}\) and \(\Delta 2\)-Rag\(^{1/-}\) mice were titrated in culture with CFSE-labelled F5 T cells and H-2\(^{b}\) DCs. Both extracts were processed by DCs and caused a comparable low level of proliferation by the F5 T cells (Fig. 2a). The level of proliferation of the F5 T cells should be related to the quantity of pituitary-derived NP peptide processed and presented by DCs. More considerable proliferation of T cells was observed when DCs were coated with 1 nM NP peptide (Fig. 2b). Pituitary extract from control Rag\(^{1/-}\) mice did not cause any F5 T-cell proliferation (Fig. 2a).

Second, a semiquantitative RT–PCR was used to measure NP mRNA levels in the pituitaries of the 48-Rag\(^{1/-}\) and \(\Delta 2\)-Rag\(^{1/-}\) mice. While the bioassay suggested that pituitaries from the two strains contained similar quantities of NP protein, the semiquantitative RT–PCR demonstrated considerably lower levels of NP mRNA in the \(\Delta 2\)-Rag\(^{1/-}\) strain (Fig. 2c).

\(\Delta 2\)-Rag\(^{1/-}\) F5 double-transgenic mice have a normal growth phenotype

We previously reported that 48-Rag\(^{1/-}\) mice are normal, but that 48-Rag\(^{1/-}\) F5 mice, which are double transgenic for the secreted form of NP and the F5 TCR, show an early massive destruction of GH-producing cells and develop a dwarf phenotype.\(^2\) We now show that the growth phenotype of both \(\Delta 2\)-Rag\(^{1/-}\) and \(\Delta 2\)-Rag\(^{1/-}\) F5 mice was normal (Fig. 3a). However, T-cell infiltration was seen in the pituitary of some adult \(\Delta 2\)-Rag\(^{1/-}\) F5 mice (Fig. 3c), and when GH levels were measured in 16-week-old \(\Delta 2\)-Rag\(^{1/-}\) F5 mice, they were significantly \((P = 0.003)\) lower than in age-matched Rag\(^{1/-}\) F5 control mice (Fig. 3b). In rodents, growth becomes GH dependent at \(\approx 3\) weeks of age. Thus, even with severe GH deficiency from birth, growth defects only become apparent during the third postnatal week, and animals with more slowly developing GH deficiency can achieve normal size in adulthood.\(^2\) The difference in growth phenotypes between 48-Rag\(^{1/-}\) F5 mice and \(\Delta 2\)-Rag\(^{1/-}\) F5 mice could be explained by the lower level of antigen expression combined with its intracellular context in the \(\Delta 2\)-Rag\(^{1/-}\) F5 mice. Both parameters might result in delayed T-cell recognition and activation, resulting in a delayed onset and severity of GH deficiency.

Non-secreted NP appears to take longer to reach peripheral T cells when compared with secreted NP (Fig. 3d), as shown by the fact that CFSE-labelled F5 T cells transferred into T-cell-replete \(\Delta 2\)-B10 hosts of different ages only divided in the lymph nodes of mice more than 2 weeks old (Fig. 3d). This delay in activation of F5 T cells may explain the normal growth of \(\Delta 2\)-Rag\(^{1/-}\) F5 mice. The gradual accumulation of T cells in the pituitaries of these mice eventually overcomes the ability of the somatotroph population to proliferate in response to somatotroph damage, so that GH deficiency becomes manifest in adulthood. In contrast, the pituitaries of 48-Rag\(^{1/-}\) F5 mice expressing the secreted form of the NP antigen are infiltrated by T cells as early as 2 weeks after birth (J. de Jersey, unpublished observation). Transferred CFSE-labelled F5 T cells divided in the lymph nodes of 2-week-old 48-B10 hosts (Fig. 3d).

**Figure 2.** (a) **In vitro** proliferation of carboxy fluorescein diacetate succinimidyl ester (CFSE)-labelled F5 T cells in response to cross-presentation of \(\Delta 2\)-Rag\(^{1/-}\), 48-Rag\(^{1/-}\) and Rag\(^{1/-}\) pituitary extracts by bone marrow-derived H-2\(^{b}\) dendritic cells (DCs). (b) **In vitro** proliferation of CFSE-labelled F5 T cells incubated with DCs and 1 nM ASNENMDAM peptide. (c) Semi-quantitative PCR analysis of influenza nucleoprotein A/NT/60/68 (NP) transgene expression. RNA was extracted from the pituitaries of 48-Rag\(^{1/-}\) and \(\Delta 2\)-Rag\(^{1/-}\) mice and reverse transcribed, as described. Dilutions of cDNA were amplified with primers specific to NP and growth hormone (GH). The GH semiquantitative PCR was used to control for the concentration of cDNA in each sample.

**Pituitary pathology is less severe on a Rag\(^{1/-}\) background**

In our previous study, the marked and early-onset pituitary damage occurred in Rag-negative mice ('48-Rag\(^{1/-}\) F5') that
Figure 3. Characteristics of double-transgenic \( \Delta 2 \)-Rag-\(^{−/−} \) F5 mice. (a) Growth of male \( \Delta 2 \)-Rag-\(^{−/−} \) F5 (■) mice compared with F5 (○) and 48-Rag-\(^{−/−} \) F5 (×) mice. Growth data for the 48-Rag-\(^{−/−} \) F5 mice have been previously published\(^{20} \) and are included here for comparison only. (b) Pituitary GH levels in individual 16-week-old male \( \Delta 2 \)-Rag-\(^{−/−} \) F5 (■; \( n = 4 \)) and Rag-\(^{−/−} \) F5 (○; \( n = 6 \)) mice. The mean value for each group is indicated by a horizontal line. (c) High CD44 expression in pituitary-infiltrating CD8\(^{+} \) T-cell receptor (TCR)\(^{+} \) cells isolated from a 12-week-old \( \Delta 2 \)-Rag-\(^{−/−} \) F5 mouse. (d) In vivo proliferation of transferred, CFSE-labelled F5 T cells in the cervical lymph nodes of B10, 48-B10 and \( \Delta 2 \)-B10 mice of various ages. B10 mice (\( n = 5 \)), 48-B10 (13 days old; \( n = 8 \)) and \( \Delta 2 \)-B10 mice (14 days old; \( n = 7 \), 23 days old, \( n = 5 \); and >35 days old, \( n = 10 \)) were injected intravenously (i.v.) with \( 10 \times 10^6 \) T cells. The percentage of transferred T cells that had undergone one or more divisions was determined by fluorescence-activated cell sorter (FACS) analysis, 4 days after cell transfer. Numbers indicate the proportion of mice in each group in which the F5 T-cell proliferation was observed.

48-Rag-\(^{−/−} \) F5 mice were bred with B10 mice to generate two different strains: 48-B10-F5 and 48-B10. 48-B10-F5 mice were Rag-\(^{−/−} \) and heterozygous for the transgenic F5 TCR. 48-B10 mice no longer had a transgenic TCR and were also Rag-sufficient. We previously reported that the pituitaries of 16-week-old 48-B10-F5 mice were GD-deficient (3% of normal GD levels), but the deficiency was far less severe than that seen in age-matched 48-Rag-\(^{−/−} \) F5 mice (undetectable levels)\(^{20} \). 48-B10 mice, without any transgenic T cells, had normal levels of pituitary GH (Fig. 4).

This result suggested that the degree of autoimmune pathology was related to T-cell precursor frequency and/or lymphopenic status. When the absolute number of F5 T cells was quantified with a dimeric major histocompatibility complex (MHC) reagent, the Rag\(^{+} \) B10-F5 mice contained a greater number of F5 T cells than age- and gender-matched Rag-\(^{−/−} \) F5 counterparts (Fig. 4a). A high degree of allelic exclusion was observed in B10-F5 mice, with \( \approx 96\% \) of lymph node CD8 T cells bearing the transgenic F5 TCR (Fig. 4b). Therefore, a reduced frequency of F5 T cells was not likely to be the reason for the lesser severe phenotype observed in 48-B10 mice. Other probable factors include the lack of lymphopenia in 48-B10-F5 mice that had normal-sized spleens. Thus, activated F5 T cells had less ‘space’ to fill in these mice. Additionally, it is conceivable that some of the F5 TCR-bearing CD8 T cells had a second TCR, possibly reducing the functional capacity of these dual-receptor cells\(^{29,30} \). The mean fluorescence intensity of F5 TCR staining in B10-F5 mice (397 ± 22) was slightly reduced compared to that of Rag-\(^{−/−} \) F5 mice (433 ± 51). The normal pituitary phenotype in 48-B10 mice suggests that their NP-specific T-cell precursor frequencies are too low to allow for functional recognition of pituitary-derived NP, or that if such activation occurs, it is either controlled or is insufficient to overcome the normal mechanisms that maintain an adequate number of GH-producing somatotrophs\(^{31} \).

CD8 T cells cause pituitary pathology after virus infection

In the absence of immunization, 48-B10 mice do not suffer any pituitary damage. Their pituitaries are free of T cells until an old age and they have a normal growth phenotype and normal GH levels (Fig. 5). When 48-B10 mice were infected with influenza virus A/NT/60/68, NP-specific T cells were detectable in the peripheral lymph nodes and spleen at day 7 after infection. This population of endogenous ASNENMDAM (NP 366–374) epitope-specific CD8 T cells was undetectable in uninfected mice. At day 7 after infection, endogenous NP 366–374-specific T cells comprised 2.2 ± 0.7% (\( n = 3 \)) of the total CD8 T-cell population in the spleens of 48-B10 mice. Six weeks after virus infection, the pituitary pathological changes were severe, and the mice showed marked weight loss.

Figure 4. Characteristics of F5 T-cell receptor (TCR) transgenic mice on a Rag-positive background. (a) Absolute numbers of F5 T cells in spleens of age- and gender-matched B10-F5 (Rag\(^{+} \), \( n = 5 \)) and Rag-\(^{−/−} \) F5 (\( n = 5 \)) mice. (b) Lymph node cells from B10-F5 (Rag\(^{+} \), \( n = 5 \)) and Rag-\(^{−/−} \) F5 (\( n = 5 \)) mice were stained with anti-CD8 (y-axis) and the major histocompatibility complex (MHC)-peptide dimer reagent [D\(^{\alpha} \):NP68(366–374)]. Plots are representative examples from each group of mice and percentages are mean values. Error bars are standard deviations.
infection, the circulating pool of NP-specific CD8 T cells had contracted and was no longer detectable by FACS analysis with the dimeric MHC reagent. However, 3.5 months after immunization, a significant reduction in pituitary GH was observed in 48-B10 mice ($P = 0.004$, Fig. 5). When the precursor frequency of NP-specific T cells was raised, by transferring GFP-F5 T cells at the time of virus infection, the reduction in GH was more severe than observed after virus infection alone (Fig. 5). In parallel experiments, the pituitaries of B10 control mice were unaffected by any of the infections and T-cell transfers (results not shown).

**DISCUSSION**

We have previously shown that in mice which express a transgene-encoded antigen in the pituitary in a secretory form, T cells are activated in the periphery and a fulminating autoimmune hypophysitis ensues. In the present study, we compared this strain with another mouse strain in which the transgenic antigen is expressed in the same cells, but in a non-secreted form. Semiquantitative RT–PCR suggested that the amount of NP mRNA was much lower in the $\Delta.2$-Rag$^{-/-}$ F5 strain. In contrast, an in vitro functional assay, measuring T-cell proliferation in response to pituitary-derived NP cross-presented by DCs, indicated that pituitary NP levels were equivalent. Despite the apparent discrepancy in antigen-expression levels, cross-presentation of antigen in vivo occurred in the lymph nodes, presumably by migratory DCs of pituitary origin, and resulted in T-cell activation in both lines, but differences in the timing of cross-presentation eventually produced two disparate phenotypes.

The different phenotypes observed in the F5 TCR transgenic models reflect the initial differences in the processing and presentation of NP to T cells. It is unlikely that the difference between the two strains is a result of the fact that secreted protein reaches lymph nodes directly, as discussed previously. The amount of soluble antigen outside the pituitary gland would be far below the threshold required for cross-presentation. The data suggest that there must be a threshold of critical antigen density in lymph nodes, in the form of DCs presenting the antigen, which, if exceeded, results in T-cell activation. The delay in T-cell activation in the $\Delta.2$-Rag$^{-/-}$ F5 mice suggests that it took longer to reach the critical antigen density in these mice. This may have been because of a lower level of antigen expression in the $\Delta.2$-Rag$^{-/-}$ F5 strain and/or the difference in antigen context. The difference in phenotype in this model has a simple endocrine explanation. To affect growth, pituitary damage must be significant and uncompensated from 3 weeks onwards. The $\Delta.2$ strain achieves a normal growth curve because T cells did not infiltrate the pituitary and cause sufficient GH deficiency in time to affect growth. In the pituitary, DCs can only be loaded with NP as the result of the steady-state turnover (apoptosis/necrosis) of NP-transgenic somatotrophs, which is not likely to exceed 2% per day, based on previous work in rats. In contrast, pituitary DCs in the 48-strain could be loaded with soluble NP from somatotrophs that secrete actively from birth and generate high local concentrations of antigen.

Interestingly, whilst the onset of pituitary pathology is delayed in $\Delta.2$-Rag$^{-/-}$ F5 mice, the autoimmune consequences are similar to those seen in the 48-Rag$^{-/-}$ F5 mice. Thus, in this case, the context of antigen expression had little influence on the predisposition to autoimmunity and CD8 T cells could be primed, albeit with different kinetics, independently of antigen context. The non-secreted form of NP must somehow access DCs that activate T cells in the lymph nodes.

Once T cells have encountered antigen, they will expand only to the extent that their surroundings will allow. The rapidity and extent to which T cells can expand is affected by the ‘space’ available to fill. In Rag$^{-/-}$ mice there is abundant space to fill and T-cell expansion is unhindered. However, in a Rag-sufficient mouse, expansion may be inhibited by the presence of T and B cells in the lymph nodes and spleen. The presence of CD4$^+$ CD25$^+$ cells may also actively inhibit CD8 T-cell proliferation. This could explain why 48-B10-F5 mice have a milder phenotype despite having a higher number of F5 TCR-bearing T cells. Whilst in this model, and in other models of spontaneous CD8-mediated autoimmunity, Rag insufficiency either allows the disease to proceed or accelerates disease onset, other models have shown the opposite effect. T-cell activation/expansion in 48-B10-F5 mice could also have been restrained because of a reduced functionality of F5 T cells that may express two T-cell receptors, resulting in lower levels of surface F5 TCR and thus reduced sensitivity to activation by NP.

Nevertheless, 48-B10-F5 mice are clearly autoimmune, but their GH deficiency is delayed and less severe.

Neither the 48-B10 nor $\Delta.2$ B10 mice (i.e. expressing NP but in the absence of transgenic F5 T cells) exhibit autoimmune pathology under normal circumstances. Presumably, antigen is being processed and presented by DCs in the lymph nodes of these mice, but the number of NP-specific T cells is vastly reduced compared with the F5 TCR transgenic strains. When the NP-specific CD8 T-cell pool was increased after infection with virus, the number of specific T cells expanded to a level sufficient to cause GH deficiency. At the height of the immune response evoked by virus, F5 T cells constituted $\approx2\%$ of the total CD8 pool. This expansion, combined with the inflammation caused by viral infection, and, of course, the possibility that
T cells with different NP-epitope specificities were also activated, would be sufficient to cause pituitary autoimmunity. It is well known that lymphocytic hypophysitis may be manifest in other autoimmune conditions, including those affecting other endocrine systems, and we showed previously that the autoimmune reaction triggered by a somatotroph-specific epitope extends to other pituitary cell types. Our present results now suggest that the pituitary gland could be susceptible to T-cell-mediated pathology after infection with a virus expressing a soluble pituitary antigen. If so, strategies for immunon contraceptive immunity based on vaccination against soluble pituitary gonadotrophins could have other unexpected endocrine consequences.

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