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Autoimmunity to DNA and Nucleosomes in Binary Tetracycline-Regulated Polyomavirus T-Ag Transgenic Mice

Signy Bendiksen,²* Marijke Van Ghelue,²† Thomas Winkler,‡ Ugo Moens,* and Ole Petter Rekvig³*§

The mechanism(s) responsible for autoimmunity to DNA and nucleosomes in SLE is largely unknown. We have demonstrated that nucleosome-polyomavirus T-Ag complexes, formed in context of productive polyomavirus infection, activate dsDNA-specific B cells and nucleosome-specific CD4⁺ T cells. To investigate whether de novo expressed T-Ag is able to terminate nucleosome-specific T cell tolerance and to maintain anti-dsDNA Ab production in nonautoimmune mice, we developed two binary transgenic mouse variants in which expression of SV40 large T-Ag is controlled by tetracycline, MUP rTA/T-Ag (tet-off), and CMV rtTA/T-Ag (tet-on) mice. Data demonstrate that MUP rTA/T-Ag mice, but not CMV rtTA/T-Ag mice, are tightly controlling T-Ag expression. In MUP rTA/T-Ag transgenic mice, postnatal T-Ag expression activated CD8⁺ T cells but not DNA-specific B cells, while immunization with T-Ag and nucleosome-T-Ag-complexes before T-Ag expression resulted in elevated and remarkably stable titers of anti-T-Ag and anti-dsDNA Abs and activation of T-Ag-specific CD4⁺ T cells. Immunization of nonexpressing MUP rTA/T-Ag mice resulted in transient anti-T-Ag and anti-dsDNA Abs. This system reveals that a de novo expressed DNA-binding quasi-autoantigen maintain anti-dsDNA Abs and CD4⁺ T cell activation once initiated by immunization, demonstrating direct impact of a single in vivo expressed molecule on sustained autoimmunity to DNA and nucleosomes. The Journal of Immunology, 2004, 173: 7630–7640.

High titers of IgG Abs to dsDNA represent a classification criterion for systemic lupus erythematosus (SLE)⁴ (1). Subpopulations of these Abs are central in the pathogenesis of SLE-associated nephritis (2–5), thus representing an important pathogenetic factor in this disorder.

Deriving from several studies during the last decade, there is increasing evidence that anti-dsDNA Abs are produced by cellular mechanisms similar to conventional anti-nonself Abs (6). This immune response is Th cell-dependent, associated with Ig class switch and affinity maturation secondary to prolonged stimulation with cognate Ags (i.e., complexes of DNA and proteins). The exact nature of the molecules or molecular complexes responsible for anti-dsDNA Ab production in vivo in SLE is not fully determined. Central candidate structures may be pure apoptotic nucleosomes (7, 8) potentially generated on background of altered chromatin degradation in context of defects in genes like those encoding DNase1 (9) or serum amyloid P (10). Alternatively, hetero-complexes of self nucleosomes and nonself DNA-binding proteins derived from bacteria or viruses may account for anti-DNA or anti-nucleosome Ab production (6, 11).

Previously, we have demonstrated that nucleosomes complexed with the polyomavirus-encoded DNA-binding protein large T-Ag have the potential to induce anti-DNA Abs (12–14). Because nucleosomes alone did not induce anti-dsDNA Abs or activated nucleosome-specific T cells in normal individuals (14–16), this directly demonstrated that nucleosomes were rendered immunogenic upon complex formation with T-Ag. This complex had the potential to stimulate DNA-specific B cells in a way analogous to a hapten (DNA/nucleosome)-carrier (T-Ag) complex.

In a clinical study, a highly significant correlation between productive polyomavirus infection and Ab production against T-Ag and dsDNA was observed (12, 13, 17). These results (discussed in Ref. 6) suggested that an infectious nonself DNA-binding protein, when binding nucleosomal DNA in vivo, could create a molecular source responsible for initiating production of anti-DNA Abs, and also potentially for maintaining this immune response. This model is principally different from that implying that human anti-dsDNA Ab production can be initiated and sustained by autologous apoptotic nucleosomes (7, 8, 18). To study this model in more detail, we decided to develop T-Ag transgenic (tg) mice that may allow full control of expression of the transgenic protein and thereby to mimic expression profiles of the T-Ag similar to those observed in context of polyomavirus infection in human SLE (13)—transient, sustained, or recurrent. Drug-inducible expression systems for transgenes have been developed. One such system using the Escherichia coli tetracycline (tet) resistance operon has been applied to tightly regulate gene expression in cell lines and in transgenic mice (19–21). Based on the tet-regulatory system, we established a binary inducible transgenic mouse model in which the conditional
expression of T-Ag can be tightly regulated through application of tet. Two tet-regulated hybrid mice were selected for the planned experiments, tet-on or tet-off, where T-Ag expression is achieved by giving tet to the mice (tet-on) or after withdrawal from the mice (tet-off), respectively.

In this study, we examined if the tet-on- and/or tet-off-conditioned T-Ag tg mouse models are valid to investigate requirements for initiation and sustained production of anti-dsDNA Abs. Three basic aims were the focus for these studies. First, we analyzed whether control of T-Ag expression in tet-on and tet-off was tight. Second, experiments were performed that focused on whether T-Ag expression alone was sufficient to initiate anti-dsDNA Abs. Third, we examined whether anti-dsDNA Ab production, once initiated either by isolated T-Ag expression or after immunization with T-Ag and nucleosome-T-Ag complexes, could be maintained by sustained T-Ag expression.

Data presented here demonstrate that the tet-off system for regulation of T-Ag expression, but not that of tet-on, was tightly controlled, and in these mice, T cells responded both to immunization with T-Ag, and also to de novo expressed T-Ag. Sole in vivo T-Ag expression did not induce anti-dsDNA Abs but activated CD8+ T cells. Once initiated by immunization, however, subsequent in vivo expression of T-Ag maintained a remarkably stable anti-dsDNA Ab production and sustained activation of CD4+ T cells.

Materials and Methods

Generation of transgenic mice

The previously described plasmid pBSLT (12) was digested with XhoI and SalI, and T-Ag full-length cDNA was isolated and subsequently cloned in the corresponding sites of the plasmid pTRE2 (Clontech Laboratories, Palo Alto, CA) to produce pTRE2-SLT. The pTRE2 plasmid contains a tetracycline-responsive TRE/CMV promoter. To generate tetracycline-responsive T-Ag transgenic mice, pTRE2-SLT was linearized with XhoI and XhoI, and the DNA fragment encompassing the TRE/CMV promoter with XbaI and NotI and the DNA fragment encompassing the TRE/CMV promoter with XbaI and NotI and the DNA fragment encompassing the TRE/CMV promoter.

Table I. Primers used to detect SV40 T-Ag, rTAT/rTAT transactivator, and APRT coding sequences by PCR.

<table>
<thead>
<tr>
<th>Target Sequence</th>
<th>Name</th>
<th>Primer Sequence</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SV 40 T-Ag</td>
<td>SVT.f</td>
<td>CTTTGGAGGCTTCTGGAGATGCAAAT</td>
<td>227</td>
</tr>
<tr>
<td></td>
<td>SVT.r</td>
<td>CATGACTCCAATAACTATTACATTCAG</td>
<td></td>
</tr>
<tr>
<td>Transactivator</td>
<td>tTA.f</td>
<td>CGCCCAGAAGCTTAGTGGTAG</td>
<td>201</td>
</tr>
<tr>
<td></td>
<td>tTA.r</td>
<td>GCTCCATCGCGATGACTTAG</td>
<td></td>
</tr>
<tr>
<td>APRT*</td>
<td>MAPRT.f</td>
<td>CAGAGAAGTGGCTCATTTGAGATG</td>
<td>386, genomic</td>
</tr>
<tr>
<td></td>
<td>MAPRT.r</td>
<td>CGTGAGCCACACAAAGGCATTAG</td>
<td>277, cDNA</td>
</tr>
</tbody>
</table>

* These primers were generated to determine whether mRNA was contaminated with genomic DNA (see legend to Fig. 1 for details).

Isolation of RNA from solid tissue samples and cDNA synthesis

RNA was isolated according to a protocol described by Vaira et al. (23). The cDNA synthesis was performed with Superscript II RNase H reverse transcriptase kit for RT-PCR (Invitrogen, San Diego, CA), according to the manufacturer’s protocol. RT-PCR was performed using the T-Ag and adenine-phosphoribosyltransferase (APRT) encoding sequence-specific primers (Table I). The APRT primers were designed to span two exons and one intron to separate genomic PCR products from those obtained from cDNA. This ensures that the T-Ag RT-PCR products amplified from tissue specimens were derived from cDNA.

Immune electron microscopy (IEM)

This was essentially done as described elsewhere (24, 25). Liver tissue was fixed by immersion in 8% formaldehyde in PBS, 2.3 M sucrose, and frozen on aluminum specimen pins by immersion in liquid nitrogen. Ultrathin sections were cut on a Reichert Ultracut S with a Reichert FCS cryochemical using a diamond knife (Drukker International, Cuijk, The Netherlands). Sections were thawed and mounted on Formvar-coated specimen grids before immunolabeling according to standard procedures. T-Ag expression was demonstrated using the mouse monoclonal anti-T-Ag Ab Ab-2 (Oncogene Research Products, Cambridge, MA), diluted 1/10 in PBS supplemented with 1% cold water fish skin gelatin (G-7765, Sigma-Aldrich, St. Louis, MO) to block for nonspecific binding of Abs. A rabbit anti-mouse IgG Ab (ICN/Chappel, Aurora, OH) was added, and binding was visualized by Protein A-gold (University of Utrecht, Utrecht, The Netherlands). Every step was washed in PBS, cold water fish skin gelatin. The grids were finally washed in distilled water and dried in 1.8% methylcellulose and 0.3% uranyl acetate. T-Ag-expressing SV-T2 (CCL 163.1; American Type Culture Collection, Manassas, VA) and nonexpressing A31 (CCL 163; American Type Culture Collection) cells were used as positive and negative controls, respectively. The specimens were examined in a Jeol 1010 transmission electron microscope (Tokyo, Japan).

Indirect immunofluorescence (IIF)

IIF was performed as described previously (26) on tissue cryosections from frozen liver samples. Sections were incubated with the anti-T-Ag Ab Ab-2 (diluted 1/10 in PBS, 1% albumin (PBSA)) and subsequently with Alexa Fluor 488-conjugated anti-mouse Fcγ Abs (Molecular Probes, Eugene, OR), diluted 1/500 in PBSA. After each Ab, the slides were washed in PBS. The specimens were analyzed using a LSM 510 Meta Zeiss confocal microscope.

Antigens

Nucleosomes and nucleosome-T-Ag complexes from the fibroblast cell lines A31 and the constitutively T-Ag-expressing SV-T2, respectively, were prepared from chromatin after cell lysates and micrococcal nuclease digestion, as described in detail elsewhere (14, 15). Characterization of the molecules and complexes used here revealed that the nucleosomes derived from A31 and SV-T2 cells, denoted nucleosomes and nucleosome-T-Ag complexes, respectively, had a nucleosomal DNA size spanning from binary CMV rTAT-Ag and MUP rTAT-Ag tg mice in the form of doxycycline hyclate (BD Biosciences, Clontech), a derivative of tetracycline, and therefore denoted tet in this study. Tet was dissolved in water supplemented with 5% sucrose at a concentration of 200 μg/ml. The animals were housed in the Animal Research Department facilities at the Faculty of Medicine, University of Tromsø, treatment and care of the animals were in accordance with the guidelines of Norwegian Ethical and Welfare Board for Research Animals, and the study was approved by the Institutional Review Board.
Approximately 200 bp up to several thousand base pairs (14, 15). In contrast to nucleosomes from A31 cells, SV-T2 cell-derived nucleosomes also contained T-Ag at a T-Ag:nucleosomal DNA ratio of ~0.5 (w/w), as determined earlier (14). Polyomavirus SV40 T-Ag was expressed in recombinant baculovirus, purified by affinity chromatography, and characterized by SDS-PAGE and Western blot using the anti-T-Ag Ab Ab-2 as previously described (14, 15). T-Ag was virtually pure and had a molecular mass of ~90 kDa and some degradation products (14, 17). Human and calf thymus (CT) dsDNA (Sigma-Aldrich) were SI nuclease digested as described previously (13, 27), while the circular cloning vector pUC18 (Amersham Pharmacia Biotech) was used without further structural modifications as described in detail elsewhere (27). The DNA preparations were devoid of proteins according to Coomasie blue-stained SDS-polyacrylamide gels (data not shown). Histones (containing H1, H2A, H2B, H3, and H4) were obtained from Boehringer Mannheim (Mannheim, Germany), and methylated RSA (mBSA) from Sigma.

**Immunization of groups of mice**

Both tet-on and tet-off mice were divided into four groups, each consisting of three mice (Table II). group 1 served as a nonimmunized, T-Ag nonexpressing control group, while group 2 consisted of nonimmunized mice forced to express T-Ag. Group 3 mice were first immunized subcutaneously with T-Ag (200 μg/ml in CFA) and 3 wk later with nucleosome-T-Ag complexes (200 μg/ml DNA) in IFA without subsequent T-Ag expression. Group 4 mice were immunized as group 3 and forced to express T-Ag ~10 wk after the first immunization. See Table II for protocol and experimental details.

**Analyses of cellular and humoral immune responses**

Each experimental group (tet-on groups 1–4 and tet-off groups 1–4), each consisting of three mice, were analyzed for the following immunological parameters (see Table II for experimental details). Cellular immune responses were analyzed in tet-on mice 12 wk (groups 1 and 2) or 36 wk (groups 3 and 4) after initiation of experimental immunization and/or T-Ag expression in vivo. For tet-off mice, cellular immune responses were determined 23 wk after initiation of the experiments for all groups of mice, if not otherwise stated in the text.

Humoral immune responses were measured over 12 (groups 1 and 2) or 36 wk (groups 3 and 4) for tet-on and over 23 wk for tet-off mice using serum samples drawn at 2- to 4-wk intervals, if not otherwise stated in the text. For some analyses, early and late serum specimens may not be included due to lack of sufficient amounts of those samples (see Fig. 4G for details).

**T cell cultures and Ag-induced proliferation**

Preparation of spleen or lymph node cells and stimulation with selected Ags was performed largely as described previously (17). The cells were cultured in Iscove’s DMEM (Life Technologies Laboratories, Grand Island, NY) supplied with 25 mM HEPES buffer, 1× nonessential amino acids (Life Technologies), 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 10% FCS (Sigma-Aldrich), in round-bottom 96-well culture plates. The T cells (5 × 10⁶ cells as spleen or lymph node cells, in 200 μl medium/well) were sham-stimulated (medium) or stimulated with 10 μg/ml T-Ag, 15 μg/ml (as DNA) nucleosomes or nucleosome-T-Ag complexes, or 20 μg/ml histones (17). All experiments were performed in triplicate. The results are given as mean cpm (±SD), or as stimulation index (SI) defined as the ratio between mean cpm of Ag- and medium-stimulated T cells. In some experiments, mAbs against CD4 and CD8 molecules (Santa Cruz Biotechnology, Santa Cruz, CA), both at final concentrations of 10 μg/ml IgG, were added in parallel triplicate medium-, T-Ag-, or nucleosome-T-Ag-stimulated cultures. T cell proliferation was measured at day 3 after Ag stimulation in vitro, if not otherwise stated in the text, by [3H]thymidine (Amersham Pharmacia Biotech) incorporation (1 μCi per well) as described previously (17).

**Measurement of anti-T-Ag and anti-dsDNA Abs by ELISA or by solution-phase anti-dsDNA Ab ELISA (SPADE)**

For detection and quantitation of Abs to T-Ag and CT dsDNA, standard indirect solid-phase ELISA was performed as described in detail previously (13, 28). To determine relative titers for each Ab specificity, the serum sample drawn at week 6 after the first immunization (3 wk after last boost injection with nucleosome-T-Ag complexes, see above) served as a reference serum for each individual mouse. The dilution of subsequently drawn serum samples giving 50% of maximum binding of the reference serum for a given Ab specificity (T-Ag or dsDNA) represented the relative titer of that serum Ab. By this approach, we could directly measure whether the Ab titers remained stable, increased, or decreased relative to expression of T-Ag. The SPADE assay measures Ab binding to DNA in solution using biotinylated DNA, as described in detail recently (27). In short, biotinylation of DNA was performed according to recommendations by the manufacturer (Pierce Chemical Company, Rockford, IL). Human dsDNA or circular pUC18 dsDNA (1 μg/ml in H₂O) were mixed with equal volumes of EZ-Link photoactivable biotin (1 μg/ml H₂O) on ice and irradiated for 3 min using a 275-W sun lamp at a distance of 8 cm from the light source. The biotinylated DNA molecules were subsequently purified by 2-butanol extraction and ethanol precipitation. SPADE was performed by mixing 0.5 μg of the biotinylated DNA molecules with 50 μl of serially diluted serum samples (1/20–1/160 in PBSA) (solution-phase step (27)). After incubation for 30 min, the mixtures were added to microtiter plates (Nunc MaxiSorp; Nunc, Roskilde, Denmark) coated overnight at 37°C with 50 μl of streptavidin (Sigma-Aldrich; 5 μg/ml in PBS). After incubation for 30 min, the plates were washed three times with PBS, 0.05% Tween 20 (PBST), and incubated 1 h with HRP-conjugated anti-mouse Fcγ Abs (Sigma-Aldrich), appropriately diluted in PBST. Subsequently, the ELISA was developed as described for the conventional ELISA (13, 28). For each biotinylated DNA, checkerboard titration, using serial dilutions of both sera and biotinylated DNA molecules, was performed to optimize the assay system (data not shown).

**Statistics**

Student’s t test was used to test for differences of means of groups, and a difference was regarded statistically significant at p < 0.05.

**Results**

Analyses of the ability of tet to control T-Ag expression in tet-on (CMV rtTA/T-Ag) and tet-off (MUP rTA/T-Ag) mice

To test the inducible expression of T-Ag in the tet-on mice, 6-wk-old animals were administered either with water or water/sucrose supplemented with tet. T-Ag expression was monitored by IIF and

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**Table II. Overview of the experimental protocols for immunization, and induced expression of the T-Ag transgene, in the tet-on and tet-off mice**

<table>
<thead>
<tr>
<th>Transgenic Mice</th>
<th>Group</th>
<th>Immunization</th>
<th>T-Ag Expression</th>
<th>Observation Time (wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tet-on CMV rtTA/T-Ag</strong></td>
<td>1</td>
<td>No</td>
<td>T-Ag (0)</td>
<td>Continuous from week 10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>No</td>
<td>T-Ag (0)</td>
<td>Continuous from week 0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>T-Ag (0)</td>
<td>Continuous from week 10</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>T-Ag (0)</td>
<td>Continuous from week 0</td>
<td>Yes</td>
</tr>
</tbody>
</table>

| **Tet-off MUP rTA/T-Ag** | 1 | No | T-Ag (0) | Continuous from birth | No | 0–23 |
| | 2 | No | T-Ag (0) | Withdrawn week 0 | Yes | 0–23 |
| | 3 | T-Ag (0) | Continuous from birth | No | 0–23 |
| | 4 | T-Ag (0) | Withdrawn week 10 | Yes | 0–23 |

*See Fig. 1 for details.

*Week 0 is defined as the time point for initiation of the experiments.*
IEM, and detection of the transcribed T-Ag encoding gene was done by RT-PCR. IEM with anti-T-Ag Abs demonstrated that T-Ag was expressed and localized to chromatin of constitutively T-Ag-expressing mouse SV-T2-positive control cells, but no T-Ag could be detected in the T-Ag nonexpressing A31-negative control cells (Fig. 1, A and B for SV-T2 and A31 cells, respectively). In the tet-on mice, T-Ag was expressed and localized to liver cell chromatin after tet administration (Fig. 1C), but also in the absence of tet exposure (Fig. 1D). Similarly, nuclear staining for T-Ag was observed by IIF in both tet-exposed and nonexposed mice (data not shown). Taken together, these results demonstrate that the tet-on mice were leaky as the T-Ag transgene was expressed even in the absence of tet. Consistent with this expression pattern, RT-PCR performed on liver and kidney mRNA demonstrated that T-Ag gene transcription was detectable in absence of tet (Fig. 1G, lanes 1 and 2 for liver and kidney mRNA, respectively).

In the tet-off mice, T-Ag expression was initiated after removing tet from the drinking water, as demonstrated by presence of chromatin-localized T-Ag as determined by IEM (Fig. 1E), while mice continuously receiving tet did not demonstrate detectable T-Ag expression (Fig. 1F). Consistent with these results, nuclear localization of expressed T-Ag was detected after removal of tet by IIF using anti-T-Ag Abs, but not in mice receiving tet (data not shown). Thus, tet-off mice receiving tet are not spontaneously expressing T-Ag, at least not at the sensitivity levels of IIF and IEM. Even at the sensitivity level of RT-PCR, T-Ag-encoding gene transcripts were undetectable in tet-off mice receiving tet when using mRNA purified from liver, muscle, and spleen (Fig. 1G, lane 5 for liver mRNA, not shown for muscle and spleen). In these mice, transcripts could, however, be recognized by RT-PCR using liver mRNA, but not mRNA from muscle or spleen, after induced T-Ag expression (Fig. 1G, lane 6 for liver, not shown for muscle or spleen). In addition, a weak transcription of the T-Ag gene was observed in the kidneys after turning the gene on (data not shown).

APRT RT-PCR using primers that allowed distinction of PCR products deriving from genomic DNA and mRNA demonstrated that the mRNA used in the T-Ag-specific RT-PCR is not contaminated by genomic DNA (see e.g., Fig. 1G, lanes 3, 4, and 7–9, where lane 9 demonstrates two bands, the lower from cDNA and the larger from genomic DNA). Thus, also determined by RT-PCR, T-Ag expression in vivo could be tightly controlled by tet in the tet-off, but not in the tet-on, system, directly demonstrating that

**FIGURE 1.** Immune electron microscopy to detect chromatin-localized T-Ag using the monoclonal anti-T-Ag Ab Ab-2. T-Ag is detected in nuclei of SV-T2 cells, constitutively expressing T-Ag (A), but not in nonexpressing A31 cells (B). In C and D, liver cells from tet-on (CMV rtTA/T-Ag) mice are examined for T-Ag expression in presence (C) or absence of tet (D). For tet-off MUP tTA/T-Ag mice, nuclear T-Ag in liver cells is detected after induced expression of T-Ag by removing tet (E), while mice receiving tet are negative for nuclear T-Ag (F). In G, RT-PCR was performed to detect T-Ag gene transcription. RT-PCR on T-Ag mRNA from liver (lane 1) or kidney (lane 2) from a tet-on mouse not receiving tet. Lanes 3 and 4 represent APRT RT-PCR on the same mRNA as in lanes 1 and 2, demonstrating bands of 277 bp deriving from cDNA, and not genomic DNA giving a band of 386 bp (see Table I for details). Lanes 5 and 6 represent T-Ag RT-PCR from T-Ag nonexpressing (lane 5) and T-Ag expressing (lane 6) tet-off mice, while lanes 7 and 8 demonstrate APRT RT-PCR on the same mRNA as in lanes 5 and 6. In lane 9, APRT RT-PCR on cDNA contaminated with genomic DNA is demonstrated. The upper band (386 bp) derives from genomic DNA, while the lower (277 bp) from cDNA.
the tet-off system is superior to the tet-on in controlling T-Ag expression.

Tet-on (CMV rtTA/T-Ag) mice are nonresponsive to immunization with T-Ag and nucleosome-T-Ag complexes as demonstrated by absence of Ab production and T cell proliferation

The tet-on mice did not produce anti-T-Ag or anti-ssDNA or anti-dsDNA Abs during the observation period, irrespective whether immunization with T-Ag and nucleosome-T-Ag complexes was followed by tet-induced T-Ag expression or not (data not shown, see Table II for experimental details), demonstrating that they were immunologically nonresponsive to T-Ag and DNA.

To test whether the absence of humoral immune responses after immunization could be due to nonresponsive T cells, spleen T cells taken from tet-on group 2 and tet-on group 4 mice were tested for proliferation in response to T-Ag and nucleosome-T-Ag complexes at the end of their respective observation periods. None of these mice contained T cells that proliferated above the levels of medium-stimulated T cells (Fig. 2, A and B for group 2 and group 4 mice, respectively). The inability of these tet-on mice to produce anti-T-Ag Abs and the nonresponsiveness of their T cells when stimulated ex vivo with T-Ag or nucleosome-T-Ag complexes (Fig. 2, A and B) is consistent with the observations that T-Ag is constitutively expressed in tet-on mice irrespective whether stimulated with tet or not (see Fig. 1, C and D). These results confirm that T-Ag expression is not controlled by the tet inducer in these mice, resulting in Ag-selective nonresponsiveness. This is further evident as tet-on mice that did not receive tet (i.e., mice not forced to express T-Ag) responded to immunization with mBSA by producing high titers of anti-mBSA Abs (data not shown), as previously observed in nonmanipulated BALB/c mice (29).

T cell responses in nonimmunized and immunized tet-off (MUP rtTA/T-Ag) mice

Tet-off mice were immunized with T-Ag and boosted with nucleosome-T-Ag complexes following by activation of T-Ag expression. Four weeks thereafter, spleen or draining lymph node T cells were analyzed for their ability to proliferate to T-Ag or nucleosome-T-Ag complexes. As demonstrated in Fig. 2, C and D, both spleen- and lymph node-derived T cells responded similarly to stimulation with T-Ag and to nucleosome-T-Ag complexes. Autoimmune nucleosome-specific T cells could also be detected among lymph node T cells (Fig. 2D). This was further analyzed using spleen cells from all four experimental groups of tet-off mice (see below and Table III for details).

Tet-off mice were separated into four groups (see Materials and Methods and Table II for details). Spleen T cells from tet-off group 1 mice (nonimmunized and no T-Ag expression) did not proliferate in response to T-Ag or nucleosome-T-Ag complexes (Fig. 2E). If nonimmunized mice were forced to express T-Ag by withdrawing tet (tet-off group 2 mice), T cells from these responded significantly to nucleosome-T-Ag complexes (SI: 4.4, Fig. 2F), and also somewhat weaker to pure nucleosomes (SI: 2.1, Table III). Notably, isolated T-Ag did not significantly stimulate these T cells, indicating that they mainly recognized peptides derived from complexed, but not free T-Ag (17), and potentially also nucleosome-derived peptides (Fig. 2D and Table III), analogous to what has been observed previously (see Discussion and Refs. 15 and 16). Similar responses to T-Ag and nucleosome-T-Ag, but not to pure nucleosomes, were observed in mice immunized with T-Ag and nucleosome-T-Ag complexes in the absence of T-Ag expression in vivo (tet-off group 3 mice, Fig. 2G).

In mice immunized with T-Ag and boosted with nucleosome-T-Ag followed by expression of T-Ag (tet-off group 4), strong T cell responses to nucleosome-T-Ag were observed (Fig. 2H), while T cells possessed delayed and prolonged proliferative responses after medium-stimulation (Fig. 3A), becoming apparent at day 4 after initiation of the in vitro T cell cultures. The reason for this delayed proliferation after medium stimulation in this particular group of mice (Fig. 3A) is unclear, but was reproduced in several experiments using both spleen and draining lymph node T cells (data not shown). One explanation for this phenomenon may be proliferation of autoimmune, nucleosome-specific T cells in response to nucleosomes released in the cultures when cells start to die (see Discussion). This response could in separate experiments be inhibited by incubation of the medium-stimulated cultures with anti-CD4 Abs (73% inhibition) but only weakly with anti-CD8 Abs (33% inhibition, Fig. 3B). Such delayed and prolonged T cell responses have previously been demonstrated for T cells taken from patients with active SLE (14, 15).

The contribution of CD4+ and CD8+ T cells in Ag-induced proliferation experiments was investigated using T cells of individual tet-off group 2 (nonimmunized) and tet-off group 4 (immunized) mice, both expressing T-Ag (Fig. 3, C and D for tet-off group 2 and 4, respectively). Proliferation of T cells from tet-off group 2 mice in response to nucleosome-T-Ag stimulation in vitro could be blocked more effectively with anti-CD8 Abs (50% inhibition) than with anti-CD4 Abs (<5% inhibition) (Fig. 3C). This contrasted the CD4/CD8 phenotype of T cells from mice immunized with T-Ag, boosted with nucleosome-T-Ag followed by induction of T-Ag expression (tet-off group 4 mice). In this experimental system, anti-CD4 Abs inhibited T cell proliferation by 74% in responses to nucleosome-T-Ag (Fig. 3D). However, because the proliferation in medium-stimulated T cell cultures was higher when measured at day 4 (Fig. 3, A and D) than at day 3 (Figs. 2H and 3A) after initiation of the cultures, these T cells may have proliferated in response to both nucleosome-T-Ag complexes added to the cultures and to nucleosomes released from dead cells in the cultures (see Discussion). Anti-CD8 Ab treatment of parallel cultures resulted in 38% inhibition of proliferation (Fig. 3D). Thus, whether T-Ag is expressed in vivo in otherwise nonimmunized T-Ag tg mice, or is expressed after immunization with T-Ag and boosted with T-Ag-nucleosome complexes, may decide whether CD8+ or CD4+ T cells are continuously stimulated by the expressed transgene.

Autoimmune T cell responses in tet-off group 1–4 (MUP rtTA/T-Ag) mice

Linked presentation of T-Ag and nucleosome-derived (histone) peptides has been shown to terminate immunological tolerance to nucleosomes (6, 15, 17). We therefore analyzed spleen T cells for responsiveness to nucleosome-T-Ag complexes, nucleosomes, and histones in tet-off group 1–4 mice. Data presented in Table III demonstrate that mice expressing T-Ag have T cells that respond to mouse nucleosomes (SI: 2.1 and 2.2, for tet-off group 2 and 4 mice, respectively). None of the groups of mice had T cells responding significantly to pure histones.

Humoral immune responses to T-Ag and dsDNA in tet-off group 1–4 (MUP rtTA/T-Ag) mice

Nonimmunized and T-Ag nonexpressing tet-off group 1 mice did not produce Abs to T-Ag or to dsDNA over the observation period (Fig. 4A). The same negative result was observed for nonimmunized tet-off group 2 mice forced to express T-Ag (Fig. 4B).
These data are consistent with the absence of T cell responses in tet-off group 1 mice (Fig. 2E) or presence of nucleosome-T-Ag-responsive T cells mainly of the CD8 phenotype in tet-off group 2 mice (Figs. 2F and 3C).

In contrast, mice immunized with T-Ag and boosted with nucleosome-T-Ag complexes produced Abs to dsDNA (Fig. 4, C and D for tet-off group 3, and Fig. 4, E and F for tet-off group 4 mice, respectively) and to T-Ag (Fig. 4G for tet-off group 3 and 4 mice).
Table III. Analyses of T cell responses (mean cpm ± SD) to nucleosome-T-Ag complexes, nucleosomes or histones in tet-off group 1–4 MUP rTA/T-Ag mice examined at the end of the observation period for each group

<table>
<thead>
<tr>
<th>tet-off Group</th>
<th>Immunizeda</th>
<th>tetb</th>
<th>Medium</th>
<th>Nucleosome-T-Ag complex</th>
<th>Nucleosomes</th>
<th>Histones</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>–</td>
<td>+</td>
<td>3274 (±1677)</td>
<td>2575 (±524)</td>
<td>2661 (±534)</td>
<td>4612 (±1363)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SL: 0.8</td>
<td>SI: 0.8</td>
<td>SI: 1.4</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>–</td>
<td>4564 (±2516)</td>
<td>20025 (±2308)</td>
<td>9483 (±2010)</td>
<td>6852 (±3809)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SL: 4.4</td>
<td>SI: 2.1</td>
<td>SI: 1.5</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>3772 (±1218)</td>
<td>11888 (±3050)</td>
<td>5003 (±1611)</td>
<td>5142 (±1332)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SI: 3.2</td>
<td>SI: 1.3</td>
<td>SI: 1.4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>–</td>
<td>4676 (±189)</td>
<td>21907 (±727)</td>
<td>10178 (±231)</td>
<td>6327 (±1667)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SI: 4.7</td>
<td>SI: 2.2</td>
<td>SI: 1.4</td>
<td></td>
</tr>
</tbody>
</table>

a The mice were immunized with T-Ag followed by nucleosome-T-Ag complexes 3 wk later as described in Materials and Methods and outlined in Table II.

b See Table II for details on time points for tet administration. Proliferation was measured when the experiments were terminated for each group.

To examine whether there are differences between tet-off group 3 and group 4 mice with respect to sustained Ab production, titers of anti-T-Ag and anti-dsDNA Abs were determined in these two groups of mice (see Figs. 4, C–G for details). As is evident, titers for anti-dsDNA Abs declined in tet-off group 3 mice (Fig. 4, C and D) from a mean titer of 963 (week 6) to 368 (week 23), and anti-T-Ag titers declined from 318 (at week 10) to 241 (week 18) (Fig. 4G). In tet-off group 4 mice, however, the anti-dsDNA and anti-T-Ag Ab titers remained stable over the observation periods (mean titers for anti-dsDNA Abs were 1657 (week 6) and 1518 (week 23) (Fig. 4, E and F) and for anti-T-Ag Abs 463 (week 10) and 510 (week 18), respectively (Fig. 4G)). For anti-dsDNA Abs, there was a significant drop in Ab titers in tet-off group 3 mice at week 16 and thereafter compared with titers in tet-off group 4 mice (p < 0.04 at week 16 and p < 0.001 at week 23). Unfortunately, due to lack of sufficient amounts of early and late serum samples, the initial titers of anti-T-Ag Abs could not be determined, and titer variations following week 6 for the anti-T-Ag Ab titers were therefore unknown. In one mouse, however, this could be determined (mouse 3 of tet-off group 3), and the anti-T-Ag Ab titer declined from 760 (week 6) to 185 (week 23). This is similar to the transient nature of anti-T-Ag Abs observed among SLE patients in which polyomavirus infection occasionally is terminated (13). Statistical calculations for differences between the groups with respect to anti-T-Ag Ab titers were therefore not performed.

Quality of the anti-dsDNA Abs produced by tet-off (MUP rTA/T-Ag) group 3 and group 4 mice

The properties of anti-DNA Abs produced in the tet-off groups 3 and 4 mice were further analyzed to determine whether they developed sufficient avidity to bind dsDNA, not only in solid-phase dsDNA ELISA, but also in solution. For such studies, we used soluble biotinylated circular pUC18 and linear human dsDNA as Ags (27). Furthermore, we searched for evidence that a prolonged immune response (as in tet-off group 4 mice (Fig. 4F)) resulted in Abs with sufficient avidity to bind e.g., circular pUC18 plasmid dsDNA in solution (27), as compared with early Abs in immunized T-Ag nonexpressing tet-off group 3 mice. By comparing anti-dsDNA Abs of each individual mouse in tet-off group 3 (Fig. 5A, sera drawn at week 6) and tet-off group 4 (Fig. 5B, sera drawn at week 23), Abs from all mice-bound CT dsDNA in solid-phase ELISA, and human dsDNA in SPADE. More importantly, all mice of tet-off groups 3 and 4 bound circular pUC18 dsDNA in solution (Fig. 5, A and B) at titers similar to those recently published for human SLE (27). Some of these tet-off group 3 and 4 Abs also bound in the Crithidia luciliae assay (data not shown), previously demonstrated to correlate with binding in pUC18 SPADE (27). Thus, the anti-dsDNA Ab responses in immunized T-Ag nonexpressing and T-Ag-expressing mice possessed sufficient avidity to bind stringent dsDNA structures in solution, requiring higher avidity than in solid-phase ELISA (30).

In summary, de novo expression of T-Ag without prior immunization initiated mainly CD8+ T cell proliferation and not Ab production, while immunization with T-Ag and nucleosome-T-Ag complexes activated T cells dominated by a CD4 phenotype. If T-Ag expression was initiated subsequent to the immunizations, such CD4+ T cells had the potential to maintain anti-dsDNA and anti-T-Ag Ab production in contrast to nonexpressing mice, where the Abs declined over time.

Discussion

Recurrent or constitutive polyomavirus infection profiles are regularly observed phenomena in SLE patients (13, 31–35). Because productive infection depends on early expression of T-Ag (36, 37), a constitutive infection pattern reflects sustained T-Ag expression in vivo, and secondary to this also a constitutive binding of T-Ag to chromatin (14). If released from dying T-Ag-expressing cells, such complexes have a demonstrable potential to induce anti-nucleosome and anti-DNA Abs (12) and to activate nucleosome-specific T cells (15, 16). This indicates that productive polyomavirus infection-induced termination of tolerance to nucleosomal ligands may be biologically relevant as a model to investigate activation of B cell and T cell autoimmunity to dsDNA and nucleosomes.

To investigate the question, whether a de novo-expressed nuclear (auto) Ag is able to initiate and maintain an anti-DNA Ab response in nonautoimmune mice, we developed an experimental animal model in which the expression of SV40 large T-Ag is under stringent control in vivo. There are several aspects that needed to be analyzed to validate the T-Ag tg mouse model for studies of DNA and nucleosome tolerance regulation: How tight is the T-Ag gene expression regulated in the Ag-specific immune system responsive or tolerized; what is the CD4/CD8 phenotype of the T cells that eventually are stimulated by the Ag-specific immune system? To answer these questions, we designed the following strategies: To determine whether a de novo-expressed T cell proliferation and not Ab production, while immunization with T-Ag and nucleosome-T-Ag complexes activated T cells dominated by a CD4 phenotype. If T-Ag expression was initiated subsequent to the immunizations, such CD4+ T cells had the potential to maintain anti-dsDNA and anti-T-Ag Ab production in contrast to nonexpressing mice, where the Abs declined over time.

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By different analytical approaches, immunofluorescence or immune electronmicroscopy on liver sections and RT-PCR to detect T-Ag gene transcripts, we observed that the tet-on tg system was not tightly regulated, and T-Ag was expressed irrespective whether the gene was activated by tet or not. This is particularly evident because T-Ag localizes to liver cell chromatin due to its nuclear localization signal and high avidity for chromatin (36), making it easy to detect expressed T-Ag by e.g., IIF and IEM.

In accordance with the tendency for spontaneous T-Ag expression in the tet-on mice, these were nonresponsive to T-Ag as demonstrated by absence of T-Ag-induced T cell proliferation and Ab production after experimental immunization with T-Ag and nucleosome-T-Ag complexes. This is consistent with earlier observations demonstrating that the tet-on system in other contexts may be leaky (38–40) but in contrast to observations obtained by Kistner et al. (22), who demonstrated no expression above background of the luciferase transgene in transgenic mice with a tet-inducible transactivator system in the absence of tet. Increased residual expression of T-Ag in our rTA/T-Ag tet-on mice in the absence of tet may be explained by the site of chromosomal integration of the T-Ag transgene. This is consistent with earlier observations that unregulated basal transcription of the integrated transgene driven by promoters activated by tetracycline may result from unsuitable chromosomal integration of the transgene (41).

In contrast to these results, T-Ag expression in the tet-off system was demonstrated to be tightly controlled as determined by negative IIF, IEM, and RT-PCR assays for T-Ag detection and expression in presence of tet. However, withdrawal of tet from the drinking water of these mice activated the T-Ag-encoding gene, and both T-Ag mRNA, and nuclear localized T-Ag in liver cells could be detected, similar to what has been described by Manickan et al. (19). Because both systems use the same T-Ag tg mouse, the tTA/T-Ag system must be more effective than the rtTA/T-Ag system in controlling T-Ag expression (see Results). In these tet-off mice, T-Ag expression without preceding immunization with T-Ag and nucleosome-T-Ag-activated CD8+ nucleosome-T-Ag-specific T cells but no Ab production. Immunization of the mice with T-Ag and nucleosome-T-Ag complexes before induced T-Ag expression, however, resulted in activation of CD4+ T cells and anti-T-Ag and anti-dsDNA Ab production. The activated T cells did not significantly respond to isolated T-Ag, but to nucleosome-T-Ag complexes and also weakly to nucleosomes (see Fig. 2 and Table III). This may indicate that the activated T cells recognize nucleosome-bound T-Ag-derived peptides, but not peptides derived from isolated T-Ag, equivalent to a similar phenomenon previously published (17). In that work we demonstrated that monoclonal T cell lines generated from mice immunized with free T-Ag proliferated to free T-Ag, but not to T-Ag complexed with nucleosomes (17). In analogy to this, long-term T-Ag expression in the tet-off mice and subsequent in vivo nucleosome-T-Ag complex formation may well have resulted in selection of T cells specific for that complex.
and, although not formally proven, with a specificity for nucleosome-bound T-Ag-derived peptides that may differ from peptides generated from noncomplexed T-Ag (17).

The CD8/CD4 dichotomy of the T cell responses is in agreement with T cells activated by endogenously produced nonself proteins presented in context of MHC class I molecules after isolated T-Ag expression, while prior immunization directed the T cell phenotype toward a MHC class II-T-Ag-peptide complex-dependent CD4 T cell response. It is, however, unexpected that a soluble Ag or an antigenic complex given to the in vitro cell culture stimulates CD8 T cells primed in vivo by de novo expression as demonstrated here (see Fig. 3 for examples). However, it has been demonstrated that soluble Ags taken up by APC may be presented by MHC class I molecules and thus stimulate CD8 T cells (discussed in Ref. 42).

These results demonstrated that the binary tet-off tg system proved useful to investigate in more detail requirements for both induction and sustained production of anti-dsDNA Abs, and also for regulation of T cell tolerance to nucleosomes.

In the tet-off mice, production of anti-dsDNA Abs could be maintained by sole T-Ag expression after prior immunization with T-Ag and nucleosome-T-Ag complexes, while in immunized T-Ag nonexpressing mice, Abs to dsDNA declined. The titers of the Abs determined in solid-phase (conventional anti-dsDNA ELISA) and in solution-phase anti-DNA ELISA (SPADE) using linear and circular dsDNA molecules, compared well with those currently determined in autoimmune (NZB×W/F)F1 mice (manuscript in preparation), and with those determined by the same solid- and solution-phase assays recently published for human SLE-derived anti-dsDNA Abs (27), which differ significantly from the much higher titers reported for Abs to nonself proteins or to Z DNA, as discussed by Stollar (43). The results of these experiments directly demonstrated that T-Ag expression initiated a process that resulted in continuous stimulation of autoimmune dsDNA-specific B cells.
The delayed response would be consistent with the time needed to release sufficient amounts of nucleosomes to stimulate autoimmune nucleosome-specific T cells that evidently are activated in vivo in these mice (Fig. 2D and Table III).

Whether activated nucleosome-specific T cells may be maintained in vivo by autologous nucleosomes in absence of expressed T-Ag is currently under investigation by testing whether CD4+ autoimmune T cells activated by long-term T-Ag expression can be maintained by subsequent immunization with pure nucleosomes after terminating expression of T-Ag (experiments in progress). The results of the present study validate the tet-off model as useful for such experimental protocols. Furthermore, this tet-off conditioned transgenic system may have perspectives far beyond the focus of this study, allowing studies in general on the impact of infectious-derived proteins on regulation of tolerance to self constituents in context of infections.

Two independent sets of evidences have been presented that demonstrated a biological relevance for the binary tet-regulated T-Ag tg model described here. First, mice inoculated with T-Ag-expressing plasmids produced (transient) Abs to T-Ag and dsDNA, but also to the DNA-binding eukaryotic transcription factors TATA-binding protein (TBP) and cAMP-response-element-binding protein (CREB) (12, 13). In a prospective clinical study of 20 SLE patients followed over 1 year, a mirror image of these experimental results were obtained. Both within and among these SLE patients, sustained productive polyomavirus infection was the most prominent observation (13). Linked to this infection profile, Abs to T-Ag, dsDNA, TBP, and CREB were detected, identical with what we observed in T-Ag plasmid-inoculated mice (12, 13). In a cross-sectional study on healthy individuals, SLE, and related autoimmune syndromes, a strong and statistically highly significant correlation between Abs to dsDNA and T-Ag was noted (28). Those results strongly indicated that cognate interaction of B cells recognizing DNA or DNA-associated autologous proteins (e.g., histones, TBP or CREB) and T cells recognizing T-Ag had taken place as a consequence of complex formation between T-Ag and DNA/nucleosomes in vivo in the context of polyomavirus reactivations. In subsequent studies, T-Ag-specific T cells possessed the potential to proliferate when stimulated with T-Ag or T-Ag complexed with nucleosomes in vitro. In the presence of T-Ag complexed with nucleosomes, activated T-Ag-specific T cells provided help, probably by secreted IL-2 (Ref. 45, and references therein), to terminate nucleosome-specific T cell anergy. The latter T cells progressed into functional, autoimmune T cells if nucleosome-derived, autologous peptides were properly presented by the same APC (a situation consistent with determinant spreading) (6, 16). Both classes of T cells, either immune or autoimmune, may provide help for dsDNA-specific B cells to produce nephritogenic anti-dsDNA Abs (reviewed in Ref. 6). In conclusion, the present experiments and observations demonstrate that the tet-off system described here is sufficiently controlling T-Ag expression to avoid T-Ag- and nucleosome-related T cell nonresponsiveness. In these mice, sustained anti-dsDNA and anti-T-Ag Ab production could be achieved by constitutive expression of T-Ag once the Ab production was induced by immunization with T-Ag followed by nucleosome-T-Ag complexes. Based on present and previous experimental and prospective clinical results (6, 14–17, 28), this experimental system opens for further studies to describe molecular and cellular processes that are operational in both induction and sustained production of potentially pathogenic Abs to dsDNA or nucleosomes, representing important disease-modifying factors in syndromes like SLE.
CONTROLLED T-Ag EXPRESSION AND AUTOIMMUNITY TO NUCLEOSOMES

Acknowledgments
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References