

## Mice transgenic for a soluble form of murine cytotoxic T lymphocyte antigen 4 are refractory to murine acquired immune deficiency syndrome development

L. DE LEVAL,\*† S. DEBRUS,\*† P. LANE,‡ J. BONIVER & M. MOUTSCHEN\* \*Laboratory of Pathology, University of Liège, Liège, Belgium, and ‡Department of Immunology, Medical School, Birmingham University, UK

### SUMMARY

Interactions between B and CD4<sup>+</sup> T cells are central to the pathogenesis of retrovirus-induced murine acquired immune deficiency virus (MAIDS). Prompted by previous work showing that treatment with cytotoxic T lymphocyte antigen 4 immunoglobulin (CTLA4Ig) partly inhibited the disease, we studied the course of infection in mice deficient for CD28–B7 interactions (mCTLA4-Hγ1 transgenic mice). Despite a relative viral load identical to that of non-transgenic mice, the transgenic mice did not develop any of the major MAIDS symptoms (i.e. lymphoproliferation and immune anergy). The mCTLA4-Hγ1 did not however, completely inhibit B-cell activation as indicated by a slight hypergammaglobulinaemia and microscopic blastic transformation. Absence of MAIDS in transgenic mice was associated with much lower levels of both interleukin-4 and interferon-γ transcripts following viral infection. These results support the theory that the CD28/B7 costimulatory pathway is a critical determinant to MAIDS development.

### INTRODUCTION

Mouse acquired immune deficiency virus (MAIDS) is induced by murine leukaemia viruses (MuLV) present in a virus mixture recovered from a radiation-induced lymphoma of C57BL/6 mice.<sup>1,2</sup> The crucial component of this preparation is a replication-defective retrovirus, designated BM5def<sup>3</sup> or DU5H,<sup>4</sup> with a single open reading frame encoding a Pr60<sup>gag</sup> protein. The syndrome is characterized by a rapid and persistent proliferation of B and CD4<sup>+</sup> T cells, hypergammaglobulinaemia, phenotypic abnormalities of lymphocyte subsets, and increasingly severe defects of cellular and humoral immunity.<sup>5</sup> Although B cells are the main target for defective viral expression,<sup>6</sup> development of the disease is strictly dependent on the presence of functional CD4<sup>+</sup> T cells.<sup>7</sup> Mechanisms involved in CD4<sup>+</sup> T-cell contribution to MAIDS pathogenesis have been only partly elucidated. Of most importance was the demonstration that chronic T-cell activation and induction of anergy is major histocompatibility complex (MHC) class II antigen-dependent.<sup>8</sup> More recent research has focused on the costimulatory signalling pathways possibly involved in MAIDS: antibodies against certain adhesion molecules<sup>9</sup> or against CD40-ligand expressed on

T cells<sup>10,11</sup> were found to inhibit MAIDS development significantly.

Binding of the T-cell receptor CD28 to its counter-receptors B7.1 (CD80) and B7.2 (CD86) on antigen-presenting cells (APC) is now recognized as one of the most potent accessory activating signals to T cells.<sup>12,13</sup> The demonstration of an increased expression of B7 molecules on B cells in MAIDS<sup>14</sup> prompted us to analyse the effect of a functional blockade of these ligands on MAIDS development. A soluble fusion protein of cytotoxic T lymphocyte antigen 4 (CTLA4) binds to B7 with high avidity and acts as a competitive inhibitor of physiological CD28–B7 interactions.<sup>15</sup> In mice inoculated with the MAIDS-inducing preparation and treated with CTLA4 immunoglobulin (CTLA4Ig) (3 × 50 µg/week), lymphoproliferation progressed at a much slower rate than in untreated mice and the loss of *in vitro* responsiveness to mitogens was reduced.<sup>14</sup> However, the inhibitory effects of CTLA4Ig were circumvented with time, so the importance of CD28/B7 interactions for MAIDS induction was actually difficult to discern in this model. To investigate this question, we analysed the fate of MAIDS virus inoculation in mice transgenic for mCTLA4-Hγ1 [a fusion protein between mouse CTLA4 and human immunoglobulin G1 (IgG1)]. The generation of transgenic (tg) mice (called mCTLA4-hγ1 tg hereafter) has been described previously.<sup>16</sup> This system allows us to reach and maintain higher serum levels of the transgenic protein than iterative injection of the soluble CTLA4Ig to non-transgenic mice. Such serum levels are more likely to saturate binding *in vivo*.

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†The first two authors contributed equally to the work.

Correspondence: Dr M. Moutschen, CHU Sart-Tilman, 4000 Liège, Belgium.

## MATERIALS AND METHODS

### *Mice and virus*

The mCTLA4-H $\gamma$ 1 transgenic mice, produced by Peter Lane (Basel Institute for Immunology, Basel, Switzerland), were maintained in our facility by breeding transgenic males to C57BL/6 females, under pathogen-free conditions. Progeny was tested at 5 weeks of age by determining the presence in the serum of the transgenic product, using a competitive inhibition immunoassay for human  $\gamma$ -heavy chain. Transgenic animals and their control littermates were inoculated three times intraperitoneally (i.p.) at the ages of 6, 7 and 8 weeks with a MAIDS-inducing viral preparation, obtained as filtered culture medium of Du5H-transfected SIM.R fibroblasts chronically infected with the replication-competent RadLV G6T2.<sup>17</sup> This preparation was quantified by XC plaque assay<sup>18</sup> to contain 1000 plaque-forming units (PFU) of ecotropic virus/ml. At different time intervals (time 0 = first viral inoculation), mice were killed by CO<sub>2</sub> asphyxiation. At autopsy, spleen (SP) and lymph nodes (LN) (axillary, inguinal and mesenteric) were weighed on a precision scale.

### *Histopathology*

Tissue samples were fixed in 4% paraformaldehyde, embedded in glycolmetacrylate (JB Polyscience, Polylab, Antwerp, Belgium); semi-thin sections (2  $\mu$ m) were stained with haematoxylin and eosin and histoenzymological staining was performed for acid phosphatases.

### *Proliferation tests*

Single cell suspensions from SP and LN were prepared with a fitting glass homogenizer and were suspended in RPMI-1640 complete medium (Gibco, Meselbeke, Belgium). Aliquots containing  $2 \times 10^5$  cells per 200 ml were cultured in triplicate in 96-well microtest plates (Nunc, Meselbeke, Belgium) for 72 hr with concanavalin A (Con A) (Boehringer Mannheim, Mannheim, Germany), 5  $\mu$ g/ml, or lipopolysaccharide (LPS; Difco, Detroit, MI), 10  $\mu$ g/ml. During the last 4 hr of culture, cells were incubated with 0.4  $\mu$ Ci [<sup>3</sup>H]thymidine (Dupont®, NEN products, Boston, MA) and collected with a cell harvester (Skatron®, Sterling, VA) onto glass fibre filters. Incorporated precursor was counted in a scintillation analyser (Tri-Carb®, Packard, Meriden, CT).

### *Immunoglobulin serum levels*

The serum levels of immunoglobulin isotypes were measured by isotype-specific sandwich enzyme-linked immunosorbent assay (ELISA). Ninety-six-well ELISA plates were coated with antibodies specific for each murine immunoglobulin isotype (rat monoclonal antibodies to murine  $\gamma$ 1-,  $\gamma$ 2a-,  $\gamma$ 2b-,  $\gamma$ 3- and  $\mu$ -heavy chains: LO-MG1-13, LO-MG2a-7, LO-MG2b-2, LO-MG3-13 and LO-MM-3; Technofarm Biotechnology, Clichy, France) diluted at 5 or 10  $\mu$ g/ml in phosphate-buffered saline (PBS)-Tween. Thereafter, serum samples at the concentration of 1/100 (for determination of IgG2b and IgG3 isotype levels) or 1/1000 (for the other isotypes tested) in PBS-Tween were serially diluted and incubated. Horseradish peroxidase (HRPO)-labelled antibodies to murine heavy chains (LO-MG1-2, LO-MG2a-3, LO-MG2b-1, LO-MG3-7 and LO-MM-8, Technofarm Biotechnology) were added and incubated. HRPO activity was visualized with *o*-phenylenediamine and

hydrogen peroxide, with quantification at 490 nm by an ELISA reader (Dynatech Laboratories, Inc., Alexandria, VA). Concentrations of the samples were determined by comparison with standard curves generated with purified isotypes.

### *Flow cytometry analysis*

For staining,  $10^6$  cells were incubated with anti-Fc $\gamma$ RII (CD32, Fc block™, Pharmingen, San Diego, CA) to block non-specific interactions, prior to labelling for 20 min with 50  $\mu$ l of rat monoclonal antibodies against the following mouse surface antigens: Thy-1.2 (30-H12), CD4 (GK 1.5), B220 (RA3-6B2), all purchased from Pharmingen, and CD8 (53-6-72), purified in our laboratory. All incubations were carried out on ice in PBS supplemented with 2% (v/w) bovine serum albumin and 0.1% sodium azide. Antibodies were fluorescein isothiocyanate (FITC), zodoxphytan phycoerythrin- (R-PE), or biotin-labelled. When biotinylated antibodies were used, cells were washed twice and counterstained with streptavidin-PE (Becton Dickinson, Erembodegem, Belgium). After additional washes, cells were analysed on a FACStar Plus® cell sorter (Becton Dickinson). Lymphocytes were gated according to forward and side scatter dot plots.

### *Cytokine gene expression*

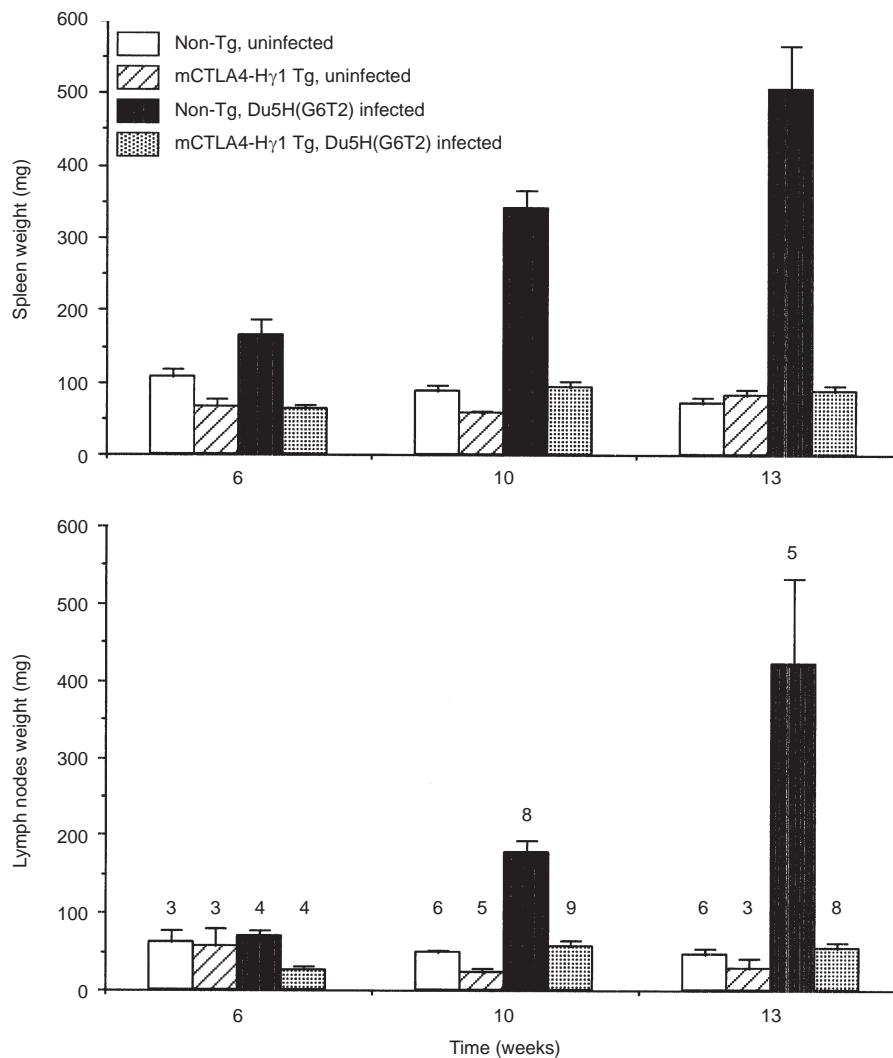
The RNase protection assays were performed on 20  $\mu$ g total RNA extracted from individual spleen samples with the Riboquant Multi probe RNase protection assay (Pharmingen) according to the manufacturer's instructions. A multiprobe set including specific templates for interleukin-4 (IL-4), IL-5, tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-13, IL-15, IL-9, IL-12, IL-6, interferon- $\gamma$  (IFN- $\gamma$ ) mRNAs and for the house-keeping genes L32 and GAPDH was used.

### *Defective virus expression*

Transcripts for Du5H gag protein in spleen cells were detected by reverse transcriptase-polymerase chain reaction (RT-PCR) technique. RNA samples were prepared following the RNazol™ B method (Biotecx, Houston, TX) and 2  $\mu$ g of individual samples were reacted with RT. Primers used for PCR reactions were 5'-CCTCTTCCTTTATGGACACT-3' and 5'-ATTAGGGGGGAATAGCTCG-3' sequences, corresponding to nucleotides 1282-1301 and 1499-1518, respectively, of the published sequence.<sup>19</sup> The DNA of pDu5H was used as a positive control. Quantification of defective gag included normalization to amplification of hypoxanthine phosphoribosyltransferase (HPRT) message. Primers used for HPRT sequence were 5'-GTTGGATACAGGCCA-GACTTTGTTG-3' and 5'-GATTCAACTTGCGCTCATCT-TAGGC-3'.<sup>20</sup> Thirty-cycle amplification PCR was verified to be below saturating conditions.

## RESULTS

Twenty-one mCTLA4-H $\gamma$ 1 transgenic mice and 17 non-transgenic littermates were inoculated with Du5H(G6T2) and assessed for MAIDS symptoms at either 6, 10, or 13 weeks after viral infection; 11 mCTLA4-H $\gamma$ 1 transgenic mice and 15 non-transgenic littermates, sham-inoculated with sterile PBS, were also analysed as non-infected controls.



**Figure 1.** The mCTLA4-H $\gamma$ 1 transgenic mice do not develop MAIDS-associated lymphoproliferation. Columns represent means of SP and LN weights  $\pm$  SEM for each experimental group at 6, 10 and 13 weeks after viral inoculation. The number of mice studied at each time-point is indicated over the top of the corresponding columns.

### Lymphoproliferation

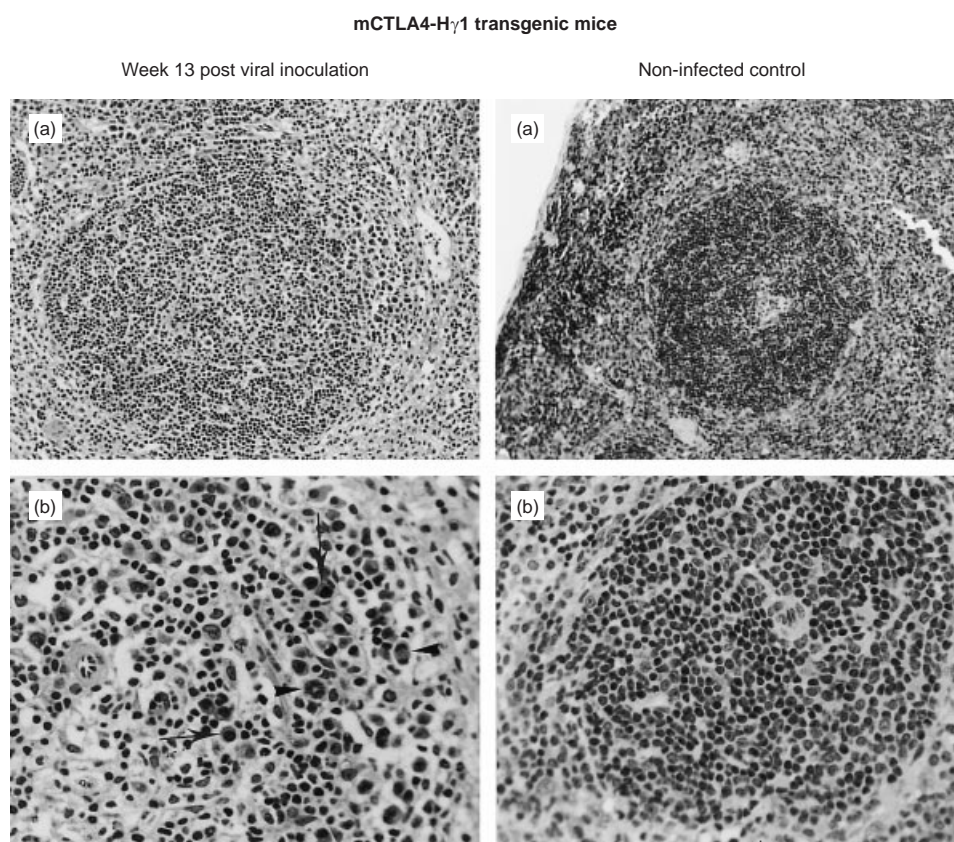
As expected, infected non-transgenic mice exhibited a progressive increase of spleen (SP) and lymph nodes (LN) weights. Figure 1 represents the means of SP and LN weight recorded from experimental animals examined at the three time-points after viral or sham-inoculation. Sham-inoculation of non-transgenic mice did not induce any variation in the weight of lymphoid organs. As previously reported by Lane *et al.* both the SP and LN weight from mCTLA4-H $\gamma$ 1 transgenic mice tended to be smaller than those of their non-transgenic littermates.<sup>16</sup> In sharp contrast with non-transgenic mice, infection of mCTLA4-H $\gamma$ 1 transgenics resulted in little or no increase in SP or LN size. Among transgenic animals examined at the 6th week, SP and LN weights from those infected were even lower than those sham-inoculated. At the 10th and 13th weeks, there was a slight increase in the weights recorded from infected transgenics when compared to uninfected ones; statistically, the difference was significant only for the values at the 10th week. At either the 10th or the 13th week, mean weights of SP and LN of infected

transgenics did not significantly differ from those of non-transgenic uninfected controls.

Despite the paucity of macroscopic changes after Du5H(G6T2) infection of mCTLA4-H $\gamma$ 1 transgenic mice, histopathological examination of the corresponding spleen specimens revealed significant microscopic changes: there was a discrete expansion and partial blastic transformation of the white pulp, taking place in the periarteriolar lymphoid sheaths (PALS), which filled with blastic cells of the immunoblastic type or with plasmacytoid features. Foci of plasmablasts were also observed in the red pulp (Fig. 2).

### *In vitro* proliferative responses to mitogens

Proliferative responses of SP cells from non-transgenic infected mice were substantially reduced at the 10th week and almost totally abrogated at the 13th week (Table 1). In sham-inoculated transgenics, responses were in the same range as in non-transgenic controls. Viral inoculation of mCTLA4-H $\gamma$ 1



**Figure 2.** Histological appearance of spleen removed from mCTLA4-H $\gamma$ 1 transgenic mice. In the left panel, MAIDS-infected tg mouse at the 13th week after viral inoculation (haematoxylin and eosin). (a) Concentric enlargement of the periarteriolar lymphoid sheath around the central arteriole ( $\times 100$ ) (b) Higher magnification of the same field showing large blastic lymphoid cells of the immunoblastic type or with plasmocytoid features (arrows); note the presence of several mitosis (arrowheads) ( $\times 250$ ). By comparison, in the right panel, spleen from a non-infected transgenic mouse contains small lymphoid nodules ( $\times 100$ ) (a), composed mainly of small lymphoid cells ( $\times 250$ ) (b).

transgenic mice did not induce any significant variation in their ability to respond to both Con A- and LPS-induced proliferation (Table 1).

#### Serum immunoglobulin isotype levels

B cell activation occurring in MAIDS is associated with hypergammaglobulinaemia.<sup>2,21</sup> Accordingly, the serum levels of IgG2a, IgG3 and IgM quantified by ELISA, were found to be several fold increased in Du5H(G6T2)-inoculated non-transgenic mice (Table 2). IgG2a mostly increased, to 20-fold the values from controls, whereas the increase in IgG3 and IgM was in the five- to 10-fold range. As previously described,<sup>22</sup> the levels of IgG2b and IgG1 were little modified after infection (not shown). Uninfected mCTLA4-H $\gamma$ 1 transgenic mice had serum levels of immunoglobulin isotypes slightly lower but in the same range as those from non-transgenics. After infection with Du5H(G6T2), transgenic mice developed moderate hypergammaglobulinaemia affecting the same subclasses as those found to be expanded in non-transgenic infected animals; the total serum concentration of immunoglobulin (G2a + G3 + M) in transgenics was however, only 25% of that of non-transgenics.

#### Phenotypic abnormalities

Phenotypic abnormalities associated with MAIDS include the expansion of large B cells expressing B220 at low density,<sup>6</sup> and the expansion of the Thy-1.2<sup>-</sup> CD4<sup>+</sup> subset.<sup>23,24</sup> At the 13th week, fluorescence-activated cell sorter (FACS) analysis confirmed these phenotypic shifts in each LN suspension from non-transgenic infected mice (not shown). By contrast, in LN suspensions of infected transgenics the blastic shift was inconstantly detected and, when present, involved only a small fraction of the total B population than in non-transgenics, and there was no expansion of the Thy-1.2<sup>-</sup> CD4<sup>+</sup> subset (not shown).

#### Semi-quantitative evaluation of cytokine gene expression

Among the various cytokines tested, only transcripts encoding sequences specific for IL-4, -5, -13 and IFN- $\gamma$  were detected in both PBS- and Du5H injected mice (Fig. 3). The levels of transcripts in uninfected transgenic and nontransgenic mice did not significantly differ. At the 13th week after viral inoculation, the main findings in non transgenic mice were a 15-fold increase of IL-4 transcripts and a 5-fold increase of IFN- $\gamma$  transcripts whereas the levels of IL-13 only slightly increased. By sharp

**Table 1.** Comparison of the proliferative response of splenocytes from uninfected or infected normal and transgenic mice to concanavalin A (Con A) and lipopolysaccharide (LPS)

mCTLA4-H $\gamma$ 1 TG	Infection	<i>n</i>	Unstimulated	Con A	LPS
<i>10th week</i>					
-	-	4	613 $\pm$ 213	24130 $\pm$ 4104	29510 $\pm$ 6515
-	+	7	431 $\pm$ 102	7340 $\pm$ 1871	15852 $\pm$ 1865
+	-	3	1395 $\pm$ 357	26 283 $\pm$ 2600	26 481 $\pm$ 9958
+	+	4	1582 $\pm$ 370	33 424 $\pm$ 2625	23 469 $\pm$ 2567
<i>13th week</i>					
-	-	2	838 $\pm$ 244	54 025 $\pm$ 5291	36 408 $\pm$ 1783
-	+	2	981 $\pm$ 228	1949 $\pm$ 1089	4616 $\pm$ 2052
+	-	1	2409	75464	33818
+	+	3	4301 $\pm$ 2000	55 347 $\pm$ 2416	24 520 $\pm$ 3022

Values (c.p.m.) given in the table represent the arithmetic means of the c.p.m. obtained from each mouse  $\pm$  SEM; *n* is the number of mice in each experimental group. For each mouse, c.p.m. values were calculated from triplicate wells (variations between triplicate wells were always less than 10%)

contrast, in infected mCTLA4H $\gamma$ 1 transgenic mice, no upregulation of IFN- $\gamma$  was detected and the level of IL-4 mRNA was only twice of that in uninfected controls (Fig. 3).

#### Defective viral gene expression.

The mRNAs prepared from spleens removed at the 10th week were examined by RT-PCR for expression of defective virus (Fig. 4). No transcripts were detected in any of the non-infected mice. Du5H was demonstrated in every infected animal; when semi-quantitatively compared to HPRT expression, no difference in the level of Du5H expression was found between mCTLA4-H $\gamma$ 1 transgenics and non-transgenics (Fig. 4).

### DISCUSSION

Pathogenesis of MAIDS clearly implies interactions between B and CD4<sup>+</sup> T cells.<sup>7</sup> The present work addressed the question of a possible involvement of CTLA4-CD28/B7 counter-receptors interactions in MAIDS development. Our results clearly demonstrate that mCTLA4-H $\gamma$ 1 transgenic mice do not develop the cardinal features of MAIDS even several months after repeated inoculations of the MAIDS virus.

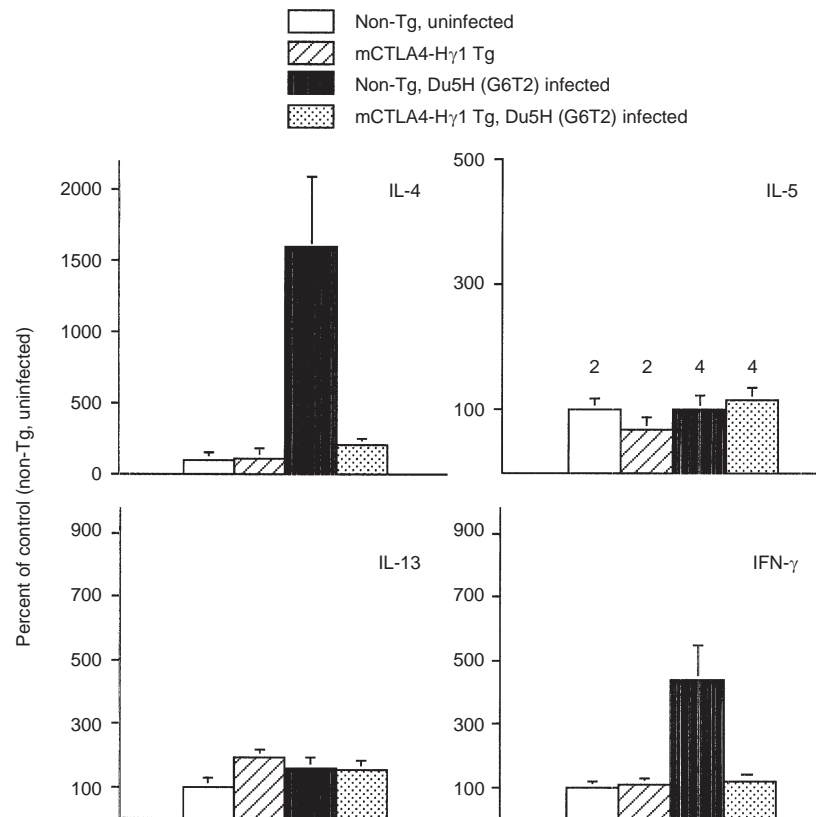
The mCTLA4-H $\gamma$ 1 transgenics were initially produced from (C57BL/6  $\times$  DBA/2)F<sub>1</sub> backcrossed with C57BL/6 mice. Since DBA/2 mice are resistant to MAIDS,<sup>25</sup> the hypothesis of a non-permissive genetic background of the tg mice must be addressed. This hypothesis can be formally ruled out for the following reasons: first, in F<sub>1</sub> crosses between resistant and sensitive strains, sensitivity to disease is dominant, rather than resistance;<sup>25</sup> second, the infected transgenic mice display high expression of defective MAIDS virus, a finding which correlates with genetic susceptibility to MAIDS;<sup>25</sup> third, the non-transgenic controls have the same genetic background and are fully susceptible to the disease.

Expression of mCTLA4-H $\gamma$ 1 is clearly associated with a much more complete and sustained protection than that obtained by iterative i.p. injections of CTLA4Ig.<sup>14</sup> This finding is likely due to a higher concentration of the fusion protein in tg mice. Despite the lack of pharmacodynamic data about the fate of injected mCTLA4Ig in mice, it can be reasonably inferred from studies on serum clearance of huCTLA4Ig injected intravenously (i.v.) into mice<sup>15</sup> that the steady-state concentration of the mCTLA4Ig obtained after repeated i.p. injections of 50  $\mu$ g three times a week is probably close to 10  $\mu$ g/ml. The serum levels of mCTLA4-H $\gamma$ 1 fusion protein achieved in tg mice are basically 10–30  $\mu$ g/ml<sup>16</sup> and can be assumed to be much higher in mice with MAIDS where the transgene was

**Table 2.** Comparison of the levels of IgG2a, IgG3 and IgM isotypes in sera from uninfected or infected normal and transgenic mice

mCTLA4-H $\gamma$ 1 TG	Infection	<i>n</i>	IgG2a ( $\mu$ g/ml)	IgG3 ( $\mu$ g/ml)	IgM ( $\mu$ g/ml)
<i>10th week</i>					
-	-	3	557 $\pm$ 145	863 $\pm$ 298	368 $\pm$ 66
-	+	7	12 695 $\pm$ 1698	4966 $\pm$ 645	3244 $\pm$ 533
+	-	4	407 $\pm$ 209	413 $\pm$ 46	177 $\pm$ 19
+	+	6	2414 $\pm$ 252	1731 $\pm$ 355	1499 $\pm$ 290
<i>13th week</i>					
-	-	3	491 $\pm$ 5	213 $\pm$ 48	364 $\pm$ 67
-	+	5	12 071 $\pm$ 1140	2986 $\pm$ 362	2412 $\pm$ 432
+	-	3	171 $\pm$ 76	233 $\pm$ 80	349 $\pm$ 31
+	+	5	2037 $\pm$ 633	790 $\pm$ 184	992 $\pm$ 148

Dosages were performed on sera from individual mice, according to the protocol described in the Materials and Methods. Values given in this table, expressed in  $\mu$ g/ml, represent the arithmetic means of individual values  $\pm$  SEM



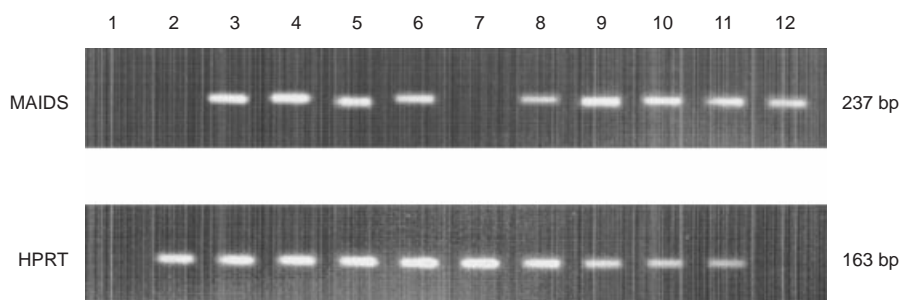
**Figure 3.** Measurements of cytokine mRNA levels with the RNase protection assay. The assays were performed on RNA extracts from SP samples of individual experimental mCTLA4-H $\gamma$ 1 tg and non-tg mice at the 13th week after infection with Du5H (G6T2) or sham inoculation with PBS. The number of mice studied in each group is indicated over the top of the corresponding column. Selected film exposures yielding similar overall signal intensities between experiments were scanned with a densitometer (PhosphorImager) to obtain absorbance values for cytokines and L32 transcripts. Quantification of cytokine mRNA levels was done by normalization to those of the L32 housekeeping gene. The values for infected non-tg, infected tg and uninfected tg mice were converted to percentage of values obtained in uninfected non-tg mice. The mean indexes ( $\pm$  SEM) from all experiments are shown.

found to be expressed by a very large number of blastic plasmacytoid B cells in the spleen (not shown).

Alternatively, the phenotype of mCTLA4-H $\gamma$ 1 mice could also contribute to their resistance to the disease, since these mice are devoid of germinal centres.<sup>16</sup> Interestingly, MAIDS is associated with the dramatic expansion of B and T cells with rare phenotypes normally restricted to germinal centres (B220<sup>dim</sup> B cells<sup>26</sup> and T CD4<sup>+</sup> Thy-1<sup>-</sup> cells<sup>27</sup>). This suggests that the abnormal interactions between B cells and CD4<sup>+</sup> T cells leading to MAIDS could preferentially take place in germinal centres or in a functionally similar microenvironment. Despite a high viral gene expression in the lymphoid organs of the infected mCTLA4-H $\gamma$ 1 mice, there is only a very limited expansion of B220<sup>dim</sup> B cells in these animals. This could indicate that the B-cell subset which mediates the abnormal interactions with CD4<sup>+</sup> T cells and induces their MAIDS-associated functional defects is absent or reduced in mCTLA4-H $\gamma$ 1 mice. A much lower frequency of 'MAIDS-inducing' B cells (due to absence of GC) could therefore permit a higher

protective effect of a given concentration of CTLA4Ig. Such a mechanism could also contribute to the resistance to MAIDS of other strains of mice deficient in MHC class II<sup>8</sup> or CD40<sup>28</sup> - deficient mice to MAIDS since these animals are also devoid of GC.<sup>29,30</sup>

Despite their resistance to MAIDS, infected mCTLA4-H $\gamma$ 1 mice display similar Du5H viral gene expression to infected non-transgenic mice. Similar findings have recently been reported in CD40-deficient mice infected with LP-BM5def: the absence of CD40 does not change the expression level of the defective virus.<sup>28</sup> These observations demonstrate that the infection of target cells by the defective virus and its persistence in this population is not strictly dependent on cognate T-cell help; furthermore they suggest that a large fraction of non-infected cells must participate in the lymphoproliferative process which develops in the infected non-transgenic mice. Despite the fact that Du5H viral gene expression analysis is *relative* and not absolute (i.e. mRNA is prepared from the same number of lymphoid cells in tg and non-tg animals), a strong



**Figure 4.** Relative expression of gag mRNA is identical in infected mCTLA4-Hg1 tg and non-tg littermates. The cDNA obtained from reaction of spleen RNA samples of individual mice with RT were amplified by either defective gag- or HPRT-specific primers for 30 cycles. The bands for the two products appeared on a 2% agarose gel at the expected migration points corresponding to 237 and 163 bp, respectively. Lanes 2 and 7 are, respectively, from uninfected non-tg and tg mice. Lanes 3–6 are from infected non-tg mice at either the 10th (lanes 3 and 4) or the 13th week (lanes 5 and 6) after viral inoculation. Lanes 8–11 are from infected tg mice at the 10th (lanes 8 and 9) or the 13th week (lanes 10 and 11) after viral inoculation. Lane 1 is a negative control for PCR (water). Lane 12 is a specificity control for PCR using J1 plasmid containing the sequence of Du5H. No amplification was obtained after PCR reaction of RNAs samples (not shown).

proliferative advantage of infected cells over non-infected cells in the non-transgenic mice would have led to a higher viral gene expression in the latter animals.

Another important issue, which was not addressed here, is the expression of the Pr60<sup>gag</sup> protein in the infected mCTLA4-Hg1 tg mice. Type 1-like cytokines have been involved in human immunodeficiency virus type 1 (HIV-1) post-transcriptional activation,<sup>31</sup> it is therefore conceivable that mCTLA4-Hg1 tg mice which secrete less IFN- $\gamma$  than their non-transgenic counterparts in response to retroviral infection are characterized by much lower Pr60<sup>gag</sup> protein expression despite a similar relative viral gene expression.

The mCTLA4-Hg1 tg mice have poor antibody class switching in response to T-dependent antigens, in correlation with the absence of GC development.<sup>16</sup> In our model, mCTLA4-Hg1 did not prevent infected mice from developing slight hypergammaglobulinaemia, involving both the IgM and IgG isotypes. Whereas CD28 is required for germinal centre reactions, the classical site for T-dependent antibody responses, MAIDS-associated hypergammaglobulinaemia may partly depend on other extrafollicular B-cell differentiation pathways, possibly independent from CD28/B7-mediated signals. A previous report on antiviral immune responses in mCTLA4-Hg1 transgenic mice suggested that highly replicating viruses, such as lymphocytic choriomeningitis virus (LCMV), might compensate for the decrease in T help caused by the blocking effects of soluble CTLA4.<sup>32</sup> Recently, the heat-stable antigen (HSA) was shown to be involved in the antigen-induced immunoglobulin class switch occurring in mice deficient in CD28.<sup>33</sup>

The efficacy of CTLA4Ig fusion protein for the inhibition of certain auto-immune diseases for the prevention of graft rejection has been largely ascribed to a modulation of cytokine secretion, most often a reversal from a T helper type 1 (Th1) to a Th2 pattern.<sup>34,34</sup> Interestingly, in the case of MAIDS infection, the resistance of the mCTLA4-Hg1 tg mice is associated with an almost complete abrogation of the up-regulation of both IFN- $\gamma$  (a Th1 cytokine) and IL-4 (a Th2 cytokine) normally found in susceptible nontg mice. Whereas it was claimed several years ago that IL-4 plays a major role in MAIDS pathogenesis,<sup>35</sup> subsequent studies favoured the

hypothesis that high-level expression of this cytokine was an epiphenomenon of disease.<sup>36</sup> If this holds true, a low level of expression of IL-4 in infected tg mice was rather expected. The role played by IFN- $\gamma$  in MAIDS is complex: several studies have shown that constitutive expression of this cytokine contributes to the progression of the syndrome. Accordingly, mice deficient in IFN- $\gamma$  or treated with a neutralizing antibody to IFN- $\gamma$  develop disease with a delayed time-course.<sup>37</sup> Surprisingly, overexpression of this cytokine by treatment with high-dose IL-12 renders mice resistant to disease.<sup>38</sup> It was therefore proposed that at high concentrations, IFN- $\gamma$  can directly inhibit B-cell proliferation and limit the development of MAIDS.<sup>37</sup> Since treatment with CTLA4Ig has been shown to increase IFN- $\gamma$  secretion in several models characterized by an *in vivo* Th2 switch,<sup>39,40</sup> it was interesting to assess the secretion of this cytokine in the infected transgenic animals.

Our results clearly show that the disease resistance conferred by mCTLA4-Hg1 tg expression is certainly not linked to a protective effect of IFN- $\gamma$  overexpression. Further experiments consisting of administration of rIFN- $\gamma$  to mCTLA4-hg1 tg mice may possibly contribute to a better understanding of the role of this cytokine in MAIDS.

Several models of T-cell anergy are characterized by incomplete signalling (i.e. TCR occupancy without appropriate costimulatory signals). Although previous reports have suggested that MAIDS could be due to similar mechanisms, Andrews *et al.* recently demonstrated that anergy induced by LP-BM5 infection cannot be reversed by providing costimulation via CD28 and IL-12.<sup>41</sup> Our results are quite compatible with this finding and suggest that at some point a complete activation of T cells must occur. T-cell refractoriness *in vitro* could therefore reflect chronic overactivation rather than an incomplete, or intrinsically abnormal activation process.

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Centre Anticancéreux près l'Université de Liège. L.dL, S.D. and M.M. are, respectively, research assistant, senior research assistant and research associate of the Fonds National de la Recherche Scientifique (FNRS).

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