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The *Yaa* gene-dependent B-cell deficiency worsens the generalized lymphadenopathy and autoimmunity of C57BL/6-*gld* male mice

N. ROSENBLATT,* K.-U. HARTMANN† & F. LOOR* *Laboratoire d'Immunologie, Université de Strasbourg, Strasbourg, France and †Experimentelle Immunologie Institut, Philipps Universität, Marburg, Germany*

SUMMARY

The BXSB mice are unique among murine models for systemic lupus erythematosus in that males are much more severely affected than females. The BXSB male disease is associated with a Y-chromosome-linked gene, which is an autoimmunity accelerator gene (*Yaa*). The *Yaa* mutation affects the B-cell subset, which becomes hyper-responsive to T-cell signals. The *Yaa* mutation was combined to the generalized lymphadenopathy disease (*gld*) gene in order to know whether an additional intrinsic B-cell defect might enhance gld disease in the male mice. The B6-*gld*-*Yaa* male mice were shown to display earlier and exacerbated lymphoproliferative and autoimmune features. It appeared that the milder gld syndrome observed in B6-*gld* male mice with a normal Y-chromosome was dependent on the mechanisms of B-cell activation and that the B cells could also accelerate the lymphoproliferation and the differentiation of T cells into Thy-1$^+$ B220$^+$ cells.

INTRODUCTION

During the last few decades, the discovery of adequate murine models has facilitated the study of systemic lupus erythematosus (SLE). Several mutant genes appear to be involved in the development and progression of murine SLE, such as the lymphoproliferation (*lpr*) and generalized lymphoproliferative disease (*gld*) genes.

The *gld* and *lpr* mice have been shown to develop very similar autoimmune and lymphoproliferative syndromes, characterized by B-cell hyperactivity, with hyperglobulinaemia and production of autoantibodies, and by spleen and lymph node enlargement.[1] This lymphoproliferation is due to the accumulation of an unusual subset of T-cell receptor (TCR)α/β T cells, principally characterized by their aberrant expression of the B-cell form of the CD45 marker (B220). This Thy-1$^+$ B220$^+$ cell population is made of two subsets: a predominant CD4$^-$ CD8$^-$ double-negative (DN) population (Thy-1$^+$ B220$^+$ CD4$^-$ cells) and a minor CD4^{dull+} population (Thy-1$^+$ B220$^+$ CD4$^+$ cells).[2] The mechanisms underlying the development of Thy-1$^+$ B220$^+$ cells remained rather undefined until recently. The hypothetical targets of *lpr* and *gld* mutations were thought of as being molecules involved in sequential steps of an intercellular metabolic pathway.[3] The *lpr* mutation was shown to affect the structural gene for the mouse Fas cell-surface protein, which can mediate apoptosis,[4] leading to the suggestion that the target of the *gld* mutation might be the ligand for the Fas antigen. Recently, the Fas ligand was shown to be a transmembrane protein belonging to the tumour necrosis factor (TNF) family[5] and the *gld* mutation was characterized as a point mutation in the gene segment encoding the C-terminal region of the Fas ligand.[6]

Both T and B cells are intrinsically defective in *lpr* mice,[7,8] a feature in line with the fact that normal T and B human cells express the Fas antigen,[9] whereas the cellular compartments affected by the *gld* mutation have not yet been defined. We and others have shown that the *gld* defect is contained within the T-cell compartment,[10,11] especially in activated mature T cells,[11] but, according to others, the gld syndrome would be due to a defect extrinsic to the T cells.[12] Nevertheless, all results available so far would indicate that *gld* B cells would be normal[10–12] and that *gld*-induced autoimmunity might result from abnormal interactions between T and B cells.

The severity of the lpr and gld diseases differs depending on strain background and sex. Thus, MRL-*lpr* mice develop severe glomerulonephritis in comparison with AKR/J-*lpr*, C3H/HeJ-*lpr* or C57BL/6-*lpr* mice.[13] The influence of sex hormones in immune responses and particularly in autoimmune diseases is also a well-known feature. In (NZB/NZW)F_1 mice, a murine model for human SLE, females develop an earlier and more severe disease in comparison with males[14] and it has been suggested that oestrogen hormones are an accelerator of the lupus disease in (NZB/W) and MRL-*lpr* mice, whereas androgen hormones would have protective properties.[15–17] In the non-autoimmune C57BL/6 (B6) genetic background, the

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Abbreviations: +, normal wild-type gene; B6, C57BL/6; BSA, bovine serum albumin; BSS, balanced salt solution; Con A, concanavalin A; gld, generalized lymphadenopathy disease; LNC, lymph node cells; lpr, lymphoproliferation; LPS, lipopolysaccharide; rIL-2, recombinant interleukin-2; SC, spleen cells; xid, X-chromosome linked immunodeficiency; Yaa, Y-chromosome linked autoimmune accelerator.

Correspondence: Dr F. Loor, Laboratoire d'Immunologie, Université Louis Pasteur, BP 24, F 67401 ILLKIRCH Cedex, France.

gld and *lpr* females also develop a more severe and earlier disease than the males.[18]

BXSB mice are unique among murine models for SLE in that males are much more severely affected than females. A Y-linked autoimmune accelerator gene (*Yaa*) has been shown to determine the early-onset B-cell hyperactivity and autoimmunity of BXSB strain male mice.[19] The *Yaa* gene was described as an accelerator of autoimmune disease rather than an inducer of disease, since the BXSB Y-chromosome was able to induce autoimmunity only in mice potentially capable of developing the disease. Thus, the action of the *Yaa* gene appears to depend on its interaction with the abnormal autosomal genomes present in autoimmune prone mice.[19–21] Furthermore, the *Yaa* mutation seems to affect the B-cell subset, since the BXSB-*xid*-*Yaa* male mice do not display an autoimmune phenotype.[22] The selective activation of *Yaa* B cells might thus be due to their hyper-responsiveness to T-helper signals.[23]

Whether the gld syndrome could be exacerbated in B6-*gld* homozygous males by the *Yaa* gene was thus an open question, particularly in order to know whether a B-cell subset deficiency might increase the *gld*-induced autoimmunity and lymphoproliferation in male mice. Homozygous B6 *[gld Yaa]* male mice were thus constructed and studied for their autoimmune and lymphoproliferative disorders in comparison with B6 *[gld Y]* mice, bearing the normal B6 Y-chromosome, and with normal B6 mice with or without the BXSB Y-chromosome (B6 *[+ Yaa]* and B6 *[+ Y]* male mice).

Our results show that the *Yaa* mutation increases the autoimmune and lymphoproliferative disorders in B6-*gld* male mice. This suggests that an intrinsic defect of the B-cell subset can, surprisingly, also act on the T-cell lymphoproliferation and differentiation.

MATERIALS AND METHODS

Mice

All mice used in this study were congenic/consomic homozygous C57BL/6 (B6) males obtained from mice bred in our animal colony from background strains whose breeding pairs originated from the Jackson Laboratory (Bar Harbor, ME). These mice (all B6) were thus *[gld Y]* mice (*gld*-congenic B6 males with the normal B6 Y-chromosome), *[+ Yaa]* mice (normal B6 males consomic for the Y-chromosome derived from the SB/Le males *[Yaa]*), and *[+ Y]* mice (normal B6 males with the normal B6 Y-chromosome). All mice were kept in isolators under positive pressure (ESI, Cachan, France) and were provided with sterilized food, water, and bedding; however, their maintenance should be considered conventional rather than specific pathogen-free.

B6 [gld Yaa] *strain construction*

The development of B6 *[gld/gld Yaa]* males (*[gld Yaa]* mice) was performed as follows. B6 *[gld/gld X]* females were mated with B6 *[+/+ Yaa]* males, providing a F_1 generation where all males had the *[gld/+ Yaa]* genotype. They were then backcrossed to B6 *[gld/gld X]* females, providing a F_2 generation whose males were for one half *[gld/+ Yaa]* and for the other half *[gld/gld Yaa]*. Assignments of the F_2 male mice to these two genotypes were made on the basis of lymphoproliferation. All male mice were individually palpated when aged 3 months. The males which showed lymph node enlargement were considered to be homozygous for the *gld* gene. From then on, the B6 *[gld Yaa]* strain was developed by crossing *[gld/gld Yaa]* males with *[gld/gld X]* females. Two generations of *[gld/gld Yaa]* male mice were maintained to allow the development of the B6 *[gld Yaa]* strain. The progeny of the third generation was used for this study. Maintenance of the B6 *[gld Yaa]* congenic strain was thus similar to the B6 *[gld Y]* one, which was maintained by crossing B6 *[gld/gld Y]* males with *[gld/gld X]* females.

Follow-up of the animals

Every month, the male mice were bled by retro-orbital sinus puncture for serological analyses. Mice were killed when 8–9, 16–17 or 32 weeks old. At each time, the spleen and a set of lymph nodes (four cervicals and two inguinals) were removed and cell suspensions prepared; splenomegaly and lymphadenopathy were quantified by counting the numbers of spleen cells (SC) and lymph node cells (LNC).

Mitogen responses. The proliferative responses of SC to concanavalin A (Con A; Pharmacia, Uppsala, Sweden), to Con A plus recombinant interleukin-2 (rIL-2; gift of Dr M. Schreier, Sandoz, Basel) and to bacterial lipopolysaccharide (LPS, from *Escherichia coli*; Difco, Detroit, MI) were investigated in these male mice as described earlier.[24] The effect of rIL-2 on the proliferative response to Con A was measured by the ratio, R, of the [Con A + rIL-2]-response to the [Con A alone]-response.

Lymphocyte subset analyses. The SC and LNC were analysed by flow cytometry. They were first washed twice in a balanced salt solution containing 1% bovine serum albumin (BSS-BSA) before lysing erythrocytes with buffered ammonium chloride. After lysing, the cells were washed in BSS-BSA and resuspended at $2{\cdot}5 \times 10^6$ cells/ml. Aliquots of 200 μl of the cell suspensions were incubated with fluorescein isothiocyanate- (FITC) or phycoerythrin (PE)-conjugated rat anti-mouse monoclonal antibodies [FITC-labelled anti-Ly-5 (B220), FITC-labelled anti-Ly-2 (CD8a), PE-labelled anti-L3T4 (CD4), PE-labelled anti-Thy-1.2; Medac, Hamburg, Germany)] for 20 min on ice in the dark. After three washes, the cells were resuspended in a FACScan buffer and analysed by using a FACScan (Becton Dickinson, Mountain View, CA).

The B220 and Thy-1 single-positive cells were representative of the B-lineage and the T-lineage cells, respectively, whereas the Thy-1^+ $B220^+$ double-positive cells were counted separately as an unusual T-cell subset.

Serological analyses. The immunoglobulin isotype levels were quantified by ELISA as described previously.[25,26] Briefly, for all immunoglobulin isotypes except IgG2a, the specific immunoglobulin isotypes were captured by specific (coated) anti-isotype antibody, and the bound immunoglobulin molecules were then detected by specific (soluble) anti-isotype biotin-labelled antibody, followed by alkaline phosphatase-coupled avidin and the enzyme substrate.[25] For the measurement of the IgG2a, both antibody reagents used as capture and as biotin-coupled antibody were specific for the $IgG2a^b$ allotype, because B6 IgG2a (b allotype) is not recognized by most xenogeneic anti-IgG2a reagents.[26]

The total serum immunoglobulin levels were calculated as the sum of the levels of the six major immunoglobulin isotypes. The sera from nine or ten individual mice of each *[+ Y]*, *[+ Yaa]*, *[gld Y]* and *[gld Yaa]* strain were studied individually

for 16-week-old mice [data presented as means ± an index of individual variability, calculated as a standard deviation (SD) of the mean], and as pools (made by mixing identical serum samples from each individual mice) at all other ages studied. The anti-single-stranded DNA antibody titres were measured in individual sera by an ELISA procedure, as described elsewhere.[27] The anti-ssDNA content of each serum was expressed as a percentage of the content of a reference B6-*lpr* mouse serum pool (a mixture of 18 sera from 4–8-month-old B6-*lpr* mice that had been selected for their high titre in anti-ssDNA antibody). The procedure to detect the isotypes of the anti-ssDNA antibodies was similar to the anti-ssDNA detection procedure until the serum deposit. The sera (at a 1/100 dilution) were added at 37° for 2 hr and the further steps were carried out as for the isotype quantification. The results were expressed as percentages of the level of the reference B6-*lpr* serum pool. Only the three isotypes of the anti-ssDNA antibodies which showed large differences between the strains are shown in the results.

Statistical analyses

The Mann–Whitney–Wilcoxon test was used. Differences were regarded as significant for $P \leqslant 0{\cdot}05$. Similarities were regarded as significant for $P > 0{\cdot}1$.

RESULTS

Viability

Twenty-two male mice of each group were compared for life expectancy. The *[gld Yaa]* mice had a short life expectancy, 11/22 dying before 34 weeks and only 7/22 mice surviving over 50 weeks. In contrast the *[gld Y]* mice displayed a longer survival, only 7/22 mice dying before 34 weeks and 10/22 mice surviving over 50 weeks. The higher death rate of the *[gld Yaa]* mice occurred within 15–22 weeks of age and not later on. Death of *[+ Y]* and *[+ Yaa]* mice in the colony during that period was below 10% (Fig. 1).

Lymphoproliferation

Only mouse strains which displayed high SC and LNC numbers when 30 weeks old, were analysed at earlier times.

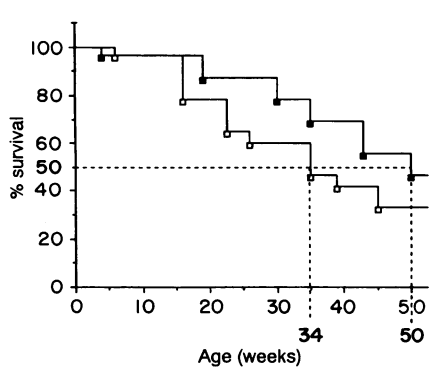

Figure 1. Survival of *gld* mice with or without the *Yaa* gene. The survival curves were compared on 22 mice each of *[gld Yaa]* (□) and *[gld Y]* (■) strains. The dotted line shows the age in weeks at which half of the *[gld Yaa]* and *[gld Y]* mice survived.

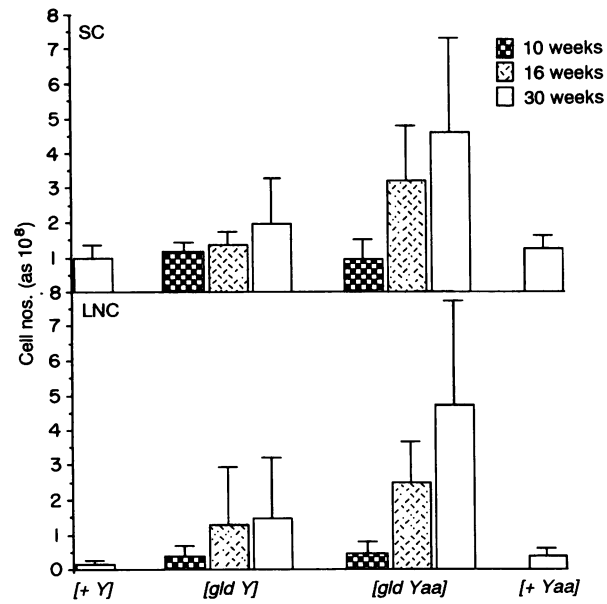

Figure 2. SC and LNC numbers in the four B6 congenic strains. Only the mouse strains showing splenomegaly and/or lymphadenopathy of 30-week-old mice were analysed, when 10 and 16 weeks old (in parentheses are the numbers of individually tested mice at each time): *[+ Y]* (18) mice, *[gld Y]* (11, 18, 16) mice, *[gld Yaa]* (10, 17, 14) mice and *[+ Yaa]* (14) mice. The columns represent the mean cell number in each group and the bars the individual variability index (calculated as a standard deviation of the individual values).

Thirty-week-old *[gld Yaa]* mice showed a severe splenomegaly ($462 \pm 271 \times 10^6$ cells) in comparison with *[gld Y]* ($196 \pm 133 \times 10^6$ cells; $P < 10^{-5}$), *[+ Yaa]* ($126 \pm 35 \times 10^6$ cells; $P < 10^{-5}$) and *[+ Y]* ($99 \pm 37 \times 10^6$ cells; $P < 10^{-5}$) mice. The *[+ Yaa]* mice showed larger SC numbers than the *[+ Y]* mice but these two strains were not statistically different from each other ($P = 0{\cdot}09$).

The 30-week-old *[gld Y]* mice displayed a lymphadenopathy ($146 \pm 175 \times 10^6$ cells) in comparison with *[+ Y]* ($15 \pm 11 \times 10^6$; $P < 10^{-2}$) and *[+ Yaa]* ($38 \pm 24 \times 10^6$; $P = 0{\cdot}38$) mice, though the LNC numbers recovered from the *[gld Yaa]* mice were still larger ($470 \pm 303 \times 10^6$ cells; $P < 10^{-3}$). Thus, as a mean, the lymphadenopathy of 30-week-old mice was about three times stronger in *[gld Yaa]* than in *[gld Y]* mice. However, the 30-week-old *[gld Yaa]* males displayed splenomegaly and lymphadenopathy similar to those displayed by the 30-week-old *[gld X]* females ($474 \pm 291 \times 10^6$ SC, $P = 0{\cdot}85$, and $553 \pm 323 \times 10^6$ LNC, $P = 0{\cdot}38$).

The enlargement of the *[gld Yaa]* spleen and lymph nodes occurred particularly between the ages of 10 and 16 weeks (3·4× for SC and 5·4× for LNC), whereas analyses of age-matched *[gld Y]* mice gave corresponding increases of only 1·1× for SC and 3·5× for LNC during the same lapse of time. Furthermore, the SC and LNC numbers were, respectively, 1·6× and 1·7× higher in 16-week-old *[gld Yaa]* mice than in 30-week-old *[gld Y]* mice, underlining the earlier size increase of the former. Thus, *[gld Yaa]* mice showed a particularly severe and early enlargement of the spleen and lymph nodes when compared with *[gld Y]* mice (Fig. 2).

Table 1. Cell phenotypes* in the spleen and lymph nodes of 7–8 month-old *[+Y], [gld Y], [gld Yaa]* and *[+Yaa]* B6 mice†

Strain	Mouse nos.‡	$CD4^+$	$CD8^+$	Thy-1^+	$B220^+$	Thy-1^+ $B220^+$	$CD4^+$ $B220^+$
Spleen							
[+Y]	6	17±2	7±2	28±5	57±6	1±0·5	0·5±0·5
[gld Y]	5	14±8	5±4	16±8	42±9	11±5	2±2
[gld Yaa]	5	7±2	3±2	16±3	25±10	29±10	5±2
[+Yaa]	7	13±4	5±2	27±5	54±7	1±1	0±0·5
Lymph node							
[+Y]	6	25±6	16±5	49±12	40±11	2±1	1±0·5
[gld Y]	5	11±8	5±3	16±7	36±6	36±8	4±1
[gld Yaa]	5	10±5	5±1	19±4	22±10	49±7	9±2
[+Yaa]	7	18±2	16±4	46±5	45±5	1±0	1±0·5

*Thy-1^+ $B220^+$ and $CD4^+$ $B220^+$ were the double-positive cells; $CD4^+$, Thy-1^+ and $B220^+$ represent the percentages of the single-positive $CD4^+$, Thy-1^+ and $B220^+$ cell populations excluding the double-positive $CD4^+$ $B220^+$ and Thy-1^+ $B220^+$ cell populations, respectively.

†The percentages of positive cells expressing the marker were determined in individual mice and are expressed as mean ± individual variability (calculated as a standard deviation).

‡Numbers of mice analysed individually.

Determination of cell phenotype

The phenotypes of the cells responsible for the splenomegaly and lymphadenopathy of the 7-month-old *[gld Yaa]* and *[gld Y]* mice were studied by flow cytometry analyses of their SC and LNC suspensions (Table 1). In both *[gld Yaa]* and *[gld Y]* mice, the spleen and lymph node enlargement was linked to an accumulation of numerous T cells with the unusual Thy-1^+ $B220^+$ phenotype. Nevertheless, their percentages were, in spleen and lymph nodes respectively, 2·6× and 1·4× higher in *[gld Yaa]* than in *[gld Y]* mice. The increased numbers of Thy-1^+ $B220^+$ cells were, in part, due to an increase of their Thy-1^+ $B220^+$ $CD4^+$ subset, which represented up to 9% of *[gld Yaa]* LNC. The percentages of the single-positive $B220^+$ SC and LNC were reduced 2× and 1·6×, respectively, in *[gld Yaa]* in comparison with *[gld Y]* mice.

Proliferative responses of SC to LPS and to Con A

Only mouse strains showing a defective response with 30-week-old mice were studied at an earlier time. With 30-week-old mouse SC, the B-cell proliferative responses to LPS (Fig. 3) were significantly reduced for *[gld Yaa]* mice in comparison with *[gld Y]* mice ($P < 10^{-3}$), the latter being in the normal range in comparison with *[+ Y]* mice ($P = 0{\cdot}12$) and *[+ Yaa]* mice ($P = 0{\cdot}25$).

With 30-week-old mouse SC, the T-cell proliferative responses to Con A (Fig. 3) were reduced for both *[gld Y]* and *[gld Yaa]* mice. The latter mice being already affected when 16 weeks old, the *[gld Y]* and *[gld Yaa]* mice were statistically different ($P < 10^{-3}$) when tested at both ages (16 and 30 weeks old).

Addition of exogenous rIL-2 led to some enhancement (R) of the proliferative response of Con A-pretreated SC from both *[gld Y]* mice ($R = 1{\cdot}22 \pm 0{\cdot}41$) and *[gld Yaa]* mice ($R = 2{\cdot}74 \pm 1{\cdot}35$). While those enhancement factors restored a response in the normal range for *[gld Y]* mice, they did not for *[gld Yaa]* mice whose proliferative response levels remained at only a fifth of the levels found with normal *[+ Y]* mice (data not shown).

Interestingly, for 30-week-old mice, the Con A responses shown by *[gld Yaa]* spleen cells were 10-fold lower than those of *[gld Y]* SC, although the samples contained the same proportion (16%) of single-positive Thy-1 cells. Similarly, their LPS responses were fourfold lower than those of *[gld Y]* SC,

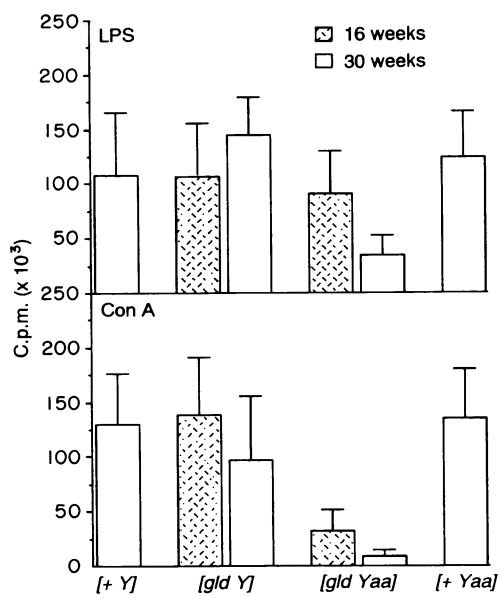

Figure 3. SC proliferative responses to LPS and to Con A of the four B6 congenic strains. Only the mouse strains showing a defective B-cell or T-cell activity with 30-week-old mice were analysed when 16 weeks old. Equal numbers of mice were tested at both ages within each strain, but B-cell response assays were less numerous than T-cell ones [in parentheses are the numbers of individually tested mice for LPS responsiveness (first number) and for Con A responsiveness (second number)]: *[+ Y]* mice (10, 16), *[gld Y]* mice (16, 19), *[gld Yaa]* mice (5, 12) and *[+ Yaa]* mice (10, 10). The columns represent the mean c.p.m activity in each group and the bars the individual variability index (calculated as a standard deviation of the individual values).

although the simple positive B220 cells were only slightly less represented in the *[gld Yaa]* samples (25%) than in the *[gld Y]* (42%) samples.

Increase and distribution of serum immunoglobulin isotypes with ageing

The sera of *[gld Yaa]*, *[gld Y]* and *[+ Yaa]* mice were analysed by ELISA tests at various ages. For the *[+ Y]* mice, only serum data obtained at 2–3 and 7–8 months of age are shown as the immunoglobulin levels increased linearly with time. The whole serum immunoglobulin levels of all mice, except the *[+ Yaa]* mice, increased with ageing: 1·5× for *[+ Y]*, 2·7× for *[gld Y]* and 6·9× for *[gld Yaa]* male mice, between 2–3 and 7–8 months. The mean serum immunoglobulin level of 32-week-old *[gld Yaa]* mice (31·8 mg/ml) was 2·9× higher than that of age-matched *[gld Y]* mice, but similar to that of the age-matched *[gld X]* females (35·1 mg/ml). With 2–3- and 7–8-month-old mice, the serum immunoglobulin levels of *[+ Y]* and *[+ Yaa]* mice were similar (no statistical differences), thus showing that *[+ Yaa]* mice could not develop hyperglobulinaemia like *[gld Y]* or *[gld Yaa]* mice. The *[gld Yaa]* mice developed a severe hyperglobulinaemia quite early in life. Indeed, the serum immunoglobulin levels were 2·3× and 2·7× higher in 16-week-old *[gld Yaa]* mice than in age-matched *[gld Y]* and *[+ Yaa]* mice, respectively. Already at this age, the *[gld Yaa]* mouse population was statistically different ($P < 10^{-3}$) from the *[gld Y]* and *[+ Yaa]* mouse populations (Fig. 4).

The typical distribution of the *[gld Y]* immunoglobulin isotypes[28] was confirmed by the high percentage (35%) of the T-independent immunoglobulins (IgM + IgG3) and the low percentage of IgG1 (7%). This characteristic 'gld-like' immunoglobulin isotype distribution was also seen in the sera of the 16-week-old *[gld Yaa]* mice with 29% T-independent immunoglobulins and 4% IgG1. However, the distribution of the *[gld Yaa]* immunoglobulin isotypes then changed with ageing. From 16 to 32 weeks of age, IgM decreased from 19% to 5%, while the IgG2 (IgG2a + IgG2b) increased from 66% to 81% of the whole serum immunoglobulin. These changes in the immunoglobulin isotype distribution also reflected a change in the absolute concentrations of immunoglobulin isotypes: the

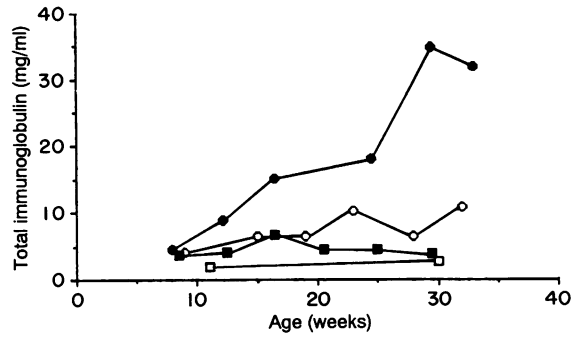

Figure 4. Serum immunoglobulin concentrations in the four B6 congenic strains. Total immunoglobulin concentrations, with ageing, in serum pools from *[gld Yaa]* (●), *[gld Y]* (○), *[+ Y]* (□) and *[+ Yaa]* (■) mice.

Table 2. Distribution of the six major immunoglobulin isotypes in sera of *[gld Yaa]* mice in comparison with their *[+ Y]*, *[gld Y]* and *[+ Yaa]* mouse controls*

	Age (months)	T-independent		T-dependent				Total immunoglobulin
		IgM	IgG3	IgG1	IgG2b	IgG2a	IgA	
[+ Y]	3	0·14	0·14	0·22	0·81	0·54	0·07	1·92
		7	**7**	**12**	**42**	**28**	**4**	
	7	0·29	0·35	0·28	0·80	1·01	0·12	2·85
		10	**12**	**10**	**28**	**35**	**5**	
[gld Y]	2	0·14	0·21	0·80	1·73	1·14	0·08	4·10
		3	**5**	**20**	**42**	**28**	**2**	
	4	0·31	0·32	0·49	2·14	1·65	0·06	4·97
		6	**7**	**10**	**43**	**33**	**1**	
	8	2·88	0·91	0·81	2·89	3·22	0·16	10·87
		27	**8**	**7**	**27**	**30**	**1**	
[gld Yaa]	2	0·21	0·23	0·70	1·88	1·53	0·03	4·58
		5	**5**	**15**	**41**	**33**	**1**	
	4	2·19	1·08	0·48	3·49	4·21	0·15	11·6
		19	**10**	**4**	**30**	**36**	**1**	
	8	1·46	2·65	1·36	17·71	8·01	0·64	31·83
		5	**8**	**4**	**56**	**25**	**2**	
[+ Yaa]	3	0·36	0·19	0·87	1·53	1·06	0·09	4·10
		9	**5**	**21**	**37**	**26**	**2**	
	7	0·33	0·32	0·62	1·16	1·34	0·12	3·89
		9	**8**	**16**	**30**	**34**	**3**	

*The levels of each immunoglobulin isotype and the total immunoglobulin levels are expressed in mg/ml and the numbers in bold represent the levels of immunoglobulin isotype expressed as percentage of the total immunoglobulin levels.

Table 3. Evolution with ageing of the anti-ssDNA titres in the sera of *[gld Yaa]* mice and their *[gld Y]* and *[+ Yaa]* B6 mouse controls*

Mice	Serum no.†	3–7 weeks	10–12 weeks	15–18 weeks	22–25 weeks	28–30 weeks	33–37 weeks
[gld Y]	12–20	9±10	15±14	25±12	36±27	49±30	35±22
[gld Yaa]	14–23	5±3	46±36	87±49	111±29	125±30	125±34
[+ Yaa]	12–16	9±12	12±11	18±13	23±19	22±15	17±12

*The titres are expressed as the percentage of the standard serum anti-ssDNA titre (reference *lpr* pool); the values were the mean of individual mouse values ± the variability index calculated as a standard deviation. All the values for the *[+ Y]* B6 mouse controls were ⩽10%.

†Numbers of sera tested.

level of IgM decreased from 2·2 to 1·5 mg/ml, whereas the cumulated levels of IgG2a and IgG2b increased from 7·7 to 25·7 mg/ml, IgG2b alone representing 17·7 mg/ml in 32-week-old *[gld Yaa]* sera (Table 2). Thus, the *[gld Yaa]* B cells might have 'switched' from IgM to IgG2b production.

Anti-ssDNA titres

The profile of evolution of the serum titres of anti-ssDNA antibody was studied with time. In comparison with male mice of the other strains, the *[gld Yaa]* mice showed, from 10 weeks after birth, higher anti-ssDNA titres, which increased with time until the mice were 25 weeks old, at which time they remained stable. The anti-ssDNA titres of 25-week-old *[gld Yaa]* mouse sera were three- and fivefold higher than those of *[gld Y]* and *[+ Yaa]* mice, respectively (Table 3). Furthermore, the *[gld Yaa]* males also displayed higher anti-ssDNA titres than the *[gld X]* females (not shown); for 30-week-old mice, *[gld X]* females had a mean anti-ssDNA titre of 75, in comparison to 125 for *[gld Yaa]* males. Thus, the *[gld Yaa]* mouse population was found to be statistically different ($P < 10^{-3}$) from the *[gld Y]*, *[+ Yaa]* and *[gld X]* populations.

The high *[gld Yaa]* anti-ssDNA titres were due to increases of the anti-ssDNA IgG2a, IgG2b and IgM isotypes. In comparison with age-matched *[gld Y]* and *[+ Yaa]* sera, 34-week-old *[gld Yaa]* anti-ssDNA titres were, respectively, 1·8× and 1·7× higher for IgM, 4× and 6× higher for IgG2a, and 3× and 5·7× higher for IgG2b (data not shown).

DISCUSSION

In comparison with B6-*gld* females, B6-*gld* males with the normal C57BL/6 Y-chromosome developed a mild form of gld disease. Here, we report that the association of *Yaa* gene with the *gld* gene worsened the gld syndrome in the B6-*gld* males. Indeed, these *[gld Yaa]* mice developed a marked autoimmune and lymphoproliferative syndrome, which was both earlier and larger than those developed in *[gld Y]* mice and similar to those developed in the B6 *[gld X]* females. Such an exacerbated syndrome might result from some synergistic interactions of the *Yaa* and *gld* genes, since neither *[gld Y]* mice nor *[+ Yaa]* mice showed features similar to those of *[gld Yaa]* mice. In view of the marked synergism observed, its potential mechanism is intriguing and worth discussing further.

A known effect of the *Yaa* gene was to be an accelerator of autoimmune disease, since the *Yaa* gene was able to induce autoimmunity only in mice with an autoimmune genetic background.[19–21] Similarly, the *[gld Yaa]* mice displayed an increased autoimmunity in comparison with *[gld Y]* mice. Indeed, the *[gld Yaa]* mice developed a severe autoimmunity with high serum levels of immunoglobulins and anti-ssDNA antibodies, presumably reflecting increased plasma cell numbers and/or B-cell hyperstimulation. Support for such a B-cell hyperactivation and differentiation was found in the defective proliferation responses of *[gld Yaa]* B cells to LPS, which were markedly decreased in 30-week-old *[gld Yaa]* mice. Though this age-dependent decrease of LPS responses could partly be due to a dilution of normal T and B cells by the increasing numbers of Thy-1$^+$ B220$^+$ cells, this did not seem sufficient to account for the very low levels of proliferation observed. Thus, the low LPS response of *[gld Yaa]* B cells *in vitro* might be consistent with their selective activation *in vivo* leading to their exhaustive activation.

Thus far, two alternative mechanisms were proposed to interpret the effects of the *Yaa* mutation on the B-cell subset, both of which suggested the expression of a peculiar *Yaa* gene-encoded component on the B-cell surface.[29] In one hypothesis, a subset of mouse B cells (those which can be deleted by the *xid* mutation) would display a membranous '*Yaa*' gene-encoded component which would function as an intercellular adhesion-like molecule; this would favour the interactions between autoantigen-specific T-helper cells and autoreactive 'Yaa$^+$ B cells' and their selective activation towards autoantibody production. In the other hypothesis, the Yaa$^+$ B cells would present a peptide derived from the *Yaa* gene product in association with their major histocompatibility complex (MHC) class II molecules; a Yaa peptide recognition by Yaa-specific T-helper cells would then induce the polyclonal activation of Yaa-peptide presenting B cells, leading to hyperglobulinaemia with high titres of natural antibodies. In both hypotheses, the B cells would thus become hyper-responsive to T-cell signals.[23]

Along the same lines, one may speculate that *[gld Yaa]* B cells would be further exposed to, and activated by, some additional gld-specific factor, somewhat similar to the 'L-BCDF', a factor claimed to mediate *lpr* B-cell hyperactivation in the *lpr* mice, the nature of which has remained elusive.[30]

We show here a novel effect of the *Yaa* mutation, the induction of a larger and accelerated lymphoproliferative syndrome in *gld* mice. This was an unexpected consequence of the *Yaa* mutation since BXSB male mice develop only moderate lymphoid proliferation consisting predominantly of B cells,[31] whereas the *[gld Yaa]* mice developed a massive lymphoproliferation due to the accumulation of the two unusual T-cell subsets known to cause the *[gld Y]* splenomegaly and lymphadenopathy: the Thy-1$^+$ B220$^+$ CD4$^-$ and

Thy-1$^+$ B220$^+$ CD4$^+$ subsets.[2,32] Furthermore, the *[gld Yaa]* mice showed no increase of B cells but, on the contrary, a considerably reduced percentage of the B220$^+$ single-positive cells was observed in their spleen and lymph nodes in comparison with *[gld Y]* mice. As suggested above, the presence of such a reduced B220$^+$ cell subset might reflect a terminal differentiation and maturation of B cells into plasma cells. Indeed B cells have been shown to lose the high molecular weight forms of the leucocyte common antigen (LCA; B220) as they differentiate into antibody-producing cells,[33,34] and the anti-B220 antibody we used (RA3–6B2) actually recognized its highest molecular weight form.[35]

Since an early decrease in immediate B-cell precursors was found in BXSB male mice,[36] a B-cell precursor deficiency might also account for another B-cell depletion and a decreased B220$^+$ cell frequency. This apparent decline of B-lineage lymphopoiesis in the central lymphoid tissues of the bone marrow was parallel to a B-cell hyperactivation in the peripheral lymphoid organs, as also suggested by our study. There could be physiological mechanisms[36] through which extensive self-renewal of mature B cells during immune responses or autoimmunity leads to down-regulation of new B-cell formation in marrow.

The earlier and stronger lymphoproliferative *[gld Yaa]* disease might also be explained by a T-cell involvement, especially the CD4$^+$ T cells, known to be involved in other autoimmune and lymphoproliferative diseases. Thus, treatment of MRL-*lpr* mice with monoclonal anti-Thy-1·2 or anti-L3T4 (CD4) antibody could reduce the lymphoproliferative and autoimmune disorders.[37,38] The *[gld Yaa]* mice might show a higher CD4$^+$ T-cell activity than *[gld Y]* and *[+ Yaa]* mice. Thus, as suggested above, autoreactive *Yaa* B cells would induce a proliferation of interacting autoreactive CD4$^+$ T cells. Increased CD4$^+$ T-cell numbers might also come from an apoptosis defect of the *gld* T cells, resulting from the *gld* gene alteration. Such an apoptosis defect could result from a deficiency in an apoptosis-inducing ligand. This might be the ligand for the Fas antigen (mutated in *lpr* mice),[3,4] since the *gld* mutation was shown to affect directly the Fas ligand-encoding gene.[6] Both aforementioned mechanisms might thus lead to increased CD4$^+$ T-cell numbers and to earlier and more severe lymphoproliferation. In both *lpr* and *gld* mice, the Thy-1$^+$ B220$^+$ subset seemed to be continuously derived, via a Thy-1$^+$ B220$^+$ CD4$^+$ intermediate, from activated CD4$^+$ memory T cells with specificities for self or foreign antigen.[32,39]

In conclusion, the *[gld Yaa]* male mice developed an exacerbated lymphoproliferative and autoimmune syndrome, which was the result of the synergistic interaction of the *Yaa* and *gld* gene. Our results show that the association of the *Yaa* mutation, causing an intrinsic B-cell defect, to the *gld* mutation, which affected only the T cells, could worsen the autoimmune, but also lymphoproliferative, disease. Thus, we demonstrated that the B cells could act on the T-cell differentiation and proliferation. Which molecules are involved in this B–T-cell co-operation has yet to be elucidated. Recently, the interactions between the CD80 and CD28 were shown to allow peripheral T cells to respond to an activated B cell by dividing and producing cytokines required for T-cell differentiation.[40] The *[gld Yaa]* mouse should be an interesting model to unravel further mechanisms of B–T-cell interaction.

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