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# Immunoregulatory role of Jα281 T cells in aged mice developing lupus-like nephritis

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Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the emergence of autoreactive T cells. Humans and mice with SLE have reduced numbers of CD1d-restricted invariant natural killer T (iNKT) cells, suggesting a key role for these cells in its immunopathogenesis. This subset uses an invariant TCR constituted by Vα14Jα281 chains paired with some Vβ domains. The regulatory role for iNKT cells in non-autoimmune mice was suggested by our previous results showing that aged Jα281 knockout (KO) mice produce anti-dsDNA. Here we show that old Jα281 KO mice have proteinuria and antibodies against dsDNA and cardiolipin. Histological analysis of Jα281 KO mice revealed glomeruli damage and deposition of C3c and IgG, mainly of the $IgG_3$ subclass. In spleens of aged Jα281 KO mice there is an increase of activated marginal zone B cells. The evolution of lesions may depend on the age-associated increase of autoantibodies production, preferentially $IgG_3$, mainly secreted by marginal zone B cells. Our results provide the first evidence of a lupus-like syndrome in non-autoimmune mice, supporting an age-related immunoregulatory role of $J\alpha281^+$ cells, probably associated with the activation of marginal zone B cells.

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## Introduction

Systemic lupus erythematosus (SLE, lupus) is an autoimmune disease characterized by the loss of tolerance to a variety of self antigens. Development of SLE is associated with the emergence of autoreactive Th cells [1–4] accompanied by a reduction of regulatory T cells [5–7]. However, the nature and specificity of regulatory T cells that inhibit autoantibodies production and development of lupus remain largely undefined. Some regulatory T cells that are γδ T cells or $V_H$ peptide-reactive $CD8^+$ T cells have been reported to be capable of inhibiting autoantibodies secretion by ablating autoreactive B cells[3, 7]. Recent reports show that humans and mice with lupus and related autoimmune diseases have reduced numbers of invariant natural killer T (iNKT) cells [8–11], and that their number recovers during the improvement in disease activity [10]. These

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**Abbreviations: α-GalCer:** α-galactosylceramide · **BUN:** blood urea nitrogen · **CL:** cardiolipin · **CLS:** chronic lesions score · **GAS:** glomerular activity score · **iNKT:** invariant NKT · **KBS:** kidney biopsy score · **MZ:** marginal zone · **SLE:** systemic lupus erythematosus · **TIAS:** tubulointerstitial activity score

observations suggest that iNKT cells may be an essential part of a possible regulatory T cell network that inhibits the induction of SLE.

Murine iNKT cells coexpress NK cell markers (*e.g.*, CD161) and T cell markers (*i.e.*, an invariant Vα14Jα281 TCR chain paired predominantly with Vβ8 chains) and are mostly $CD4^+$ or double negative. They recognize, in association with CD1d [12], an endogenous ligand [13], which is mimicked by a glycolipid, α-galactosylceramide (α-GalCer) [12, 14, 15]. CD1d-reactive iNKT cells, which can be traced using α-GalCer/CD1d tetramers [16–19], appear to play protective roles against a variety of immune-mediated conditions, including autoimmune diabetes [20–22].

NZB/NZW $F_1$ and MRL-*lpr/lpr* mice, which spontaneously develop autoantibodies and nephritis, are useful models for studying the pathogenesis of SLE [2, 3, 4, 7, 23, 24]. Recently, the introduction of induced models of lupus, generated in otherwise normal mouse strains by exposure to hydrocarbon oils such as pristane, has promoted further investigations on the mechanisms responsible for the outcome of SLE [25, 26]. Mechanisms by which pristane induces lupus-like autoimmunity are poorly understood. In CD1d KO mice with BALB/c background [27], the enhanced effect of CD1d deficiency on the development of nephritis, autoantibodies production, and cytokine responses was shown in pristane-treated animals [26]. The previous study indicates that CD1d deficiency exacerbates pristane-induced lupus and that pristane inoculation in CD1d-sufficient mice numerically and functionally suppresses CD1d-expressing DC and iNKT cells. In addition, CD1d deficiency enhances the numbers of marginal zone (MZ) B cells, which are crucial for autoantibodies production [28]. These findings suggest an immunoregulatory role for CD1d-restricted T cells in lupus [28]. In lupus prone strains, the immunoregulatory activity of iNKT cells varied over their life-time, revealing a marked increase in the potential of these cells to contribute to the production of IFN-γ with advancing age [29].

Since autoantibodies secretion increases with age in CD1d KO mice (S. A. Porcelli, personal communication), we speculate that it could be useful to study renal functions and autoantibodies production in aged Jα281 KO mice lacking iNKT cells, to test if this cell subset is also responsible for the regulation of autoimmune response in not lupus-prone mice. Our findings provide the first evidence that $J\alpha281^+$ cell deficiency is associated with an increased autoantibodies secretion and the development of autoimmune kidney damage.

## Results

### Development of lupus-like nephritis in Jα281 KO mice

To determine whether iNKT cells are involved in the development of age-associated lupus-like nephritis, we analyzed renal function in C57BL/6 and BALB/c Jα281 KO and wild-type mice. We tested 20 sex-matched mice per group at 12 and 24 months of age. Proteinuria was more severe in aged Jα281 KO mice than in the control littermates (Fig. 1A, B), independently from the genetic background. At 12 months, 50% of Jα281 KO mice developed mild proteinuria, whereas in the wild type only 40% had this grade of proteinuria on either the C57BL/6 (Fig. 1A) or BALB/c background (Fig. 1B).

The frequency of moderate to severe proteinuria was high (80%) at 24 months of age in C57BL/6 (Fig. 1A, left panel) and in BALB/c (Fig. 1B, left panel) Jα281 KO mice. None of the wild-type mice developed moderate or severe proteinuria at 24 months ($p=0.0001$). Blood urea nitrogen (BUN) was also elevated in old Jα281 KO mice (Fig. 1C, D), suggesting an advanced renal disease; 80% of Jα281 KO mice but none of the wild-type mice had elevated BUN (>15 mg/100 mL, $p=0.002$). Of the Jα281 KO mice, 20% had a severely elevated BUN levels (>30 mg/100 mL). Here again no significant differences were seen between the BALB/c and the C57BL/6 genetic background. Fig. 1E and F show the results of survival of aged Jα281 KO compared to wild-type mice: while 80% of aged Jα281 KO died within month 28 only 20% of wild-type mice died before 30 months.

At 24 months of age, when renal function is severely compromised in Jα281 KO mice, animals were killed and renal histology was analyzed in sections obtained from ten kidneys per group (Fig. 2A, B). Mild and focal mesangioproliferative glomerulonephritis was found in 60% of wild-type mice, 40% of wild-type mice had no evidence of nephritis by light microscopy, and none of the wild-type mice had evidence of diffuse proliferative or chronic lesions. However, of the Jα281 KO mice, 50% developed diffuse proliferative glomerulonephritis with fibrous crescents, glomerulosclerosis, tubular atrophy and interstitial fibrosis, 25% showed mild to moderate mesangioproliferative lesions, and the remaining 25% of these mice had mild focal or no lesions. The composite kidney biopsy score (KBS) was increased in Jα281 KO mice ($p<0.05$). Further histological analysis revealed an increase in glomerular activity score (GAS) as well as in chronic lesions score (CLS) in Jα281 KO animals.

 

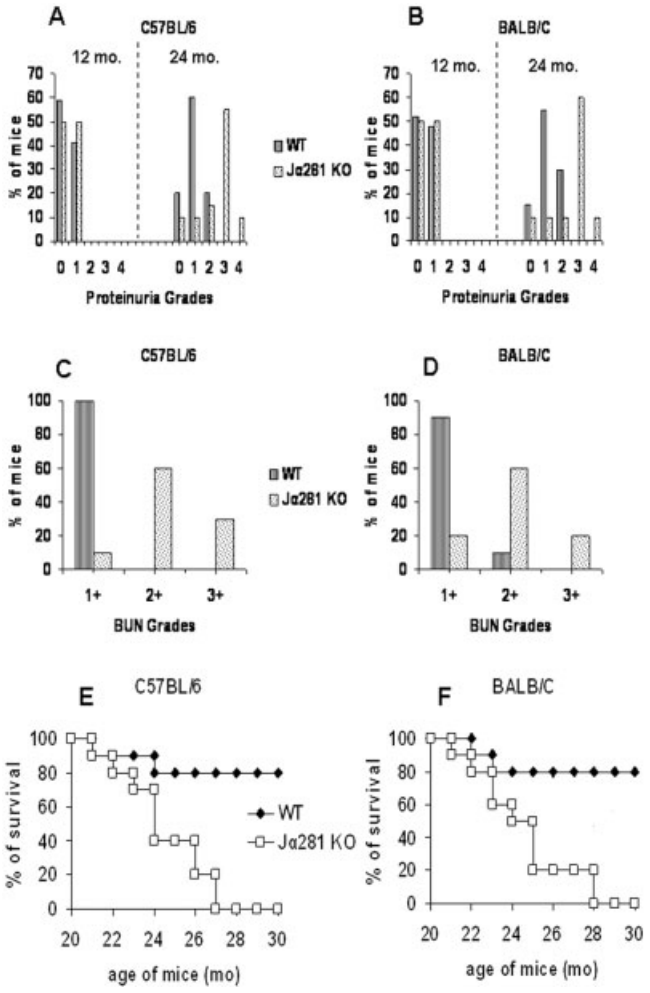


**Figure 1.** Jα281 cell deficiency accelerates nephritis in aged mice. C57BL/6 (A) and BALB/c (B) Jα281 KO ($n$=20) and wild-type mice ($n$=20) were monitored for proteinuria. Results are shown as the percentage of mice with 0+ to 4+ proteinuria at 12 and 24 months (0+, 0 mg/100 mL; 1+, 30 mg/100 mL; 2+, 100 mg/100 mL; 3+, 300 mg/100 mL; and 4+, >2000 mg/100 mL). In (C) and (D) BUN levels in 24-month-old Jα281 KO($n$=20) and wild-type ($n$=20) mice were shown. Results are shown as the percentage of mice with normal (1+, 5–15 mg/100 mL) or elevated (2+, 15–26 mg/100 mL and 3+, 30–40 mg/100 mL) BUN. In (E) and (F) percentages of survival were recorded comparing groups of 20 Jα281 KO and wild-type C57BL/6 (E) or BALB/c mice (F). Survival was detected from 20 to 30 months of age.

## Jα281 cell deficiency is associated with autoantibody production

It has been observed that anti-dsDNA production increases in CD1d KO mice with age (S. A. Porcelli, personal communication). We studied IgG anti-dsDNA in sera obtained from 24-month-old C57BL/6 and BALB/c Jα281 KO mice. Results shown in Fig. 3A represent the mean of ten tested mice. Aged C57BL/6 or BALB/c Jα281 KO mice had six- to sevenfold more anti-dsDNA than wild-type mice. As anti-dsDNA antibodies in lupus-prone strains were frequently associated with high levels of IgG anti-cardiolipin (CL), we tested sera of aged mice (24 months) for the presence of IgG anti-CL (Fig. 3B). Independently from the genetic background, Jα281 KO mice developed 13-fold more anti-CL IgG than age-matched wild-type mice. Moreover, anti-nuclear antibodies were increased in sera of aged Jα281 KO mice when compared to sera of wild-type mice (data not shown).

Thus, compromised renal functions and histological features correlate with increased anti-dsDNA and anti-CL in aged Jα281 KO mice, suggesting that the lack of Jα281$^+$ cells may cause the expansion and activation of B cells producing autoantibodies.

## IgG and C3c deposition in glomeruli of Jα281 KO mice

Previously reported data suggest that aged Jα281 KO mice develop a lupus-like nephritis, probably due to autoantibodies deposition. To confirm this hypothesis,

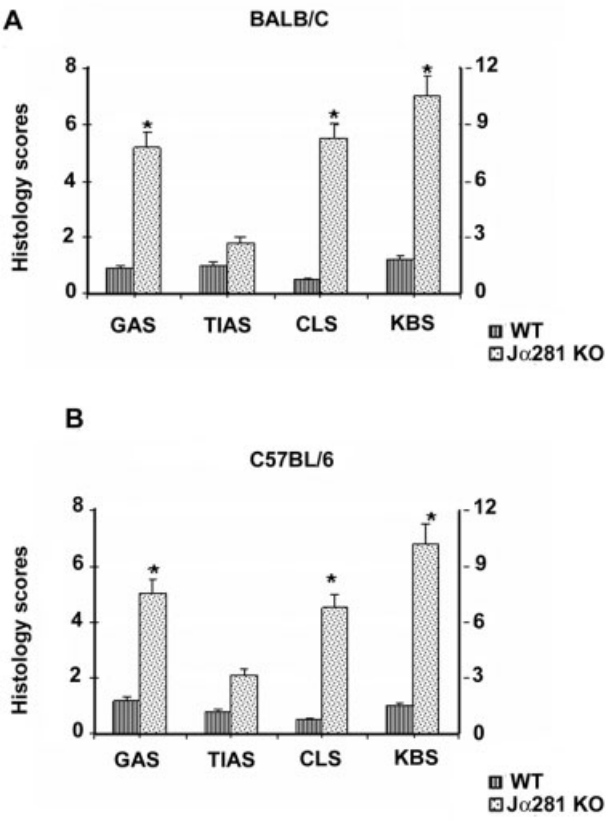


**Figure 2.** Kidney histology in aged Jα281 KO mice. (A) Comparison of histological scores between ten kidneys obtained from 24-month-old wild-type and ten Jα281 KO BALB/c mice. (B) Data obtained in wild-type and Jα281 KO C57BL/6 mice. Individual components (GAS, TIAS, and CLS) and a composite KBS (see the *Materials and methods*) in 24-month-old mice are shown as the mean ± SE scores (*, $p<0.05$, Mann-Whitney $U$ test).

 

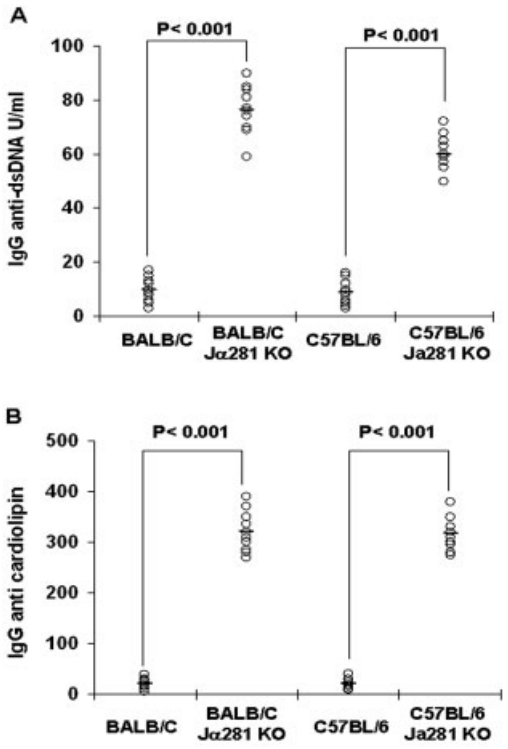


**Figure 3.** Anti-dsDNA and anti-CL autoantibodies in aged Jα281 KO mice. Sera were obtained from aged C57BL/6 and BALB/c wild-type and Jα281 KO mice (24 months). Anti-dsDNA (A) and anti-CL (B) IgG were assessed by ELISA as described in the *Materials and Methods*. Data from individual mice and means are reported in the graphics. Statistical comparison from experimental and control groups was done using Student's *t*-test.

we tested kidney sections of old Jα281 KO mice to investigate IgG deposition. A common feature of each strains analyzed was the expansion of mesangial area due to aging. Fig. 4A shows glomerulosclerosis associated with hypertrophy and increasing cell numbers in Jα281 KO kidneys (panels II and IV), while only the mesangial area is expanded in glomeruli of aged wild-type mice (panels I and III). Glomerulosclerosis is accompanied by immunoglobulin deposition, as shown in panel VI, while no IgG deposition was detected in wild-type mice (panel V). To characterize antigen specificity of IgG present in the glomeruli, anti-dsDNA IgG assays in kidney eluates of Jα281 KO and wild-type mice were performed. As shown in Table 1, anti-dsDNA IgG were found in glomeruli of aged Jα281 KO mice, whereas these autoantibodies were undetectable in kidney eluates of wild-type mice. A deeper immunohis-

**Table 1.** Anti-IgG detected in renal eluates of aged mice

| Samples | Anti-dsDNA IgG (μg/g tissue)[a)] |
|---|---|
| Jα281 KO BALB/C | 0.12 |
| BALB/C | N.D.[b)] |
| Jα281 KO C57BL/6 | 0.12 |
| C57BL/6 | N.D. |

[a)] The concentration of anti-dsDNA IgG are expressed as μg/g of kidneys for renal eluates.
[b)] N.D.: not detectable.

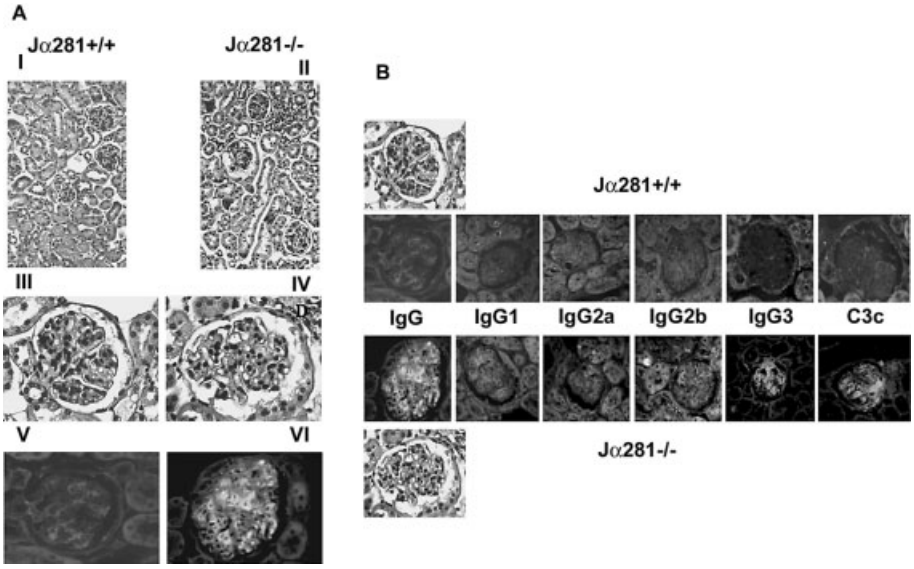


**Figure 4.** IgG3 and C3c deposition in glomeruli of aged Jα281 KO mice. (A) Kidney sections of aged Jα281 KO and wild-type C57BL/6 mice were stained by H&E methods and analyzed by light microscope at 10× and 40× magnification. Four representative photos are shown to describe damaged glomeruli (I, II, III and IV). FITC-labeled anti-mouse-IgG was added to slides to find immunoglobulin deposition in damaged glomeruli (40× magnification) (V and VI). Similar results were obtained in BALB/c background mice. (B) Kidney sections of aged Jα281 KO and wild-type C57BL/6 mice were stained by H&E and analyzed by light microscope at 40× magnification. FITC-labeled anti-mouse-IgG, $IgG_1$, $IgG_{2a}$, $IgG_{2b}$, $IgG_3$ and $C3_c$ were added to slides to detect subclasses of IgG and $C3_c$ deposition in glomeruli (40× magnification).

 

tochemical analysis of glomeruli of aged Jα281 KO and wild-type mice was shown in Fig. 4B. Glomeruli of Jα281 KO mice were brightly stained by anti-mouse IgG and a similar staining was obtained using anti-mouse C3c and $IgG_3$, but not anti-$IgG_1$, -$IgG_{2a}$ and -$IgG_{2b}$. Instead, no staining was detected in glomeruli of wild-type mice.

## Expansion of MZ B cells in Jα281 KO mice

In conditions of NKT cell deficiency such as in CD1dKO and pristane-injected wild-type mice, the lack of NKT cell-mediated regulation of MZ B cells may result in expansion and activation of this subset of B cells, which is enriched in autoreactive cells [30].These cells secrete mainly $IgG_3$ autoantibodies [30]. A role of these B cells in the development of lupus was suggested [30]. To test if MZ B cells secreting $IgG_3$ are present in aged Jα281 KO mice, we performed cytofluorimetric analysis to test if MZ B cells are expanded and/or activated in Jα281 KO mice. The results reported in Fig. 5 show that $B220^+$ $CD21^{high}$ $CD23^{low}$ cells, a phenotype corresponding to MZ B cells [30], were increased by fourfold (Fig. 5A) in the aged Jα281 KO mice when compared to wild-type mice, regardless of the genetic background of mice. Moreover, a significant proportion of the expanded MZ B cells in aged Jα281 KO mice expressed CD69 (Fig. 5 B) and CD25 (Fig. 5C), indicating that they were in an activated status. Additionally, while serum $IgG_1$ and $IgG_{2a}$ levels were similar between the Jα281 KO and control animals (data not shown), serum levels of $IgG_3$ isotype were significantly increased in Jα281 KO mice as compared to wild-type mice (Fig. 5D). Interestingly, sera of Jα281 KO mice had anti-dsDNA $IgG_3$ antibody levels greater than those detected in aged wild-type mice (Fig. 5E). This evidence suggests that, in aged mice, $J\alpha281^+$ cells regulate the activation of MZ B cells that secrete $IgG_3$, which is the main subclass of autoantibody produced in this experimental model.

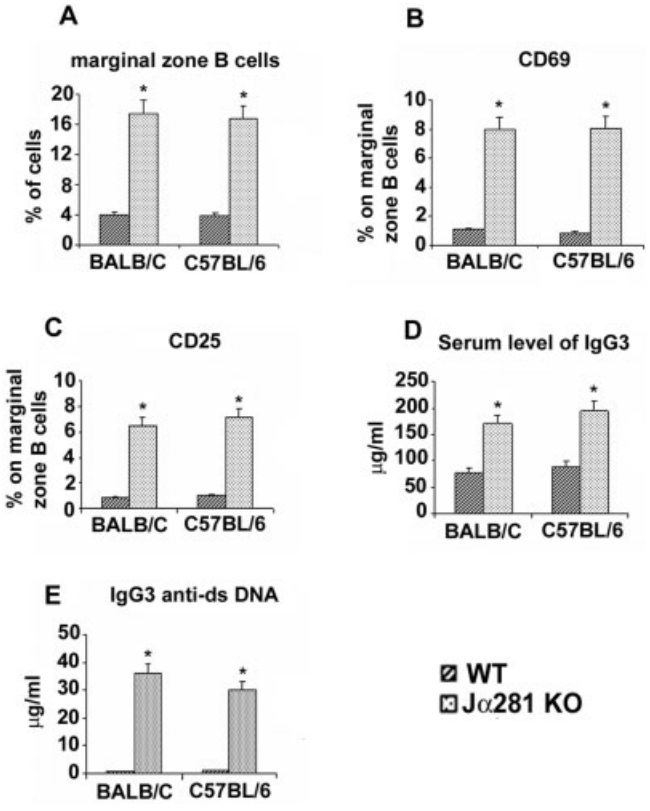


**Figure 5.** Effect of Jα281 deficiency on MZ B cells. (A) Jα281 KO and wild-type aged (24 months old) mice on BALB/c and C57BL/6 background were killed and their spleen cells were stained with B220, CD21 and CD23 and analyzed by flow cytometry. MZ B cells ($B220^+CD21^{high}CD23^{low}$) are shown as percentages in spleen cells. Results represent three independent experiments ($n$=2–4 mice/group). Percentages of $CD69^+$ (B) and $CD25^+$ (C) cells after gating on $B220^+$ $CD21^{high}$ $CD23^{low}$ were detected by FACS analysis, and results reported are the means of three different experiments. (D) Serum total $IgG_3$ levels (mean ± SE, µg/mL) in aged Jα281 KO and wild-type C57BL/6 and BALB/c mice were tested ($n$=13–16 mice/group, $p<0.02$). (E) Serum IgG3 anti-dsDNA antibody levels (mean ± SE, µg/mL) were tested in old Jα281 KO and wild-type C57BL/6 and BALB/c mice ($n$=13–16 mice/group, $p<0.02$).

## Discussion

In this paper we report that lupus-like nephritis, which is absent in aged BALB/c and C57BL/6 mice, is quite severe in Jα281 KO mice. Anti-dsDNA and anti-CL antibodies are detected in Jα281 KO but not in wild-type mice. The exacerbation of disease activity could be associated with the expansion of MZ B cells, responsible for the production of autoantibodies. Our observations suggest a regulatory role of CD1d-restricted cells and support the possibility that their depletion or reduction in functions may be responsible for lupus development. Indeed, patients with lupus and other systemic autoimmune diseases have reduced numbers of iNKT cells [9–11]. The NK/iNKT cell lineage marker, CD161, is significantly decreased in the peripheral blood cells of patients with SLE, as revealed in a recent gene expression study [31]. Importantly, in humans, lupus disease activity appears to inversely correlate with the number of circulating iNKT cells [10].

The present study indicates that the immunoregulatory function exerted by iNKT cells is evident in aged mice. It has been previously reported that α-GalCer-mediated function of iNKT cells is age-dependently upregulated [32]. This is partially attributable to the fact that systemic responses to α-GalCer are correlated with age-dependent increase of the number of liver mononuclear cells, even if the proportion of iNKT cells does not differ between young and old mice [33]. It is unknown why the ligand-mediated function of iNKT cells is enhanced in age-dependent manner; however, it

 

is possible that iNKT cells strengthen their function with age to survey malignant tumors, activated normal cells, and microbial infections.

Old CD1d KO BALB/c mice (>1.5 years old) have also increased serum anti-dsDNA antibody levels as compared with age-matched controls (S. A. Porcelli, personal communication). A more direct evidence of Jα281$^+$ cell involvement in the regulation of lupus-like disease comes from our study in which aged C57BL/6 or BALB/c Jα281 KO mice have elevated anti-dsDNA, anti-CL autoantibodies and renal $IgG_3$ and C3c deposition. In MRL-*lpr/lpr* mice, however, CD1d deficiency does not lead to either worse kidney damage or increases in anti-dsDNA antibody production [34]. The regulatory effects of CD1d-restricted events on nephritis may require intact Fas signaling, which is absent in MRL-*lpr/lpr* mice, or the anti-apoptotic effects of mutant Fas ligand are able to bypass the role of CD1d-reactive T cells. Furthermore, mechanisms of tissue damage seem to be different among the clinical manifestations of lupus in the same or in different animal models of lupus[35, 36], which probably represent several subsets of this heterogeneous disease in humans. For example, the correlation between the presence of anti-dsDNA-antibodies and kidney disease found in mouse strains was detected in almost 50% of patients affected by SLE with these clinical manifestations [37]. It is, therefore, not surprising that different mechanisms may operate in the development of lupus in various animal models. Some functionally distinct subsets of iNKT cells could promote autoimmune responses [35] while others could inhibit autoimmune disease[38, 39]. For example, implantation of transgenic T cells expressing TCR-α and –β chain genes from a T cell clone, which is CD1d specific but does not express the invariant Vα14 NKT TCR, induces a lupus-like disease in irradiated BALB/c nude recipients, whereas another subset of CD1d-reactive T cells prevents the development of lupus in the same model [40].

Finally, MZ B cells are expanded and activated in Jα281 KO mice and, in agreement with data in literature [30], we found increased serum levels and anti-dsDNA $IgG_3$ in Jα281 KO mice. These observations lead us to speculate that Jα281$^+$ T cells may regulate the expansion and activation of MZ B cells *via* CD1d, which is highly expressed on these cells. The lack of iNKT cell-mediated regulation of MZ B cells may, in turn, result in the expansion and activation of this subset of B cells, which are enriched in autoreactive cells [30, 40]. Studies have suggested a role of these B cells in the development of lupus [30, 40].

In summary, Jα281$^+$ cell deficiency in aged BALB/c and C57BL/6 mice is associated with lupus-like disease, presumably through one or more of the following possible mechanisms: (i) decreased T cell production of IL-4 along with stable IFN-γ production; (ii) decreased TNF-α production by T cells in the disease induction phase; (iii) a decrease in DC subsets that participate in the establishment of immune tolerance and regulation, and (iv) an expansion and activation of MZ B cells secreting autoantibodies. Our findings in aged Jα281 KO mice with a non-autoimmune background may be relevant to the understanding of immunopathogenetic mechanisms of spontaneous age-related lupus development. Patients with SLE also have a reduction in iNKT cell numbers, which appear to correlate with lupus disease activity [10]. Interestingly, it has been reported that the iNKT cell ligand, α-GalCer, protects BALB/c and SJL/J mice against pristane-induced lupus nephritis and protects MRL-*lpr/lpr* mice against spontaneous inflammatory dermatitis [5].

We do not know if our findings are clinically relevant in the analysis of age-associated lupus-like disease. However, Peralbo *et al.* [41] found that, in humans, ageing was associated with a significant decline in the percentage and proliferative response of peripheral blood iNKT cells. Given the important immunoregulatory role of iNKT cells, these alterations in their number and function could contribute to the deleterious autoimmune response in the elderly causing MZ B cell expansion, increased autoantibody production and lupus-like disease.

Taken together, these findings corroborate the overall conclusion that Jα281 T cells play a suppressive role in the age-related lupus-like nephritis. Further studies on the role of these cells and mechanisms by which they regulate the development of lupus nephritis in experimental models could be very useful for a deeper understanding of the pathogenesis of autoimmune diseases.

## Materials and methods

### Mice

C57BL/6 and BALB/c mice were purchased from Harlan Nossan (Correzzana, MI, Italy). Jα281-deficient mice were generous gift of Prof. M. Taniguchi (RIKEN Research Center for Allergy and Immunology, Yokohama City, Kanagawa, Japan). Mice that lack the Jα281 gene segment are devoid of Vα14Jα281$^+$ NKT cells while having the other lymphoid cell lineages intact [42]. Homogeneous populations were established by backcrossing heterozygous mice to C57BL/6 or BALB/c mice for more than eight generations. The resultant heterozygous mice were bred to obtain homozygotes [42]. In each experiment, age- and sex-matched control mice were used at 12–24 months of age.

 

### Assessment of lupus-like disease

Kidney damage was assessed in Jα281 KO and wild-type mice [2, 7]. Proteinuria was measured on a 0–4+ scale using a colorimetric assay strip for albumin (Albustix; Bayer, Elkhart, IN): 0, absent; 1+, ≤30 mg/100 mL (mild); 2+, 100 mg/100 mL (moderate); 3+, 300 mg/100 mL (severe); and 4+, >2000 mg/100 mL (severe). BUN levels were measured by impregnating Azostix strips (Bayer) with a drop of fresh blood and using the following scale: 1+, 5–15 mg/100 mL (normal); 2+, 15–26 mg/100 mL (mild); and 3+, ≥30 mg/100 mL (severe). Percentages of survival was assessed in groups of ten mice from 20 to 30 months of age comparing surviving Jα281 KO *versus* control littermates.

### Kidney histological analysis

Paraffin-embedded sections of kidney fixed in 4% paraformaldehyde were stained with hematoxylin and eosin (H&E), periodic acid-Schiff, and Masson's trichrome. Stained sections were scored for the following features on a 0–3 scale by three of us in a blind fashion [28]: (i) GAS that included glomerular proliferation, karyorrhexis, fibrinoid necrosis, cellular crescents, inflammatory cells and hyaline deposits; (ii) TIAS that included interstitial inflammation, tubular cell pyknosis, nuclear activation, cell necrosis and cell flattening, and epithelial cells or macrophages in tubular lumens; and (iii) CLS that included glomerular scars, glomerulosclerosis, fibrous crescents, tubular atrophy, and interstitial fibrosis. The raw scores assigned by various readers were averaged to obtain a mean score for each of the individual features. The mean scores for individual features were summed to obtain the three main scores (GAS, TIAS and CLS) and then all three scores were summed to determine a composite KBS.

### ELISA assay for detection of autoantibodies against dsDNA

Plates (96 wells; Costar, Copenhagen, Denmark) were coated with dsDNA obtained from calf thymus (100 μg/mL; Sigma-Aldrich, Milan, Italy) overnight at 4°C. After blocking of nonspecific binding sites with 10% FCS, serum samples were added. Bound antibodies were detected with alkaline phosphatase-conjugated goat anti-mouse IgG, $IgG_1$, $IgG_{2a}$ and $IgG_3$ (Fisher, Pittsburgh, PA). Reactions were colorimetrically developed with *p*-nitrophenyl phosphate (Sigma-Aldrich) and $A_{405}$ was measured with an ELISA reader. Anti-dsDNA antibody titers are expressed as units per milliliter using a reference-positive standard of pooled serum from MRL-*lpr/lpr* mice. To test anti-dsDNA IgG in kidney eluates and serum anti-dsDNA $IgG_3$ absorbances of samples were compared to a standard curve of mouse IgG and $IgG_3$ from 100 to 0.1 μg/mL using dsDNA-coated plates.

### ELISA assay for detection of CL antibodies

Each well of a polystyrene microtiter plate (96-well; Luxlon, Nemours, France) was coated with 50 μL bovine heart CL (10 μg/mL) diluted in absolute ethanol. The plate was incubated overnight at 4°C to allow the alcohol to evaporate. After blocking with 10% FCS, the plate was washed with PBS and incubated with sera diluted in FCS. The plate was washed three times and then incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (Rockland, Gilbertsville, PA). Positive mouse sera were defined as those giving an $A_{405}$ reading greater than the mean value plus 2 SD of sera from 60 normal control male mice. Results were reported as last dilution of sera that were scored as positive using criteria previously described.

### ELISA assay for detection of serum $IgG_3$

To determine the concentration of $IgG_3$, ELISA plates were coated with isotype-specific polyclonal antibody (Southern Biotechnology Associates or Bethyl Laboratories, Birmingham, AL) in carbonate buffer, pH 9.5. HRP-conjugated isotype-specific polyclonal antibodies were used for detection of $IgG_3$ (Southern Biotechnology Associates and Bethyl Laboratories). Concentrations of $IgG_3$ were determined using OptEIA kit and with pair of $IgG_3$-specific mAbs (BD Pharmingen, San Diego, CA). Tetramethylbenzidine was used as a substrate (KPG Laboratories). Absorbance at 450 nM was determined in an ELISA reader (Molecular Devices), at a sensitivity of 1 μg/mL. To test anti-dsDNA $IgG_3$, sera were added to dsDNA coated plates. Anti-dsDNA $IgG_3$ were detected as previously described.

### Immunohistochemistry

Tissue samples were fixed in 4% buffered formalin and paraffin embedded. Samples were cut at 5-μM thickness, stained with H&E, and examined by light microscope (Olympus, Hamburg, Germany). To detect IgG subclasses and/or C3c in sections prepared as previously described, sections were dewaxed, rehydrated and permeabilized using PBS, 0.1% Triton X-100 (Sigma, St. Louis, MO). Slides were then incubated with FITC-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG3 and C3c following procedures suggested by manufacturer (Sigma, MO). Slides were analyzed by fluorescence microscope (Olympus).

### Antibody elution from kidneys

Kidney tissue of ten mice per group was weighed, pooled, minced and collected in 500 μL PBS containing protease inhibitor cocktail (Roche, Mannheim, Germany). The mixture was centrifuged at 3000 rpm. for 5 min, and supernatants were collected. Pellets were washed and resuspended in 250 μL elution buffer consisting of 0·1 M glycine-HCl, 0·15 M NaCl, pH 2·5 and sonicated directly (Branson, Boom, Meppel, The Netherlands) on ice, with three bursts of 30 s, followed by overnight rotation at 4°C. Samples were centrifuged for 10 min at 10 000 rpm; supernatants were adjusted to pH 7·0. For further analysis, all samples were standardized based on their initial weight.

### Analysis of splenic MZ B cells

Mice were killed by $CO_2$ asphyxiation. Spleens were removed aseptically, and RBC were lysed with ammonium chloride. Spleen cells were washed with PBS-1% FCS, pH 7.2 buffer, and

 

Fc receptors were blocked with antibody against CD16/32 (clone 93; BioLegend, San Diego, CA). The $B220^+CD21^{high}CD23^-$ subset (MZ B cells) was detected by staining spleen cells from Jα281 KO and wild-type mice with FITC-labeled anti-CD21 (clone 7E9, *e*Bioscience, San Diego, CA), PE-labeled anti-CD23 (clone B3B4, *e*Bioscience), PE-labeled Cy7 anti-B220 (clone RA3–6B2, *e*Bioscience) and by gating on $B220^+CD23^-CD21^{bright}$ (MZ-B cells). To detect activation markers, MZ B cells were stained using APC-labeled CD25 (Clone PC61.5, *e*Bioscience) or allophycocyanin-labeled CD69 (Clone H1.2F3, *e*Bioscience). Stained cells were analyzed on a FACScan (BD Biosciences, San Diego, CA). Data were processed using CellQuest software (BD Biosciences).

### Statistical analysis

Levels of antibodies and renal scores were compared using Student's *t*- or Mann-Whitney *U* test. Proteinuria and BUN grades were compared using the two-sided Fisher's exact test.

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