Immunology and Cell Biology (2002) **80**, 436–440

Research Article

TNF contributes to the immunopathology of perforin/Fas ligand double deficiency

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Summary Perforin (pfp)/Fas ligand (FasL) double-deficient mice have previously been shown to be infertile, lose weight and die prematurely due to tissue destruction caused by a significant inflammatory infiltrate of monocytes/macrophages and T cells. Herein we have compared disease progression in mice additionally deficient in the inflammatory mediator TNF. Unlike pfp/FasL double-deficient mice ($TNF^{+/+}$ $pfp^{-/-}$ gld), mice lacking functional TNF, FasL and pfp ($TNF^{-/-}$ $pfp^{-/-}$ gld) were comparatively fertile, with the majority of mice not suffering severe pancreatitis or hysterosalphingitis in the first 5 months of life. The mean lifespan of $TNF^{-/-}$ $pfp^{-/-}$ gld mice was 217 ± 79 days compared with 69 ± 10 days for $TNF^{+/+}$ $pfp^{-/-}$ gld mice and the majority of moribund $TNF^{-/-}$ $pfp^{-/-}$ gld mice appeared to die as a result of severe pancreatitis, suggesting that loss of TNF was not completely protective. At 8 weeks of age, characteristics associated with the gld phenotype, such as expansion of $B220^+$ $CD4^-$ $CD8^-$ T cells, lymphadenopathy and hypergammaglobulinemia were comparable between $TNF^{+/+}$ $pfp^{-/-}$ gld and $TNF^{-/-}$ $pfp^{-/-}$ gld mice, although the lymphoid organs of $TNF^{+/+}$ $pfp^{-/-}$ gld mice contained greater numbers of $B220^+$ $CD4^-$ $CD8^-$ T cells, macrophages and T cells. We conclude that TNF is necessary for the full manifestation of immune dysregulation caused by pfp/FasL-deficiency, in particular in the early and overwhelming tissue infiltration and destruction caused by inflammatory cells.

Key words: apoptosis, immunodeficiency diseases, inflammation, rodent, transgenic/ knockout.

Introduction

Cytotoxic effector mechanisms used by lymphocytes include granule exocytosis mediated by perforin and granzymes[1] and death receptor-induced apoptosis mediated by some members of the TNF superfamily, including Fas ligand (FasL) and TNF.[2,3] Mice mutant or deficient for members of each of these cytotoxic pathways have been invaluable for dissecting the function of these molecules. Fas[4] and FasL[5] mutant, lpr and gld mice, respectively, are characterized by lymphadenopathy, splenomegaly, expansion of $B220^+$ $CD4^-$ $CD8^-$ (DN) T cells, hypergammaglobulinemia and strain-dependent autoimmune disease. Where autoimmune disease is not fatal, a significant proportion of ageing gld mice develop plasmacytoid tumours.[6] These features and additional observations[7–10] including humans with mutant Fas[7] indicate a critical role for FasL in homeostatic control of B cells, T cells, macrophages and other APC.[11]

Perforin deficiency in the mouse is not accompanied by changes in the composition of lymphocyte compartments[12] but these mice do have a decreased ability to clear some pathogens[12–14] and an increased susceptibility to tumours[15,16] particularly spontaneous lymphoma.[17] Humans with mutant pfp suffer from familial haemophagocytic lymphohistiocytosis (FHL), an autosomal recessive immune disorder characterized by uncontrolled activation of T cells and macrophages and overproduction of inflammatory cytokines.[18] With similar features, it has been well documented that a large proliferative expansion and persistence of antigen-reactive T and B cells and APC occurs in pfp-deficient mice challenged with lymphocytic choriomeningitis virus (LCMV). These mice also experience reductions in bone marrow haematopoietic precursor cells, probably due to enhanced haemophagocytosis.[12,19,20] Further supporting a role for pfp in homeostasis of the immune system was the observation that mice doubly deficient for pfp and FasL develop significant tissue infiltrates of destructive macrophages and $CD8^+$ T cells.[21] Such mice die early of severe pancreatitis and the females are infertile and suffer hysterosalphingitis. Perforin-based effector systems are therefore involved not only in host protection from pathogens, but also in the down-regulation of cellular immune activation.

Tumour necrosis factor, the protoype member of the superfamily, and its receptors, TNFR1 and TNFR2, have been deleted by homologous recombination and the mouse lines generated are normal with respect to the size and composition of their lymphocyte compartments. The major impact TNF and TNFR1 mutations have in an unchallenged mouse is an absence of B cell follicles and a defect in the formation of germinal centres[22–26] and these phenotypes can be attributed to defects in induction of lymphocyte-homing chemokines.[27] Recently, we and others have shown that loss of TNF[28] or monocyte chemotactic protein (MCP-1)[29] can ameliorate lymphoaccumulation in FasL/Fas mutant mice. Herein, we demonstrate that TNF is necessary for the early and overwhelming

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Received 4 April 2002; accepted 14 May 2002.

tissue infiltration and destruction caused by inflammatory cells in mice with pfp and FasL-deficiency.

Materials and Methods

Mice

Inbred C57BL/6 (WT) mice were purchased from The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia. C57BL/6 gld (gld; FasL mutant; breeding colonies obtained from Jackson Laboratories, BarHarbor, USA), C57BL/6 $TNF^{-/-}$($TNF^{-/-}$)[26] and C57BL/6 $TNF^{-/-}$ gld ($TNF^{-/-}$ gld) mice were obtained from the Centenary Institute of Cancer Medicine and Cell Biology, Sydney, Australia and were bred at the Austin Research Institute Biological Research Laboratories, Heidelberg, Victoria, Australia.[28] C57BL/6 perforin-deficient ($pfp^{-/-}$) mice[12] were obtained from Dr Guna Karupiah, John Curtin School of Medical Research, Canberra, Australia. Perforin$^{-/-}$ gld mice were bred from an intercross of $TNF^{+/+}$ $pfp^{-/-}$ $gld^{+/-}$ females bred to young $TNF^{+/+}$ $pfp^{-/-}$ gld males and mice were phenotyped as described.[17,30] Tumour necrosis factor$^{-/-}$ $pfp^{-/-}$ gld mice were derived from the relevant double-deficient mice and maintained by inbreeding $TNF^{-/-}$ $pfp^{-/-}$ gld pairs. Mice of both sexes were used in all experiments that were performed according to animal experimental ethics committee guidelines.

Organ weights

Mice were weighed and then sacrificed at the ages indicated. Organs were excised and weighed wet as follows: spleen, pancreas, axillary lymph nodes (aLN) (two randomly chosen). Organ weights of at least six mice of each genotype were used per time point. These results were recorded as the mean weight (percentage of total body weight) ± SD. Statistical significance was determined using the Student's *t*-test.

Histology and immunofluorescence

A full autopsy was performed at sacrifice and routinely spleen, pancreas, lymph nodes and uterus were examined by histology after fixing these tissues in formalin and on occasions also fresh frozen. The preparation and staining of sections for histology were carried out by Dr Duncan MacGregor of the Department of Anatomical Pathology, Austin and Repatriation Medical Centre, Heidelberg, Australia. Fluorescence immunohistology was performed on frozen sections. Spleen, pancreas or uterus specimens were embedded in Tissue Tek OCT compound (Sakura, USA) and snap frozen in the gas phase of liquid nitrogen. Six-micrometre sections were cut from tissue blocks and thaw-mounted onto glass slides. Sections were air-dried for 1 h, fixed in ice-cold acetone for 10 min and stored at – 20°C. Before staining, sections were rehydrated with phosphate-buffered saline (PBS), and then endogenous biotin staining blocked using DAKO Biotin Blocking System (DAKO, Carpinteria, USA). Non-specific staining by Fc portions of the primary antibodies was then blocked using Fc Block (clone 2.4G2, PharMingen, San Diego, CA, USA). Slides were incubated with each antibody layer for 30–45 min at room temperature in a humidified chamber, then washed with three changes of PBS (5 min) before addition of the next layer. Primary antibodies used were anti-B220 (biotinylated; RA3–6B2), anti-CD4 FITC (clone RMA4.5), anti-CD8α (biotinylated 53–6.7), anti-Mac-1α FITC (M1/70) and anti-TCRβ APC (H57-597) (all PharMingen). Biotinylated antibodies were visualized using the secondary fluorochrome streptavidin Alexa 594 (Molecular Probes, Eugene, USA). Sections were also stained with isotype controls, secondary alone or unstained to serve as controls for non-specific staining. After the final wash, slides were mounted and examined under a confocal fluorescence microscope (Leica, Wetzlar, Germany).

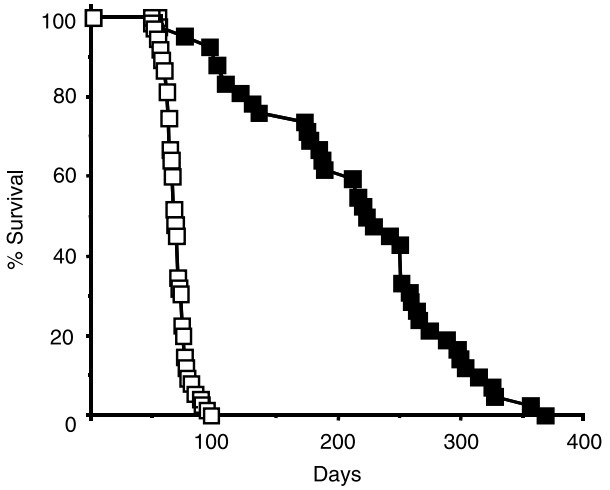

Figure 1 Kaplan-Meier survival plot of $TNF^{+/+}$ $pfp^{-/-}$ gld (□) and $TNF^{-/-}$ $pfp^{-/-}$ gld mice (■). Mean lifespan ± SD for $TNF^{+/+}$ $pfp^{-/-}$ gld mice was 69 ± 10 days (n = 75) and mean lifespan ± SD for $TNF^{-/-}$ $pfp^{-/-}$ gld mice was 217 ± 79 days (n = 42).

Flow cytometry

For multiparameter analysis of splenic lymphocytes, cells were stained with anti-B220-FITC (RA3–6B2) and anti-CD3-PE (1782) in the presence of Fc Block (clone 2.4G2) (all PharMingen), to prevent non-specific staining by Fc portions of the primary antibodies. Analysis was performed on a FACSCalibur (Becton Dickinson). Statistical significance was determined using the Student's *t*-test.

Results

TNF promotes infertility and reduced lifespan of $TNF^{+/+}$ $pfp^{-/-}$ gld mice

Tumour necrosis factor$^{-/-}$ $pfp^{-/-}$ gld mice were generated to characterize the role TNF plays in the immunopathology of disease in mice deficient for both pfp and FasL. Breeding was performed using females under the age of 4 months and the litter sizes were small; 3.7 ± 2.1 pups ($TNF^{-/-}$.$pfp^{-/-}$.gld) compared to 6.4 ± 2.7 pups with the intercross performed to yield $TNF^{+/+}$ $pfp^{-/-}$ gld progeny. As previously described[21] female $TNF^{+/+}$ $pfp^{-/-}$ gld mice themselves were completely infertile. Haematoxylin and eosin staining showed the uterus and ovary (data not shown) of young breeding $TNF^{-/-}$ $pfp^{-/-}$ gld females to be normal, with a lack of mononuclear cell infiltration and inflammation (see below). The $TNF^{+/+}$ $pfp^{-/-}$ gld disease is characterized by mice becoming ill, losing weight and dying prematurely. The mean life expectancy of $TNF^{+/+}$ $pfp^{-/-}$ gld mice in our conventional clean mouse facility was 69 ± 10 days (Fig. 1). A previous report by Spielman and colleagues indicated that a similar strain died at approximately 105 days, not surprisingly suggesting that variations in animal facility conditions (environmental) might influence

Table 1 Organ weights as a percentage of body weight

	*TNF$^{-/-}$pfp$^{-/-}$gld	*TNF$^{+/+}$pfp$^{-/-}$gld	*gld	*wt
Pancreas	0.56 ± 0.10%	0.51 ± 0.19%	0.51 ± 0.07%	0.51 ± 0.06%
Spleen	1.19 ± 0.46%	2.84 ± 0.98%	0.44 ± 0.05%	0.44 ± 0.06%
Axial lymph node	0.046 ± 0.016%	0.153 ± 0.11%	0.030 ± 0.016%	0.008 ± 0.003%

*n = 6

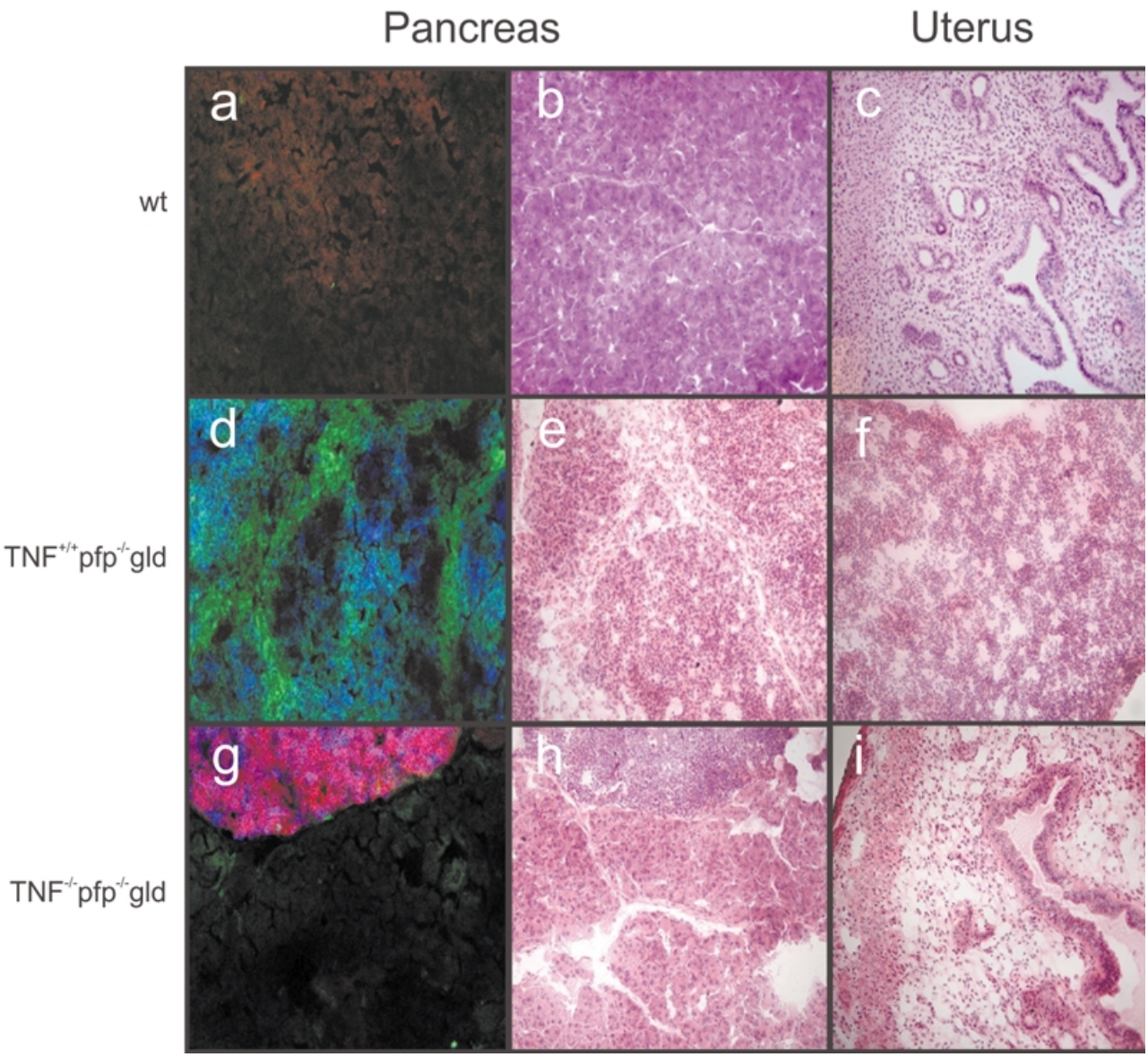

Figure 2 Comparison of the lymphocyte infiltrate into the pancreas and uterus of TNF$^{+/+}$ pfp$^{-/-}$ gld, TNF$^{-/-}$ pfp$^{-/-}$ gld and C57BL/6 WT mice. Representative haemotoxylin and eosin and immunohistological staining on pancreas from 8-week-old C57BL/6 WT (a,b), TNF$^{+/+}$ pfp$^{-/-}$ gld (d,e) and TNF$^{-/-}$ pfp$^{-/-}$ gld (g,h) mice or uterus from 8-week-old C57BL/6 WT (c), TNF$^{+/+}$ pfp$^{-/-}$ gld (f) and TNF$^{-/-}$pfp$^{-/-}$ gld (i) mice. B220 staining is red (Alexa 594), Mac1 staining green (FITC) and TCR αβ staining blue (APC). Original magnifications: × 20.

the inflammatory disease in this strain.[21] By contrast, our TNF$^{-/-}$ pfp$^{-/-}$ gld mice survived considerably longer (mean life expectancy = 217 ± 79 days) (Fig. 1). Tumour necrosis factor$^{-/-}$ pfp$^{-/-}$ gld mice also did not lose weight with age (mean weight at death was 25.9 ± 6.4 g) compared to TNF$^{+/+}$ pfp$^{-/-}$ gld mice (15.5 ± 2.8 g). At 10 weeks (approximate age of death of TNF$^{+/+}$ pfp$^{-/-}$ gld mice), TNF$^{-/-}$ pfp$^{-/-}$ gld mice weighed 24.2 ± 3.0 g.

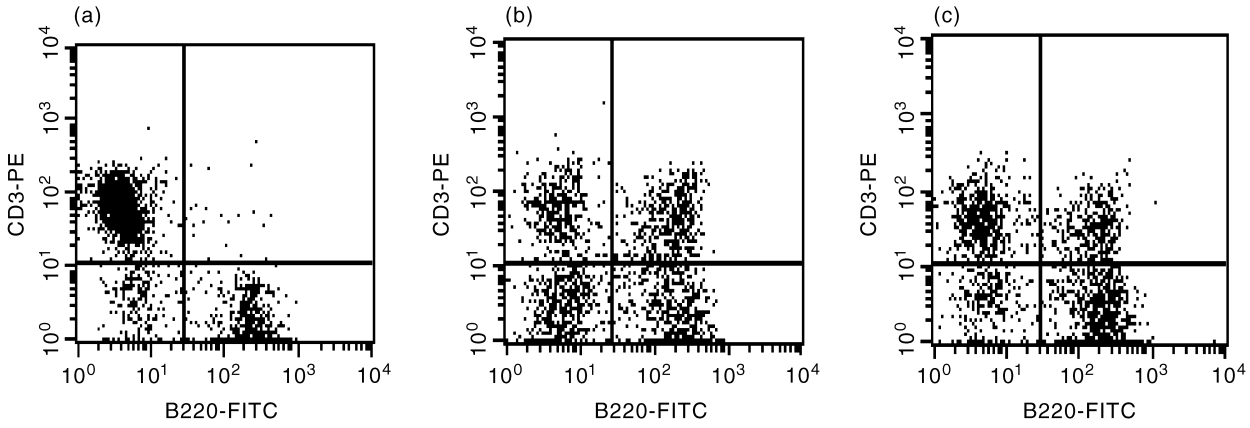

Figure 3 B220 *v*. cluster determinate 3 (CD3) expression on splenic lymphocytes. Cell suspensions from the spleens of (a) (1.0%), WT (b) (22.7%), $TNF^{+/+}$ $pfp^{-/-}$ gld and (c) (17.3%), $TNF^{-/-}$ $pfp^{-/-}$ gld mice were red blood cell depleted and labelled with mAb specific for B220 or CD3. Each dot plot shows B220 (FL1) *v*. CD3 (FL2) expression on lymphoid-gated spleen cells. The $TNF^{+/+}$ $pfp^{-/-}$ gld splenic lymphocytes contain 22.7% $B220^+CD3^+$ cells (b) and the $TNF^{-/-}$ $pfp^{-/-}$ gld splenic lymphocytes contain 17.3% $B220^+CD3^+$ cells (c). These data are representative of 7 ($TNF^{+/+}$ $pfp^{-/-}$ gld), 4 ($TNF^{-/-}$ $pfp^{-/-}$ gld) and 4 (C57BL/6 WT) mice examined. PE, phycoerythrin.

TNF promotes the onset of pancreatitis and hysterosalphingitis in the $TNF^{+/+}$ $pfp^{-/-}$ gld mouse

At 8 weeks, mean pancreas weights (relative to body weight) did not differ between $TNF^{+/+}$ $pfp^{-/-}$ gld and $TNF^{-/-}$ $pfp^{-/-}$ gld mice (Table 1), however, histolopathology indicated that a large proportion of the pancreatic tissue was infiltrated with lymphocytes or destroyed specifically in $TNF^{+/+}$ $pfp^{-/-}$ gld mice (mean pancreatic tissue area intact = $15.7 \pm 23.1\%$) (Fig. 2 b,e,h). A similar pattern of lymphocyte infiltration was observed in the uterus of 8-week-old female $TNF^{+/+}$ $pfp^{-/-}$ gld mice, compared with the relatively normal tissue in $TNF^{-/-}$ $pfp^{-/-}$ gld mice (Fig. 2 c,f,i). Immunohistology further confirmed the massive infiltrate of CD8+ T cells and Mac1+ macrophages as well as a low number of Mac1+ T cells in the pancreas of $TNF^{+/+}$ $pfp^{-/-}$ gld mice (Fig. 2d). Similarly, a large infiltrate of CD4+ T cells, CD8+ T cells and Mac1+ macrophages as well as fewer Mac1+ T cells and B220+ T cells in the uterus of $TNF^{+/+}$ $pfp^{-/-}$ gld mice by 8 weeks of age (data not shown). By contrast few, if any, lymphoid accumulations were detected in the pancreas or uterus of $TNF^{-/-}$ $pfp^{-/-}$ gld mice, although the pancreatic LN of these mice were clearly composed of β220+TCRb+ cells which are characteristic of the gld disease (Fig. 2g). It was noted that $TNF^{-/-}$ $pfp^{-/-}$ gld mice developed equivalent pancreatic infiltrates and tissue damage when moribund (> 30 weeks), suggesting that while the absence of TNF significantly delayed the onset of pancreatitis in the $TNF^{-/-}$ $pfp^{-/-}$ gld mice, pancreatitis eventually developed and was probably a major cause of morbidity in these mice.

TNF contributes to the lymphadenopathy and splenomegaly associated with $TNF^{+/+}pfp^{-/-}$ gld disease

Gld mice are characterized by an accumulation of $B220^+CD4^-CD8^-$ double negative (DN) T cells in the spleen and lymph node between 8 and 20 weeks of age[28] and develop hypergammaglobulinemia, strain-dependent systemic autoimmune disease and die prematurely. Recently, we have shown that TNF is essential for the lymphoaccumulation and mortality associated with gld.[28] In this study we compared the phenotype of lymphoid populations in $TNF^{+/+}$ $pfp^{-/-}$ gld and $TNF^{-/-}$ $pfp^{-/-}$ gld mice at 8 weeks. Between 15 and 30% of total spleen cells were observed to be $B220^+CD4^-CD8^-$ T cells, suggesting an acceleration of the DN T cell accumulation in the lymphoid organs of both $TNF^{+/+}$ $pfp^{-/-}$ gld and $TNF^{-/-}$ $pfp^{-/-}$ gld mice (Fig. 3). At this time point, as previously reported[21] lymphoaccumulation was reduced in gld mice (11%) and not evident in WT, $TNF^{-/-}$ and $pfp^{-/-}$ mice (< 2%) (data not shown). However, the spleen and axial LN weights were greater in the $TNF^{+/+}$ $pfp^{-/-}$ gld mice than the $TNF^{-/-}$ $pfp^{-/-}$ gld mice (Table 1), suggesting that even as early as 8 weeks TNF was important in the lymphoaccumulation associated with the $TNF^{+/+}$ $pfp^{-/-}$ gld disease. Histopathology of the lymphoid tissues from both strains demonstrated significant disruption of the normal architecture as early as 8 weeks (data not shown), supporting the aberrant lymphoaccumulation indicated by flow cytometry and organ weights. Serum IgG concentrations were also elevated above normal in $TNF^{-/-}$ $pfp^{-/-}$ gld and $TNF^{+/+}$ $pfp^{-/-}$ gld mice (data not shown) at 8 weeks, consistent with the hypergammaglobulinemia observed in gld mice.[31]

Discussion

Perforin, FasL and TNF are all important molecular effectors for the homeostatic control of the lymphoid system. In this study we have demonstrated that TNF is necessary for the full manifestation of immune dysregulation caused by pfp/FasL-deficiency, in particular in the early and overwhelming tissue infiltration and destruction caused by inflammatory cells. It had previously been demonstrated in $TNF^{+/+}$ $pfp^{-/-}$ gld mice that infiltrating T cells and monocytes/macrophages cause overt pathological changes in several organs.[21] Depletion of macophages in these mice using carrageenan reversed the tissue expansion of both monocytes/macophages and T cells

and restored fertility in female mice. Monocytes/macrophages may produce nitric oxide or TNF as effectors of tissue destruction; however, it would appear from our study that the major role of TNF is to enable the early infiltration of inflammatory T cells and macrophages into affected organs. It remains to be tested whether monocytes/macrophages are the source of TNF that drives the infiltration of pathogenic cells in the $TNF^{+/+}$ $pfp^{-/-}$ gld mice.

Acknowledgements

We thank Marie Snook and the staff of the ARI-BRL and PMCI animal facilities for their maintenance and care of the mice in this project. MJS is currently supported by a National Health and Medical Research Council of Australia (NHMRC) Principal Research Fellowship. This work was supported by a NHMRC Project Grant.

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