Supplemental figure legends

Supplemental Figure 1. Fas expression by BMDC. Fas was expressed by BMDC from a B6 mouse and further upregulated by exposure to LPS or a type I Interferon. BMDC from a FasKO B6 mouse were used in the same experiment as controls. Thin lines: no anti-Fas antibody added. Although FasKO BMDC staining with anti-Fas was slightly shifted compared to 'no antibody' controls, it was definitely very small compared to the shift in B6 BMDC and was insensitive to treatments.

Supplemental Figure 2. Targeting of the Fas gene.

A. Overall strategy. Mice with Exon IX of the *Fas* gene flanked by loxP sites (floxed) are termed FasKI (Fas knock-in) mice. The final targeting construct contained homology regions (light blue boxes, 9.3Kb NotI/BamHI fragment on the 5' end and 1.4Kb BamHI fragment on the 3'end), floxed Exon IX (dark blue box), floxed *neo* gene (green box), and the *thymidine kinase* gene (*TK*, gray box). About 200 clones were screened for proper recombination on both ends. Neo was removed by transient transfection of pIC-Cre plasmid with low Cre activity and the proper clones were identified by Southern blotting and PCR. Mice were then derived by blastocyst injection of targeted ES cells.

B. Southern hybridization analysis of ES cell DNA cut with Kpn I shows the presence of KI in ES clone D12. Hybridization with probe 1 (from intron VIII) shows the integrity of the insertion on the 5'end of FasKI. Hybridization with probe 2 highlights the 17Kb band reflecting the presence of the *neo* gene (intact 15 Kb genomic band from the untargeted allele and from feeder fibroblasts serves as an internal control). The 3.3 Kb band shows

addition of the *loxP* site on the 5' end of Exon IX. Probe 2 hybridizes with two bands because KpnI has a site corresponding to the middle of the probe. The 3.2Kb band is a genomic band from the untargeted allele and feeder cell DNA.

C. Removal of *neo* by transiently transfected Cre recombinase. Hybridization with KpnI-digested ES cell DNA shows deletion of *neo* in clones 1 and 2 (no 17kb band,*), but the 3.3kb band is present. The presence of KI minus *neo* was verified by PCR with two pairs of primers. Samples of "good" clones (on the left) containing FasKI (**ki**), and "bad" clones that have deleted FasKI and, thus, amplify the endogenous sequence only (**e**), along with DNA from the intact ES cell line (129) are shown. Bottom panel shows a typical genotyping PCR reaction of a litter derived from crossing two heterozygous FasKI mice. The top band (400bp) is a knock-in band; the bottom band (320bp) is a wild type (wt) band. ki- homozygous FasKI; wt- wild-type; h-heterozygous mouse. The litter shows Mendelian distribution of genotypes. Molecular markers: 100bp ladder, top band=500bp, bottom band=200bp. The absence of the *wt* allele in some animals proves that the gene was targeted correctly.

Supplemental Figure 3. Expression of CD11c-Cre-IRES-GFP transgene in NK cells. Because CD11c can be found on NK cells, we investigated GFP expression by cells carrying NK cell markers in CD11c-Cre (4097) mice (left panels) and CD11c-Cre.FasKI mice (right panels). Shown is the % of $CD11c^+$,GFP^+ splenocytes among cells gated as $DX5^+$ (top) or double-positive for DX5 and NK1.1 (bottom). No significant differences in % (and absolute numbers, not shown) were observed between the two strains. The total proportion of $CD11c^+$,$DX5^+$ cells in collagenase-treated spleens was 0.2% and

0.15% for CD11c-Cre and CD11c-Cre.FasKI, respectively.

Supplemental Figure 4. An explanation of how the ANA scoring system works. ANA score was determined individually for each experiment with the same negative control sera from 8wk old B6 mice and positive control sera (*lpr/lpr* sera that gave a saturating staining) to minimize variation between experiments. The score depends on the *brightness* of staining. All nuclei test cells were stained, but with different intensity. **A.** Shown are results of titration of two ANA-positive sera. A drop in brightness from 10 to 2 (5 times) corresponds to dilution by a factor of about 30 (on the top of the initial sera dilution of 1:100). Multiple digital images were ranked from dimmest to brightest and given a numeric brightness score (shown in the top left corners of images). Automated measurements are difficult due to the presence in some sera of antibodies to other cellular organels in addition to nuclear staining (**B**), or due to brighter staining of intranuclear compartments, such as nucleoli (**C**).

Supplemental Figure 5. Production of anti-nuclear antibodies in mice with deletion of Fas in DC, B cells and T cells.

ANA scores were determined in sera of female (red bars) and male (blue bars) mice of indicated ages. Black bars are respective Cre-negative FasKI mice. n, number of Cre^-FasKI/Cre^+FasKi mice in a group. Mean ANA scores are shown as a ratio of Cre^- to Cre^+ mice.

Analysis of ANA production in mice lacking Fas in DC, B cells and T cells revealed that: 1) mice with Fas deletion in APC showed higher titers of ANA compared

to strains with Fas deletion in T cells [*p* values comparing ANA scores in older females of 4097 CD11c-Cre.FasKI, 4272 CD11c-Cre.FasKI, CD19-Cre.FasKI and Lck-Cre.FasKI were respectively 0.01, 0.01 and 0.08 (due to larger individual variation in CD19-Cre.FasKI mice)]; 2) there was no statistical difference between end-point (old) males and females in all types of Cre transgenic FasKI mice; and 3) there was a gender difference in young CD19-Cre.FasKI mice ($p=0.02$) with females producing more ANA.

While the output of autoreactive T cells is likely to be similar in all mice, the extended presentation of multiple autoantigens by persistent Fas-negative APC may give their T cells additional advantage over T cells with deletion of Fas, explaining the higher ANA concentrations in mice with Fas-negative APC.

It is possible (although more discrete kinetic studies are needed to confirm the point) that differences in autoimmune manifestations previously reported for $B6^{lpr/lpr}$ mice (Theofilopoulos and Dixon, 1985; Warren et al., 1984a; Warren et al., 1984b) are based on the properties of Fas-negative B cells.

Supplemental Figure 6. Hyperimmunoglobulinemia in mice lacking Fas in APC.

A. IgM and IgG hyperglobulinemia in 4097 CD11c-Cre.FasKI mice. Ig levels were determined by ELISA in female and male (5-6 animals per group) 20+ wk old mice. The differences between genders were not significant.

B. IgM and IgG detection by ELISA in sera of CD19.Cre-FasKI mice (blue lines and bars) or their Cre-negative littermates (red lines and bars). Left panels show the difference in relative concentration of Ig (OD in ELISA assay, lines represent individual female mice >25 wks of age); right panels show the difference in absolute concentrations

(mg/ml±SE) of IgM or IgG (3-6 animals per group).

Supplemental Figure 7. Design of the experiment shown in Figure 4 B and C. F1(Ly5.1xLy5.2) B6 hosts received $5x10^5$ $CD8^+$ OT-1 wild-type cells and an equal number of $CD8^+$ OT-1 GZB-Cre.FasKI cells. One day after transfer, recipients were immunized with 2000 cfu of Lm-OVA. Splenocytes from immunized mice were analyzed on d7 and d14 by flow cytometry for both donor populations distinguished by staining for $Ly5.1^+$ (OT-1) or $Ly5.2^+$ (OT-1 GZB-Cre.FasKI) or double $Ly5.1^+Ly5.2^+$ (host) T cells.

Supplemental Figure 8. Additional analysis of mice lacking Fas in T cells. In our experiments, Fas deficiency in T cells did not lead to changes in animal viability or damage of internal organs as previously reported (Hao et al., 2004).

A. In female Lck-Cre.FasKI mice some loss of weight was observed, which became statistically significant only at one year of age. However, the same weight was found in age matched $B6^{lpr/lpr}$ females, indicating that the cause of the weight loss is not the attack on internal organs by T cells with up-regulated FasL [as previously suggested (Hao et al., 2004)] because *lpr/lpr* mice do not have a receptor for FasL. There was no significant loss of spleen mass or splenomegaly in Lck-Cre.FasKI mice, while $B6^{lpr/lpr}$ mice developed splenomegaly with the expected time course.

B. Purified T cells from Lck-Cre.FasKI mice (Fas-negative) were compared for FasL expression with purified T cells from Cre-negative littermates (Fas-positive). There was a very slight shift in FasL expression (red line in top panel), while FasKO T cells showed a shift in FasL expression (red line in bottom panel) restricted to a fraction of T cells

compared to T cells from a B6 mouse (black line).

C. Lungs were carefully analyzed for the presence of fibrosis in groups of ten Lck-Cre.FasKI and ten Cre-negative littermates along with a smaller group of age-matched $B6^{lpr/lpr}$ mice. No signs of lung fibrosis were found in any of the animals when lungs were properly inflated with the fixative and Masson Trichrome staining protocol was used (L.G.Luna. Manual of histologic staining methods for the arms forces institute of pathology. 3rd edition. The Blakiston Division, McGraw-Hill Book Co., New York,1968). Blue staining of collagen fibrils was observed only around blood vessels and bronchi, but not within the alveolar tissue. Representative images from each group of mice at two magnifications (10x and 20x objectives, respectively) are shown.

D. We have also investigated CD4-Cre.FasKI animals that we have backcrossed to the NOD mouse strain. In concordance with the findings of the Rajewsky group for B6.CD4-Cre.FasKI mice, we have also found a body weight loss in NOD.CD4-Cre.FasKI mice (although not dramatic and not accompanied by a loss of viability) statistically significant in 30-35 wk old mice; many mice (but not all, $p=0.07$) have also shown loss of spleen mass. Spleen weight was recorded for some of these animals, while some had the splenic cellularity estimated. They were combined in one graph using a % of the mean value of control (weight or cellularity). Results were: 1.0±0.1 for control and 0.67±0.1 for Cre^+ FasKI NOD mice. No lung pathology was observed in these mice either (not shown). Importantly, none of the Cre^+ mice became diabetic, while control mice showed an incidence close to 80%. Thus, in these animals, only some features of the previously reported phenotype (Hao et al., 2004) were found: some loss of weight and signs of lymphopenia.

The reasons for the discrepancies found in these two systems are not yet clear. One possibility is that Fas deletion happens at different developmental stages in CD4-Cre and Lck-Cre FasKI mice, leading to different consequences in terms of up-regulation of FasL expression. One cannot ignore a possible interference with microflora that must be different in our animal facilities. Pathogens can change the environment leading to activation of T cells and exaggerated expression of FasL. That may explain why we observed no lung pathology. Additionally, another (and attractive) point to make is that Fas actually provides T cells with a survival signal. (The anti-apoptotic action of Fas is discussed in Park SM, Schickel R, Peter ME. Nonapoptotic functions of FADD-binding death receptors and their signaling molecules. Curr Opin Cell Biol. 2005.17:610). That would explain why NOD mice with Fas-negative T cells do not develop diabetes, and possibly why B6.CD4-Cre.FasKI mice had poor viability. In addition, T cells from Lck-Cre.FasKI mice and from *lpr/lpr* mice (Figure 6B) had a tendency for a lower response to OVA compared to control mice.

Thus, the role of Fas as a survival factor for T cells requires further investigation.