

HEMATOPOIESIS

Increased Fetal and Extramedullary Hematopoiesis in Fas-Deficient C57BL/6-*lpr/lpr* Mice

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In this study, we examined the consequences of Fas deficiency on hematopoiesis in C57BL/6-*lpr/lpr* mice. We found a striking extramedullary increase in hematopoietic progenitor cells, comprising erythroid and nonerythroid lineages alike. These modifications preceded the lymphadenopathy, because early progenitors (colony-forming units-spleen [CFU-S] day 8) were already augmented in day-18 fetal livers of the *lpr* phenotype. Three weeks after birth, CFU-S increased in peripheral blood and spleen and colony-forming cells (CFU-C) began to accumulate 1 to 3 weeks later. Extramedullary myelopoiesis augmented progressively in Fas-deficient mice, reaching a maximum within 6 months. By then, mature and immature myeloid cells had infiltrated the spleen, the liver,

MICE HOMOZYGOUS for the *lpr* gene develop an age-dependent lupus-like autoimmune disease and a severe lymphadenopathy characterized by the progressive accumulation of TCR α / β ⁺B220⁺CD4⁻CD8⁻ double-negative (DN) T cells.¹ They also suffer from anomalies of the B-cell compartment, concerning both early and late differentiation stages.²⁻⁴

It has been demonstrated that the *lpr* gene codes for a mutant Fas/CD95/Apo-1, resulting from a retrotransposon insertion that leads to abnormal transcription and greatly reduced expression of the Fas receptor.^{5,6} A second murine autoimmune disease, called *gld*, has been shown to be complementary to *lpr*, because it is caused by a point mutation of the Fas ligand (FasL), rendering the protein nonfunctional.^{7,8} Once Fas/FasL interactions had been identified as one of the mechanisms by which cytotoxic T lymphocytes kill infected target cells and delete activated T lymphocytes, these 2 murine mutations have been extremely useful in exploring the Fas pathway of cell death.

Fas/CD95/Apo-1 is a transmembrane protein expressed by a variety of tissues and several mature hematopoietic lineages, such as T cells,⁹ B cells, monocytes, and granulocytes.¹⁰⁻¹² Its regulatory functions during the immune response have been documented by a number of investigators who emphasized the importance of the activation state for the susceptibility of Fas⁺ cells to apoptosis.^{9,13,14}

The present study was based on the assumption that Fas/FasL interactions might also exert some control on hematopoietic progenitor cell development. There is indeed increasing evidence in support of the notion that normal hematopoietic growth involves not only cell division and differentiation, but also programmed cell death or apoptosis.¹⁵ It has been reported that the *in vitro* deprivation of growth factors such as erythropoietin (Epo) or interleukin-3 (IL-3) causes apoptosis of bone marrow cells and of growth factor-dependent cell lines.¹⁶⁻¹⁹ In addition, there is some recent evidence for functional Fas expression on primitive hematopoietic progenitors freshly isolated from human fetal liver²⁰ and on human CD34⁺ progenitors after exposure to interferon γ (IFN γ) and/or tumor necrosis factor α (TNF α).²¹ According to a recent report, Fas is also

and the peritoneal cavity. Similar changes occurred in C57BL/6-*gld/gld* mice, indicating that they resulted from Fas/FasL interactions. Medullary hematopoiesis was not significantly modified in adult mice of either strain. Yet, the incidence of CFU-S decreased after Fas cross-linking on normal bone marrow cells in the presence of interferon γ , consistent with a regulatory function of Fas/FasL interactions in early progenitor cell development. These data provide evidence that Fas deficiency can affect hematopoiesis both during adult and fetal life and that these modifications occur independently from other pathologies associated with the *lpr* phenotype.
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displayed on murine hematopoietic progenitor cells after cytomegalovirus (CMV) infection.²²

We have recently shown that IL-3-induced histamine and cytokine production by myeloid precursors from murine spleen is greatly decreased after Fas cross-linking in the presence of IFN γ .²³ These data prompted us to evaluate the consequences of nonfunctional Fas/FasL interactions on other hematopoietic progenitor populations in Fas-deficient *lpr* and FasL-deficient *gld* mice with a C57BL/6 genetic background.

We analyzed hematopoietic changes in fetal liver, bone marrow, and peripheral tissues in relation to age and progression of lymphadenopathy. Furthermore, we addressed the question whether Fas/FasL interactions were directly involved in the regulation of hematopoietic progenitor frequencies by investigating the effect of *in vitro* Fas cross-linking on these cells.

MATERIALS AND METHODS

Animals. Specific pathogen-free, male or female C57BL/6-+/+, C57BL/6-*lpr/lpr*, and C57BL/6-*gld/gld* mice were purchased from the Jackson Laboratories (Bar Harbor, ME) and bred at the CSEAL (Orléans, France). Implantation of C57BL/6-*lpr/lpr* stage 2-cell embryos into hyper-ovulated C57BL/6-+/+ females was also performed at the CSEAL. For the generation of F2 embryos F1 (C57BL/6-+/+ \times

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C57BL/6-*lpr/lpr* mice were bred and intercrossed in our own animal facility. The genotype of fetal tissues from F2 embryos was determined as previously described.²⁴

Cytokines and antibodies. Murine recombinant (mr) IFN γ (specific activity, 5×10^6 U/mg; as measured by an antiviral assay using L-929 cells infected with EMC virus) was purchased from R&D Systems (Abingdon, UK). The following antibodies were used: hamster anti-mouse CD95(Fas, Apo-1) monoclonal antibody (MoAb; clone Jo2; unlabeled or phycoerythrin [PE]-conjugated), control hamster IgG, CD117(c-kit) (2B8), CD34 (RAM34), and anti-IgM (R6-60.2) MoAbs (all from Pharmingen, San Diego, CA); TCR α/β (H57-597), Ly-6G(Gr-1) (RB6-8C5), CD11b (M1/70), CD45R(B220) (RA3-6B2), CD4 (H129.19), CD8 (53-6.7), CD19 (1D3), TER-119, and Sca-1 MoAbs were purified from hybridoma supernatants, biotinylated, or conjugated with fluorescein isothiocyanate (FITC) in our own laboratory.

Cell preparations. Bone marrow cells were removed from femurs and tibias by flushing with ice-cold Hanks' balanced salt solution (HBSS; GIBCO, Grand Island, NY). Axillary and mesenteric lymph node cell suspensions were prepared by disrupting the organs in a Potter-Elvehjem homogenizer. Spleen cells were suspended in HBSS by gently teasing with forceps. Peritoneal cells were recovered after repeated lavage of the peritoneal cavity with HBSS. The liver was pressed on a 200-gauge stainless steel mesh and suspended in HBSS. Hepatic mononuclear cells were prepared as previously described.²⁵ After washing, the cells were resuspended in 30% to 35% Percoll solution containing 100 U/mL heparin and centrifuged at 2,000 rpm for 15 minutes at room temperature. The pellet was resuspended in red blood cell (RBC) lysis solution and then washed twice with minimum essential medium (MEM), supplemented with 1% sodium pyruvate 100 \times , 1% L-glutamine, 100 IU/mL penicillin, and 100 μ g/mL streptomycin culture (GIBCO) referred to as culture medium. Peripheral blood cells obtained from cardiac puncture were used after RBC lysis. Fetal livers were removed at day 18 of gestation and cells were suspended in culture medium by gently teasing with forceps.

In vitro colony-forming assay. Total colony-forming units-cells (CFU-C) were quantified in Complete Methylcellulose Medium with Recombinant Cytokines and Erythropoietin (MethoCult M3434; Stem-Cell Technologies Inc, Vancouver, British Columbia, Canada) or in MethoCult M3230 supplemented routinely with optimal concentrations of murine recombinant IL-3 (1 ng/mL), stem cell factor (SCF; 100 ng/mL), IL-6 (100 U/mL), and Epo (2 U/mL). In some experiments, these growth factors were serially diluted to compare the sensitivity of normal and Fas-deficient spleen cell progenitors. To evaluate the effect of DN T cells on colony formation, 10^6 lymph node cells from C57BL/6-*lpr/lpr* mice suffering from severe lymphadenopathy (~50% DN lymphocytes) were cocultured with 2×10^5 normal spleen cells in the methylcellulose assay. Furthermore, 10^7 lymph node cells/mL from Fas-deficient mice were incubated for 24 hours in culture medium supplemented with 10% fetal calf serum (FCS). Supernatants were then collected and assayed at a 5-fold dilution with or without the standard combination of growth factors for their effect on colony formation from control spleen cells. Sera from control and Fas-deficient C57BL/6 mice were tested for their colony-stimulating activity at a 10-fold final dilution.

Total and sorted cell populations were plated in a final volume of 1 mL at concentrations ranging from 5 to 500×10^3 cells per culture dish (FALCON 1008). Colonies were scored on day 7 to 8. They were analyzed in some experiments for the presence of erythroid cells.

Colony-forming units-spleen (CFU-S) assay. The spleen colony assay of Till and McCulloch²⁶ was used to determine the number of CFU-S in total peripheral blood, spleen, and bone marrow cell suspensions. In brief, syngeneic C57BL/6 hosts were exposed to a lethal dose (9.5 Gy, 0.87 Gy/min) of whole body irradiation from a ¹³⁷Cs source. Amounts of 1 to 5×10^4 bone marrow cells, 1 to 5×10^5

splenocytes, and 0.1 to 1×10^6 peripheral blood cells in 200 μ L of MEM were then injected per mouse via the retro-orbital sinus. At least 5 mice were used in each experimental group. After 8 or 12 days, recipient spleens were excised and surface colonies were counted after fixation in Bouin's solution. No endogenous colonies were detected in these conditions.

Flow cytometry analysis and cell sorting. Cell suspensions were incubated on ice in the presence of rat antimouse CD16/CD32 MoAb (1 μ g/ 10^6 cells; Pharmingen) to block Fc receptor functions before specific staining. Subsequently, cells were washed, pelleted, and labeled with the appropriate antibodies, using 3-color immunofluorescence. Biotinylated antibodies were revealed with Streptavidin-TRI-COLOR (Caltag, Tebu, Le Perray-en-Yvelines, France). Cells were analyzed in a FACScan cytofluorometer (Becton Dickinson, Mountain View, CA), using the LYSYS II software. RBCs and debris were excluded on the basis of forward and side scatter parameters, and dead cells were gated out by propidium iodide staining. At least 15,000 cells were acquired within the live gate. Lin⁻ cells designate a progenitor-enriched population expressing neither myeloid nor lymphoid lineage markers (negative for CD11b, Gr-1, CD45R, TER 119, CD4, CD8, and TCR α/β).

Before fluorescence sorting, spleen cell suspensions from C57BL/6 and C57BL/6-*lpr/lpr* mice were partially depleted for B and T lymphocytes using FITC-conjugated anti-CD4, anti-CD8, and anti-CD45R(B220) MoAbs. After 30 minutes of staining on ice, splenocytes were washed twice and incubated for a further 30 minutes with sheep antirat IgG-coated magnetic beads (Dynabeads M-450; Dynal A.S., Oslo, Norway) according to the manufacturer's instructions. Labeled cells were then withdrawn against the inner wall of the test tube using a strong magnet, and unbound cells were collected. This prepurified population was resuspended in culture medium supplemented with 2% FCS and TCR α/β ⁻ IgM⁻ cells were sorted on a FACS Vantage cell sorter (Becton Dickinson) at a flow rate of 5,000 cells per second.

Fas cross-linking. Bone marrow and spleen cell suspensions were adjusted to a final concentration of 2.5×10^6 and 10×10^6 nucleated cells per milliliter, respectively, in culture medium with 10% horse serum (GIBCO). They were plated into Falcon 3047 multiwell plates (2 mL/well) and incubated for 24 hours in the presence of control hamster IgG (5 μ g/mL), IFN γ (100 U/mL), anti-CD95(Fas) MoAb (5 μ g/mL), or IFN γ + anti-CD95(Fas) MoAb in a humidified atmosphere of 95% air and 5% CO₂. Cells were then recovered and assayed for clonogenic progenitors.

Histomorphological examination. Spleen and bone marrow cell suspensions were cytocentrifuged on glass slides and imprints were made from liver slices. All preparations were stained with May-Grünwald-Giemsa.

Statistical analyses. The standard Student's *t*-test was used to establish statistical significance.

RESULTS

Hematopoietic changes in spleen and bone marrow from Fas-deficient C57BL/6-*lpr/lpr* mice. We set out to evaluate total colony-forming cells (CFU-C) in spleen and bone marrow cell suspensions from 6-month-old Fas-deficient mice. As shown in Table 1, total CFU-C per spleen were more than 30-fold increased at this age. The number of cells per spleen attained $384.4 \pm 75.8 \times 10^6$ versus $109.4 \pm 3.8 \times 10^6$ in control mice with the same genetic background (means \pm SEM from 3 separate experiments). In contrast, the bone marrow of Fas-deficient mice was affected neither in terms of progenitor frequencies nor of cellularity (cells per femur + tibia: $39.4 \pm 7.8 \times 10^6$ in *lpr* v $40.4 \pm 3.8 \times 10^6$ in normal mice; means \pm SEM from 3 experiments). The *gld* mutation caused a similar

Table 1. Increased Numbers of Colony-Forming Cells in Spleens From Fas- or FasL-Deficient Mice

	CFU-C (×100) Per Tissue				
	C57BL/6- <i>lpr/lpr</i>		C57BL/6		C57BL/6- <i>gld/gld</i>
Spleen	1,235.2 ± 189.6	<i>P</i> < .001	35.3 ± 6.9	<i>P</i> < .001	1,103.6 ± 156.1
Bone marrow (femur + tibia)	662.1 ± 109.3	NS	626.1 ± 53.5	NS	648.5 ± 81.0

Total CFU-C were quantified in spleen and bone marrow from 6-month-old mice using MethoCult M3434 medium. Colonies were scored on day 7 to 8. Data are means ± SEM from 3 individual 6-month-old *lpr*, *gld*, and C57BL/6 mice.

Abbreviation: NS, not significant.

increase in spleen progenitor cells, indicating that both Fas and FasL are involved in this effect (Table 1).

Knowing that DN T cells accumulate in spleens from adult C57BL/6-*lpr/lpr* mice, we verified whether this population could eventually enhance colony formation by providing additional hematopoietic growth factors. To this end, we performed the clonogenic assay with or without lymph node cells of the *lpr* phenotype (10^6 /mL) composed of approximately 50% DN T lymphocytes. We found no significant difference between the 2 conditions (Table 2). Also, 5-fold diluted supernatant from these cells (10^7 /mL), removed after 24 hours of incubation, did not support colony growth or change the number of CFU-C obtained in response to the currently used growth factors. Conversely, depletion of the DN T-cell compartment resulted in a similar enrichment of CFU-C, whether it was performed on C57BL/6 or C57BL/6-*lpr/lpr* spleens (Table 2).

In the same line of evidence, we could rule out a higher sensitivity to growth factors as an explanation for enhanced colony formation, because splenocytes from *lpr* and control mice displayed a similar dose-response curve to serial dilutions of the growth factor cocktail (data not shown). Furthermore, we detected no colony stimulating activity in sera of Fas-deficient mice as compared with controls (data not shown).

Once we had established that the number of clonogenic progenitors was increased in the spleen of Fas-deficient mice rather than their cloning efficiency, we examined whether a particular subset of CFU-C was affected in preference to another. Table 3 shows that this is not the case, because erythroid and nonerythroid colonies were similarly enhanced, suggesting the expansion of common precursor cell.

We tested this assumption by measuring the number of both CFU-C and CFU-S (day 8) in spleens from Fas-deficient and control mice at different ages. It is clear from Fig 1 that CFU-S

were markedly increased in spleens of the *lpr* phenotype. This modification appeared at the age of 3 to 4 weeks, before the augmentation of CFU-C that was not significant at that time point. It is noteworthy that, in spleens from 3-week-old C57BL/6-*lpr/lpr* mice, the percentage of DN T lymphocytes was not yet enhanced versus control cells ($1.6\% \pm 0.4\%$ v $2.3\% \pm 0.1\%$; means ± SEM from 3 separate analyses). The size of the colonies generated in irradiated recipients was similar, whether donor cells were from mutated or wild-type mice.

Extramedullary increase of myeloid cells in C57BL/6-lpr/lpr mice. The early increase in spleen progenitor cells was followed by a striking extramedullary expansion of myeloid cells, as shown in Fig 2. The cytospin preparations of spleen cell suspensions (Fig 2A and B) and liver imprints (Fig 2C and D) demonstrate the marked myeloid infiltration in these organs at the age of 6 months. Hematopoietic foci comprise several differentiation stages, from precursor cells to mature monocytes and segmented granulocytes. The myeloproliferative rather than lymphoproliferative aspect of this pathology is further emphasized by differential spleen cell counts represented in Table 4.

These morphological data were validated by the phenotypic analysis of C57BL/6-*lpr/lpr* splenocytes (Fig 3). In comparison with age-matched controls, we observed a clear-cut expansion of granulocytes and monocytes, as assessed by the expression of Gr-1 and CD11b. Erythroid cells, recognized by the TER-119 MoAb, were also increased. The accumulation of hematopoietic progenitors in the spleen was confirmed by the higher number of cells displaying c-kit or CD34 and of more immature progenitors identified by Sca-1 expression on cells lacking both lymphoid and myeloid lineage markers (lin^-). B cells, evaluated by CD19 expression, were not modified, in contrast with DN T cells, which had accumulated at this age.

Origin of extramedullary hematopoiesis in C57BL/6-lpr/lpr mice. As already stated above for CFU-C, the incidence of CFU-S did not significantly change in the bone marrow of C57BL/6-*lpr/lpr* mice, relative to age-matched controls. How-

Table 2. Effect of DN T Cells on Colony Formation

Treatment	CFU-C/ 10^6 Spleen Cells	
	C57BL/6	C57BL/6- <i>lpr/lpr</i>
Standard assay	35.3 ± 8.4	358.5 ± 91.3
+ <i>lpr/lpr</i> LN cells	33.2 ± 5.9	—
+ <i>lpr/lpr</i> LN supernatant	31.9 ± 3.3	—
T- and B-cell depletion	685.3 ± 85.1	7,939.5 ± 256.4

Colony-forming cells were assayed in MethoCult M3230 medium supplemented with the standard growth factor cocktail in the presence or absence of 10^6 lymph node cells from C57BL/6-*lpr/lpr* mice (50% DN T cells) or their 5-fold diluted supernatant. After magnetic depletion of B220⁺CD4⁺CD8⁺ cells, TCR α/β -IgM⁺ splenocytes were electronically sorted as described in Materials and Methods. Data are means ± SEM from triplicate dishes. The experiment was performed with 3-month-old mice.

Abbreviation: LN, lymph node.

Table 3. Evaluation of Erythroid and Nonerythroid Progenitors in Spleens From Normal and Fas-Deficient C57BL/6-lpr/lpr Mice

	CFU-GM	CFU-Mix Per Spleen (×100)	BFU-E
C57BL/6- <i>lpr/lpr</i>	1,017.1 ± 325.0	109.3 ± 54.1	108.6 ± 38.9
		<i>P</i> < .001	
C57BL/6	29.6 ± 5.9	2.8 ± 1.9	3.0 ± 1.5

Total CFU-C were quantified in spleens from 6-month-old mice using MethoCult M3434 medium. Colonies were scored on day 7 to 8 and analyzed for the presence of erythroid cells. Data represent means ± SEM from 3 separate experiments.

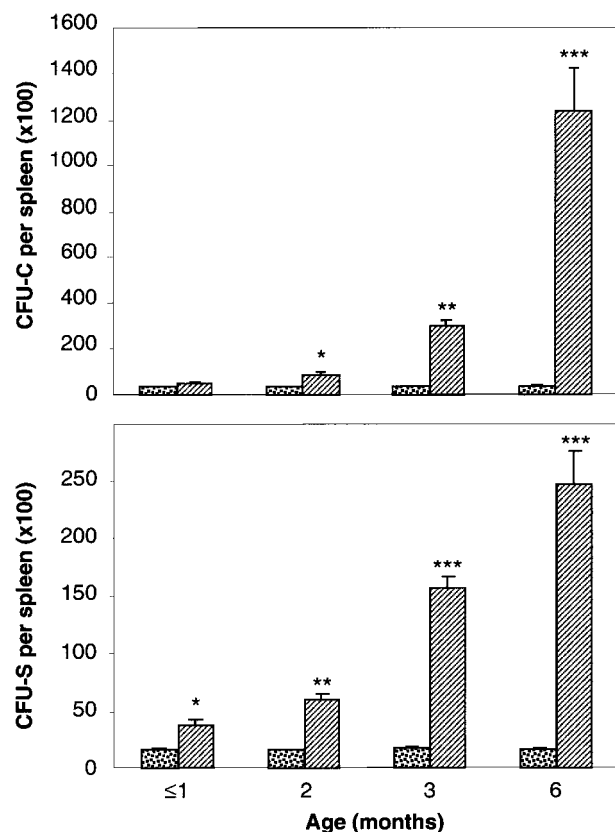


Fig 1. Age-dependent changes in the number of CFU-S and CFU-C in spleens from C57BL/6-*lpr/lpr* mice (▨), as compared with C57BL/6 controls (□). Data are means \pm SEM established from 3 individuals of the same age. CFU-S and CFU-C were determined as described in Materials and Methods.

ever, a slight transient increase in day-8 CFU-S occurred shortly after birth ($1,810.3 \pm 164.4$ CFU-S/femur + tibia in 2-week-old Fas-deficient mice ν $1,040.0 \pm 116.4$ in age-matched controls; means \pm SEM from 4 separate experiments; $P < .001$). More primitive day-12 CFU-S were also not significantly increased above normal in adult bone marrow of Fas-deficient mice, whereas they were in the spleen ($6,548.0 \pm 187.9$ CFU-S/spleen in 2-month-old C57BL/6-*lpr/lpr* mice ν $1,900.4 \pm 237.4$ CFU-S/spleen in age-matched controls; means \pm SEM from 3 separate experiments; $P < .01$). We postulated that hematopoietic changes might nevertheless occur in the bone marrow but remain inconspicuous because of a prompt emigration of these cells into the periphery. In accordance with this hypothesis, we found that CFU-S day 8 were increased in peripheral blood of 3-week-old C57BL/6-*lpr/lpr* mice, as compared with controls of the same age ($14.5 \pm 2.7 \nu$ 4.1 ± 0.8 CFU-S per 10^6 white blood cells [WBCs]; expressed as means \pm SEM from 3 separate experiments; $P < .05$).

The notion that extramedullary hematopoietic changes in Fas-deficient mice might originate from the bone marrow is also consistent with the striking accumulation of hematopoietic progenitors at several peripheral sites. Indeed, as shown in Table 5, the increase in CFU-C frequencies was also observed in the peritoneal cavity, the liver, and peripheral blood of 6-month-

old mice. Furthermore, it already took place in younger mice, concomitantly with changes in spleen (data not shown).

Hematopoietic changes during fetal hematopoiesis. The early hematopoietic modifications in Fas-deficient mice prompted us to examine the frequency of progenitor cells in fetal livers. As shown in Table 6, the incidence of CFU-S was already clearly above normal in this organ at day 18 of gestation, as compared with age-matched controls. To exclude a possible contribution of maternal growth factors to the enhanced fetal hematopoiesis, we implanted stage 2-cell embryos homozygous for the *lpr* gene into wild-type C57BL/6 females. Table 6 shows that the difference between Fas-deficient and normal fetal livers in terms of CFU-S frequencies persisted in these conditions. A similar increase occurred in homozygous *lpr/lpr* fetuses versus +/+ siblings of the same litter generated in heterozygous C57BL/6-*lpr*/+ females (Table 6). These observations indicate clearly that the maternal environment is not responsible for the increased CFU-S frequencies in Fas-deficient fetal liver. We did not observe any significant difference between fetal livers from age-matched Fas-deficient and wild-type mice in terms of cellularity (data not shown).

Effect of Fas cross-linking on hematopoietic progenitor frequencies. Previous reports on the expression of Fas on human hematopoietic progenitor cells^{20,21,27} prompted us to test the expression and functionality of the Fas antigen by cross-linking with anti-Fas MoAb. This 24-hour treatment was performed with or without IFN γ , which has been reported to increase the expression of Fas or facilitate its activation.^{20,21,27} As shown in Table 7, CFU-C frequencies were drastically diminished after treatment of total spleen cells with anti-Fas MoAb, provided that IFN γ was present during the incubation. In these conditions, Fas was effectively expressed on progenitor-enriched lin^- spleen cells (Fig 4B), as compared with freshly isolated cells, which exhibited only low background expression (Fig 4A).

Finally, we verified the effect of Fas cross-linking on medullary CFU-S and CFU-C. The results in Table 8 demonstrate that CFU-S frequencies were diminished by the ligation of Fas that required the presence of IFN γ to become effective. Interestingly, the same treatment affected bone marrow CFU-C frequencies less than their splenic counterpart (Table 7). Once again, Fas was clearly expressed on medullary lin^- cells in these conditions, whereas it was not detected before incubation (Fig 4C and D).

DISCUSSION

In the present study, we addressed the question whether, in addition to its acknowledged immunoregulatory functions, the Fas-mediated death pathway could influence early stages of hematopoietic development. For this purpose, we evaluated the effect of Fas or FasL deficiency in mice homozygous for the *lpr* or the *gld* gene, respectively, on the incidence of various progenitor subsets.

We found a marked increase in peripheral CFU-C in 6-month-old C57BL/6-*lpr/lpr* mice. At this age, lymphadenopathy and splenomegaly were well established and DNT lymphocytes had accumulated in spleen and lymph nodes. Taking into account the increase of mononuclear cells, the total number of colony-forming cells was around 30 times higher in mutant than in

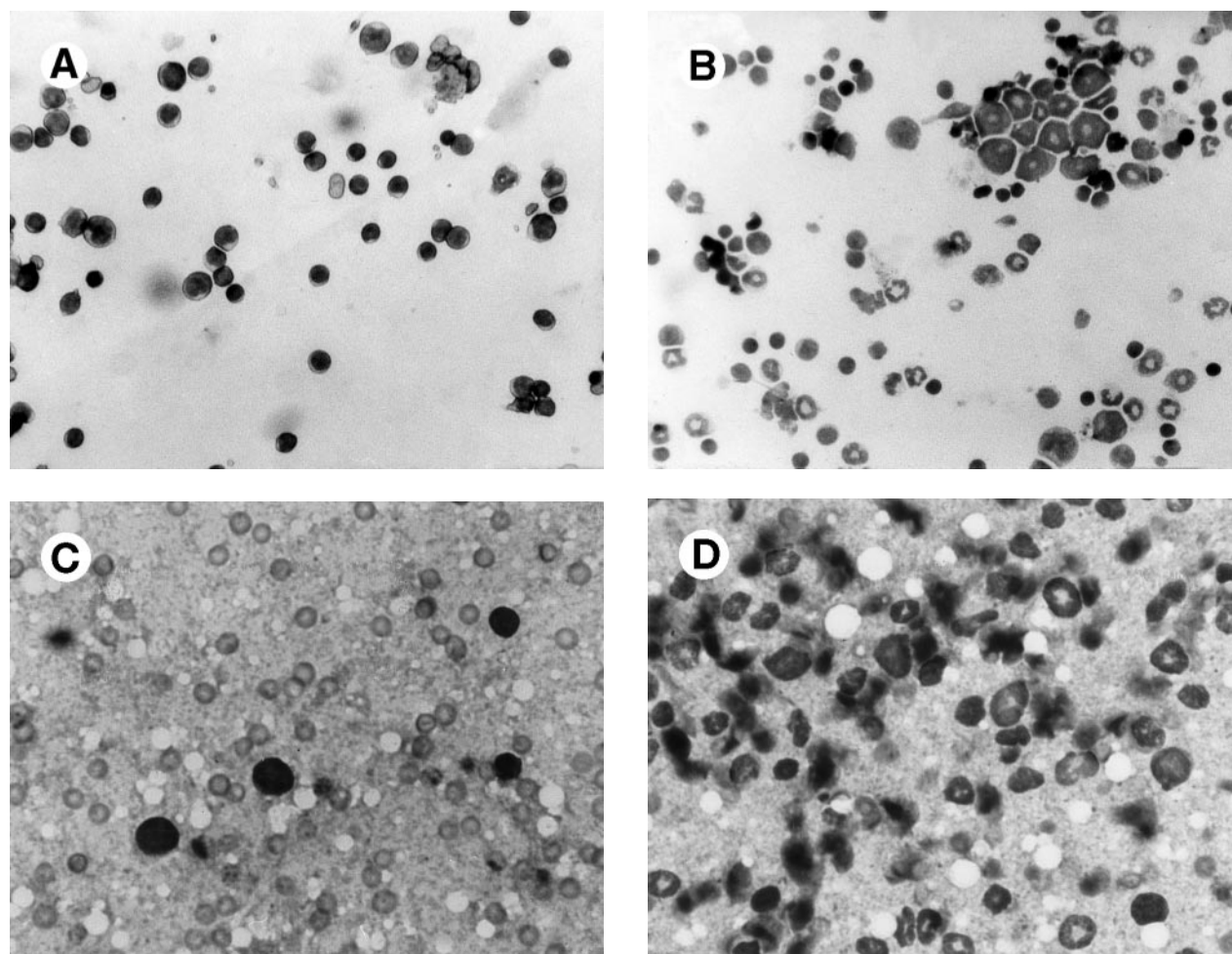


Fig 2. Histomorphological changes in spleen and liver from 6-month-old Fas-deficient mice. Cytospin preparations and liver imprints were stained with May-Grünwald-Giemsa. (A) Normal splenocytes; (B) splenocytes from Fas-deficient mice; (C) normal liver imprints; (D) liver imprints from Fas-deficient mice. Original magnification $\times 300$.

age-matched wild-type mice. A comparable increase in CFU-C took place in spleens from FasL-deficient C57BL/6 mice, proving the involvement of the Fas/FasL system in these hematopoietic changes.

As expected from the similar increase in all progenitor subsets growing in methylcellulose, the incidence of their common CFU-S precursors was also higher in Fas-deficient than in control mice. This augmentation appeared as early as 3 weeks after birth, when CFU-C were not yet modified.

Because the accumulation of DN T lymphocytes in lymph nodes and spleen is one of the most prominent features of Fas or FasL deficiency, we addressed the question of the role played by this population in the hematopoietic changes described here. DN T cells share several features of activated lymphocytes and could be a source of hematopoietic growth factors. Yet, previous reports have provided no evidence for their capacity to produce IL-3 or granulocyte-macrophage colony-stimulating factor (GM-CSF),²⁸ and in our hands, the transcripts for these cytokines

Table 4. Differential Cell Counts in C57BL/6-*lpr/lpr* Spleens Relative to Age-Matched Controls

	Lymphocytes (%)	Monocytes (%)	Granulocytes Bands/Segmented (%)	Erythroblasts (%)	Immature Myeloid (%)
C57BL/6- <i>lpr/lpr</i>	43.1 \pm 1.1 (137.9)	6.5 \pm 0.8 (20.8)	30.4 \pm 2.7 (97.3)	20.7 \pm 3.9 (66.2)	6.5 \pm 1.6 (20.8)
			$P < .001$		
C57BL/6	82.3 \pm 3.7 (90.5)	3.3 \pm 0.4 (3.6)	3.6 \pm 0.4 (4.0)	9.3 \pm 2.8 (10.2)	1.1 \pm 0.3 (1.2)

Spleen cell suspensions from 6-month-old C57BL/6-*lpr/lpr* mice and age-matched controls were cytocentrifuged and stained in May-Grünwald-Giemsa. Differential counts were performed on at least 200 cells per slide. Data are means \pm SEM from 4 different mice per group. Average cell numbers per spleen ($\times 10^6$) are indicated in parentheses.

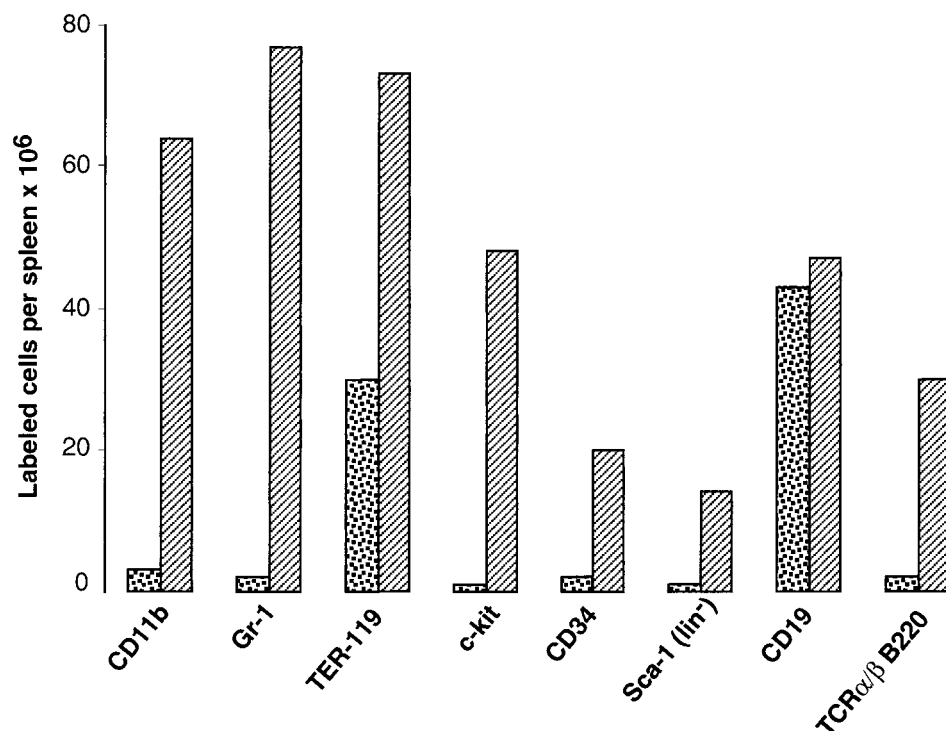


Fig 3. Phenotypic analysis of spleen cells from 6-month-old C57BL/6-*lpr/lpr* mice (▨), relative to age-matched controls (▤). Freshly isolated cells were stained with appropriate antibodies and analyzed in a FACScan cytofluorometer, using LYSYS II software. One representative experiment is shown, with 3 mice per group.

were not increased in Fas-deficient spleen cells relative to controls, as assessed by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis (G. Moreau, personal communication).

Several lines of evidence contributed to rule out the participation of DN T lymphocytes in the increased colony formation from spleen cells lacking a functional Fas receptor. First, the difference between C57BL/6 and C57BL/6-*lpr/lpr* mice in terms of colony formation remained the same after T- and B-cell depletion of the spleen, proving that these cells do not enhance to cloning efficiency by providing supplementary growth factors. Second, the addition of DN T cells from mice with severe lymphadenopathy (or their supernatants) during the clonogenic assay did not increase the number of colonies formed by normal spleen cells in response to optimal or suboptimal concentrations of growth factors and had no effect by itself. In this context it should also be noted that hematopoietic progenitors from Fas-deficient and control mice displayed the same sensitivity to growth factors.

Further evidence against the participation of DN T cells in the hematopoietic modifications resulting from Fas deficiency was

provided by the increased CFU-S in day-18 fetal livers of the *lpr* phenotype. We could exclude the maternal environment as a possible cause of this augmentation, which was observed in all Fas-deficient fetal livers, whether the fetuses were removed from females homozygous or heterozygous for the *lpr* gene or after development of homozygous *lpr/lpr* fetuses in C57BL/6 females.

Surprisingly, none of the modifications described so far took place in the bone marrow. It might be argued that medullary progenitors are protected from Fas/FasL interactions. Several mechanisms could account for such an effect, namely the expression of antiapoptotic molecules, such as Bcl-2,²⁹ or the cell cycle status of hematopoietic progenitors.³⁰ It is also possible that stromal cells prevent the activation of the Fas pathway by providing protective cytokines, such as IL-1,¹⁷ or by increasing cellular adhesion.³¹

Yet, in the light of our present data, we favor the hypothesis that bone marrow progenitors are actually increased in Fas- and FasL-deficient mice, but that they promptly emigrate into the periphery, because there are not enough hematopoietic niches to

Table 5. Increased Extramedullary CFU-C Frequencies in C57BL/6-*lpr/lpr* Mice

	CFU-C/10 ⁶ Cells			
	Spleen	Liver	WBC	Peritoneal Cells
C57BL/6- <i>lpr/lpr</i>	428.9 ± 98.3	583.2 ± 150.3	83.3 ± 7.2	92.2 ± 11.9
C57BL/6	36.9 ± 16.8	29.5 ± 9.3	14.7 ± 1.4	13.5 ± 3.3

P < .001

Cell populations were prepared from 6-month-old mice as described in Materials and Methods and assayed for CFU-C in complete MethoCult M3434 medium. Data are means ± SEM from 3 separate experiments.

Table 6. Increased CFU-S (Day 8) Frequencies in C57BL/6-*lpr/lpr* Fetal Livers (Day 18)

Genotype of Fetuses	CFU-S/10 ⁶ Cells
+/+ in +/+ mothers	90.8 ± 10.0 (6/3)
<i>lpr/lpr</i> in <i>lpr/lpr</i> mothers	302.0 ± 40.5 (6/3)*
<i>lpr/lpr</i> in +/+ mothers	247.7 ± 16.0 (8/2)*
+/+ in <i>lpr</i> + mothers	97.4 ± 10.0 (5/3)
<i>lpr/lpr</i> in <i>lpr</i> + mothers	258.8 ± 18.3 (5/3)*

Fetuses were generated as described in Materials and Methods. In parentheses is the number of fetal livers tested/number of litters. Data are means ± SEM from individual fetuses.

**P* < .001.

Table 7. Decreased CFU-C Frequencies After Fas Cross-Linking on IFN γ -Induced C57BL/6 Splenocytes

	CFU-C/10 ⁶ Cells
Control IgG	47.2 \pm 7.1
Anti-Fas MoAb	31.8 \pm 2.1
IFN γ	42.0 \pm 4.7
Anti-Fas MoAb + IFN γ	18.4 \pm 2.9*

Spleen cells (10⁷/mL) were distributed into 24-well culture plates and incubated for 24 hours in the presence of control IgG (5 μ g/mL), anti-Fas MoAb (5 μ g/mL), mrlFN γ (100 U/mL), or anti-Fas MoAb plus mrlFN γ . CFU-C were then recovered, enumerated, and assayed for colony growth in MethoCult M3434 medium. Data are means \pm SEM from 5 separate experiments.

* P < .01 between anti-Fas + IFN γ and control IgG.

maintain them. Indeed, we observed a transient increase in medullary CFU-S day 8 shortly after birth of Fas-deficient mice, in accordance with the recent report of Traver et al.,³² although it was less striking and less persistent. It is noteworthy that these investigators found no significant extramedullary modifications, possibly because the Fas mutation occurred on a different genetic background.

The appearance of hematopoietic progenitors at peripheral sites, such as liver, peritoneal cavity, and peripheral blood, that

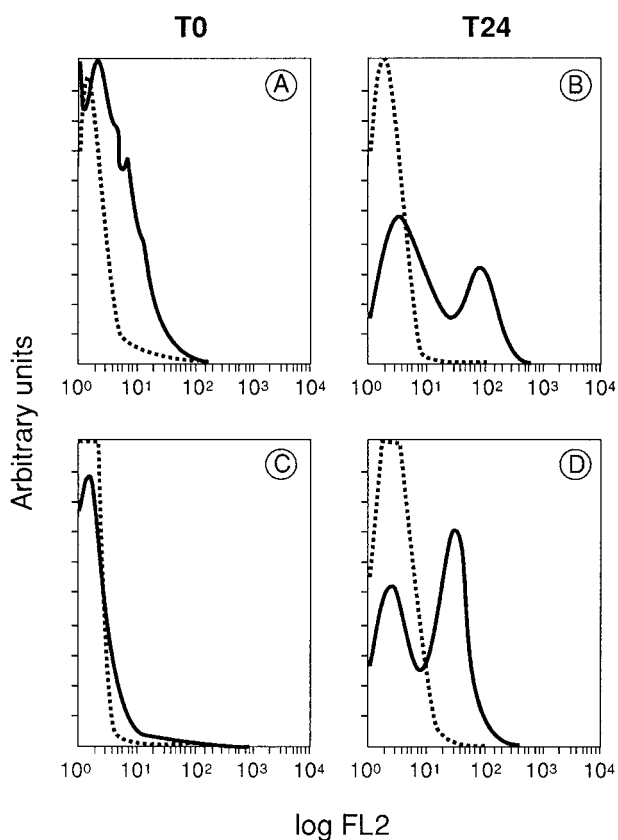


Fig 4. IFN γ -induced Fas expression on lin⁻ spleen and bone marrow cells. Flow cytometry analysis was performed on at least 20,000 events gated from the population without lineage markers. Splenocytes (A and B) and bone marrow cells (C and D) were stained directly or after 24 hours of incubation with 100 U/mL of mrlFN γ . The broken line represents control PE-conjugated hamster MoAb.

Table 8. Decreased CFU-C and CFU-S Frequencies After Fas Cross-Linking on IFN γ -Induced C57BL/6 Bone Marrow Cells

	CFU-S/10 ⁶ Cells	CFU-C/10 ⁶ Cells
Control IgG	135.4 \pm 14.5	1,742.3 \pm 176.9
Anti-Fas MoAb	153.0 \pm 21.0	1,500.0 \pm 220.7
IFN γ	126.0 \pm 14.7	1,461.0 \pm 205.3
Anti-Fas MoAb + IFN γ	39.0 \pm 11.9	934.8 \pm 164.2

Bone marrow cells (2.5 \times 10⁶/mL) were distributed into 24-well culture plates and incubated for 24 hours in the presence of control IgG (5 μ g/mL), anti-Fas MoAb (5 μ g/mL), mrlFN γ (100 U/mL), or anti-Fas MoAb plus mrlFN γ . CFU-S and CFU-C were then assayed as described in Materials and Methods. Data are means \pm SEM from 3 and 4 separate experiments for CFU-S and CFU-C, respectively. P < .01 between anti-Fas + IFN γ and control IgG for CFU-S and P < .05 for CFU-C.

are not normally involved in hematopoiesis could be interpreted as a consequence of their emigration from the bone marrow. The fact that CFU-S and, to a lesser degree, CFU-C were actually diminished after Fas cross-linking on bone marrow cells incubated in the presence of IFN γ provides another argument against an entirely protective influence of the medullary environment. It is therefore plausible that hematopoietic changes in peripheral organs originate from the bone marrow, although an independent expansion of progenitors from Fas-deficient spleens cannot be excluded. In this context, it should also be noted that increased endotoxin levels in *lpr* mice are not a likely cause for the shift towards extramedullary hematopoiesis, because colony-stimulating factors that would be induced in these conditions³³ could not be detected in sera.

The expression of Fas on hematopoietic progenitors and their decrease after Fas cross-linking suggest that the Fas pathway might have a direct regulatory effect on these cells. It has indeed been reported that human CD34⁺ cells express Fas after stimulation with IFN γ and/or TNF α or even spontaneously in the case of primitive fetal liver cells.^{20,21,26} This requirement for IFN γ was also demonstrated in our study, because cross-linking of Fas alone induced no significant decrease in CFU-C or CFU-S. It is in accordance with a recent report showing that, in addition to its upregulatory function on Fas expression,²¹ IFN γ is also capable of stimulating caspase activity.³⁴

So far, we can only speculate on the identity of the FasL⁺ cells in this particular context. Indeed, until recently, FasL expression seemed to be much more restricted to lymphoid cells than that of Fas antigen.³⁵ Natural killer (NK) cells, present both in bone marrow and spleen, express FasL spontaneously³⁶ and have been implicated in the regulation of CFU-S.³⁷ They disappear in Fas-deficient mice at about the same time, as the lymphadenopathy becomes significant.³⁸ There is also some recent evidence for FasL display on nonlymphoid cells, such as monocytes and neutrophils, which express also Fas, making them capable of regulating their own survival.^{39,40} Erythroid precursors have been reported to express FasL spontaneously,⁴¹ a feature that may also apply to other immature cells. In our hands, FasL expression could be detected in freshly isolated bone marrow cells by RT-PCR analysis (G. Moreau, personal communication).

Bone marrow stroma might contribute to Fas-mediated apoptosis by providing IFN α/β ⁴² that can replace IFN- γ during

cross-linking (data not shown). According to a recent report, Fas expression on hematopoietic progenitors can also be increased by hematopoietic growth factors,⁴³ which might eventually be secreted by medullary stroma.

In 6-month-old Fas-deficient mice, myeloid infiltration in the spleen, the liver, and the peritoneal cavity is particularly striking. The proportion of granulocytes is particularly elevated, as assessed both by morphological features and lineage markers. Because neutrophil cell death seems to be directly modulated by Fas/FasL interactions, their increased survival in the absence of functional Fas might explain this preferential expansion.³⁹

Whatever the exact mechanisms accounting for extramedullary myelopoiesis in C57BL/6-*lpr/lpr* mice, our data provide the first evidence that Fas deficiency affects the development of hematopoietic progenitors before the development of other abnormalities. Further exploration of this experimental model might eventually yield new insights into the regulation of these cells by the Fas pathway and their role in the development of the autoimmune disease.

REFERENCES

- Cohen PL, Eisenberg RA: *Lpr* and *gld*: Single gene models of systemic autoimmunity and lymphoproliferative disease. *Annu Rev Immunol* 9:243, 1991
- Dautigny N, Chabre H, Garcia C, Ezine S: Marked depletion at the late pro-B cell stage in the bone marrow of *lpr* mice correlated with the development of lymphadenopathy but not autoimmunity. *Eur J Immunol* 26:2087, 1996
- Reap EA, Piecyk ML, Oliver A, Sobel ES, Waldschmidt PL: Phenotypic abnormalities of splenic and bone marrow B cells in *lpr* and *gld* mice. *Clin Immunol Immunopathol* 78:21, 1996
- Sundblad A, Coutinho A: B lineage cell deficits in bone marrow of *lpr/lpr* mice. *Int Immunol* 8:247, 1996
- Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, Nagata S: Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature* 356:314, 1992
- Adachi MR, Watanabe-Fukunaga, Nagata S: Aberrant transcription caused by the insertion of an early transposable element in an intron of the Fas antigen gene of *lpr* mice. *Proc Nat Acad Sci USA* 90:1756, 1993
- Takahashi T, Tanaka M, Brannan CL, Jenkins NA, Copeland NG, Suda T, Nagata S: Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. *Cell* 76:969, 1994
- Lynch DH, Watson M, Alderson MR, Baum PR, Miller RE, Tough T, Gibson M, Davis-Smith T, Smith CA, Kunter K: The mouse Fas-ligand gene is mutated in *gld* mice and is part of the TNF family gene cluster. *Immunity* 1:131, 1994
- Lynch DH, Ramsdell F, Alderson MR: Fas and FasL in the homeostatic regulation of immune responses. *Immunol Today* 16:569, 1995
- Onel KB, Tucek-Szabo CL, Ashany D, Lacy E, Nikolic-Zugic J, Elkon KB: Expression and function of the murine CD95/Fas/APO-1 receptor in relation to B cell ontogeny. *Eur J Immunol* 25:2940, 1995
- Mandik L, Nguyen KA, Erikson J: Fas receptor expression on B-lineage cells. *Eur J Immunol* 25:3148, 1995
- Liles WC, Kiener PA, Ledbetter JA, Aruffo A, Klebanoff S: Differential expression of Fas (CD95) and Fas ligand on normal human phagocytes: Implications for the regulation of apoptosis in neutrophils. *J Exp Med* 184:429, 1996
- Krammer PH, Behrmann I, Daniel P, Dhein J, Debatin KM: Regulation of apoptosis in the immune system. *Curr Opin Immunol* 6:279, 1994
- Van Parijs L, Abbas AK: Role of Fas-mediated cell death in the regulation of immune responses. *Curr Opin Immunol* 8:355, 1996
- Niho Y, Asano Y: Fas/Fas ligand and hematopoietic progenitor cells. *Curr Opin Hematol* 5:163, 1998
- Koury MJ, Bondurant MC: Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells. *Science* 248:378, 1990
- Spivak JL, Pham T, Isaacs M, Hankins WD: Erythropoietin is both a mitogen and a survival factor. *Blood* 77:1228, 1991
- Rodriguez C, Lacasse C, Hoang T: Interleukin-1 β suppresses apoptosis in CD34 positive bone marrow cells through activation of the type I IL-1 receptor. *J Cell Physiol* 166:387, 1996
- Williams GT, Smith CA, Spooner E, Dexter TM, Taylor DR: Haemopoietic colony stimulating factors promote cell survival by suppressing apoptosis. *Nature* 343:76, 1990
- Barcena A, Park SW, Banapur B, Muench MO, Mechetner E: Expression of Fas/CD95 and Bcl-2 by primitive hematopoietic progenitors freshly isolated from human fetal liver. *Blood* 88:2013, 1996
- Maciejewski J, Selleri C, Anderson S, Young NS: Fas antigen expression on CD34+ human marrow cells is induced by interferon gamma and tumor necrosis factor alpha and potentiates cytokine-mediated hematopoietic suppression in vitro. *Blood* 85:3183, 1995
- Mori T, Ando K, Tanaka K, Ikeda Y, Koga Y: Fas-mediated apoptosis of the hematopoietic progenitor cells in mice infected with murine cytomegalovirus. *Blood* 89:3565, 1997
- Ben Amor A, Schneider E, Arnould A, Machavoine F, Dy M: Cross-linking of Fas mimics the inhibitory effect of anti-CD3 on IL-3 induced histamine and cytokine production by murine myeloid spleen cell precursors. *Exp Hematol* 26:903, 1998
- Singer GG, Abbas AK: The Fas antigen is involved in peripheral but not in thymic deletion of T lymphocytes. *Immunity* 1:365, 1994
- Watanabe H, Ohtsuka K, Kimura M, Ikarashi K, Ohmori K, Kusumi A, Ohteki T, Seki S, Abo T: Details of an isolation method for hepatic lymphocytes in mice. *J Immunol Methods* 146:145, 1992
- Till JE, McCulloch EA: A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res* 14:213, 1961
- Nagafuji K, Shibuya T, Harada M, Mizuno S, Takenaka K, Miyamoto T, Okamura T, Gondo H, Niho Y: Functional expression of Fas antigen (CD95) on hematopoietic progenitor cells. *Blood* 86:883, 1995
- Mori K, Kobayashi S, Inobe M, Jia WY, Tamakoshi M, Miyazaki T, Uede T: In vivo cytokine gene expression in various T cell subsets of the autoimmune MRL/Mp-*lpr/lpr* mouse. *Autoimmunity* 17:49, 1994
- Kroemer G: The proto-oncogene Bcl-2 and its role in regulating hematopoiesis. *Nat Med* 3:614, 1997
- Komada Y, Zhou YW, Zhang XL, Xue HL, Sakai H, Tanaka S, Sakatoku H, Sakurai M: Fas receptor (CD95)-mediated apoptosis is induced in leukemic cells entering G1B compartment of the cell cycle. *Blood* 86:3848, 1995
- Ginis I, Faller DV: Protection from apoptosis in human neutrophils is determined by the surface of adhesion. *Am J Physiol* 41:295, 1997
- Traver D, Akashi K, Weissman IL, Lagasse E: Mice defective in two apoptosis pathways in the myeloid lineage develop acute myeloblastic leukemia. *Immunity* 9:47, 1998
- Burgess AW, Metcalf S: Characterization of a serum factor stimulating the differentiation of myelomonocytic leukemic cells. *Int J Cancer* 26:647, 1980
- Dai C, Krantz SB: Interferon γ induces upregulation and activation of caspases 1, 3, and 8 to produce apoptosis in human erythroid progenitor cells. *Blood* 93:3309, 1999
- Suda T, Okazaki T, Naito Y, Yomoto T, Arai N, Ozaki S, Nakao K, Nagata S: Expression of the Fas ligand in cells of the T cell lineage. *J Immunol* 154:3806, 1995

36. Arase H, Arase N, Saito T: Fas-mediated cytotoxicity by freshly isolated natural killer cells. *J Exp Med* 181:1235, 1995
37. Takeda K, Dennert G: The development of autoimmunity in C57BL/6 lpr mice correlates with the disappearance of natural killer type 1-positive cells: Evidence for their suppressive action on bone marrow stem cell proliferation, B cell immunoglobulin secretion, and autoimmune symptoms. *J Exp Med* 177:155, 1993
38. Pan LZ, Dauphinee MJ, Ansar AS, Talal N: Altered natural killer and natural cytotoxic cellular activities in lpr mice. *Scand J Immunol* 23:415, 1986
39. Iwai K, Miyawaki T, Takizawa T, Konno A, Ohta K, Yachie A, Seki H, Taniguchi N: Differential expression of bcl-2 and susceptibility to anti-Fas-mediated cell death in peripheral blood lymphocytes, monocytes, and neutrophils. *Blood* 84:1201, 1994
40. Kiener PA, Davis PM, Rankin BM, Klebanoff SJ, Ledbetter JA, Starling GC, Liles WC: Human monocytic cells contain high levels of intracellular Fas ligand: Rapid release following cellular activation. *J Immunol* 159:1594, 1997
41. Dai CH, Price JO, Brunner T, Krantz SB: Fas ligand is present in human erythroid colony-forming cells and interacts with Fas induced by interferon gamma to produce erythroid cell apoptosis. *Blood* 91:1235, 1998
42. Wang J, Lin Q, Langston H, Cooper MD: Resident bone marrow macrophages produce type 1 interferons that can selectively inhibit interleukin-7 driven growth of B lineage cells. *Immunity* 3:475, 1995
43. Takenaka K, Nagafuji K, Harada M, Mizuno S, Miyamoto T, Makino S, Gondo H, Okamura T, Niho Y: In vitro expansion of hematopoietic progenitor cells induces functional expression of Fas antigen (CD95). *Blood* 88:2871, 1996