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Lymphoproliferative disorder and imbalanced T-helper response in C/EBPβ-deficient mice

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C/EBPβ is considered a key element of interleukin-6 (IL-6) signalling as well as an important transcriptional regulator of the IL-6 gene itself. We describe here how mice lacking C/EBPβ develop a pathology similar to mice overexpressing IL-6 and nearly identical to multicentric Castleman's disease in human patients, with marked splenomegaly, peripheral lymphadenopathy and enhanced haemopoiesis. Humoral, innate and cellular immunity are also profoundly distorted, as shown by the defective activation of splenic macrophages, the strong impairement of IL-12 production, the increased susceptibility to *Candida albicans* infection and the altered T-helper function. Our data show that C/EBPβ is crucial for the correct functional regulation and homeostatic control of haemopoietic and lymphoid compartments.

Key words: Castelman's disease/C/EBPβ/IL-6/gene targeting/T-helper

Introduction

Interleukin-6 (IL-6) is a pleiotropic cytokine whose production is rapidly and strongly induced by several inflammatory and infectious stimuli, and which has been implicated in a number of functions connected to immunity, haemopoiesis and inflammation, including induction of terminal differentiation of B cells to plasma cells and stimulation of immunoglobulin (Ig) secretion, stimulation of cytotoxic T-cell differentiation, induction of acute phase response genes in the liver and stimulation of the proliferation of early progenitor bone marrow cells (reviewed by Hirano *et al.*, 1990). IL-6 signalling has been shown to follow two distinct pathways, one involving tyrosine phosphorylation and activation of members of the Jak and STAT families of tyrosine kinases and transcription factors (reviewed by Ihle *et al.*, 1994), and the second leading to the activation of RAS/MAP kinases and the modification and activation of members of the C/EBP family of transcription factors (Nakajima *et al.*, 1993; Trautwein *et al.*, 1993).

Transcriptional activators and repressors of the C/EBP family constitute a large group of nuclear proteins that play important roles as regulators of cell proliferation and differentiation, and as mediators of intracellular signalling from extracellular stimuli. All C/EBP proteins [referred to here using the nomenclature introduced by Cao *et al.* (1991) of C/EBP followed by greek letters indicating the order of discovery] show a high degree of homology in the carboxy-terminal basic and leucine zipper domains, responsible for DNA binding and dimerization. Homology does not extend to the amino-terminal portion of the protein, which confers specific *trans*-activating properties. The number of C/EBP proteins is effectively higher than that of C/EBP encoding genes, because each polypeptide can form both homo- and heterodimers with the other family members.

C/EBPβ (IL-6DBP, NF-IL6) has been closely linked to IL-6 expression and signalling. Its *trans*-activating potential is enhanced by IL-6 in transfected hepatoma cells, where it acts as an inducer of acute phase response genes (Poli *et al.*, 1990), and its binding to the IL-6 gene promoter is thought to be required for transcriptional induction by IL-1/IL-6 (Akira *et al.*, 1990). In addition, C/EBPβ expression increases during differentiation of myeloid and plasma cells (Cooper *et al.*, 1992; Natsuka *et al.*, 1992; Scott *et al.*, 1992), suggesting its involvement in regulating the differentiation of myeloid and lymphoid lineages. C/EBPβ mRNA was shown to generate two proteins with opposite functions, a longer form acting as *trans*-activator and a shorter form named LIP (liver inhibitory protein) (Descombes and Schibler, 1991), which does not have a *trans*-activating domain and is believed to act as a repressor of all C/EBP factors activity.

Other members of the C/EBP family can also be modulated by IL-6: C/EBPα expression is negatively regulated in hepatoma cells (Isshiki *et al.*, 1991) and C/EBPδ mRNA is strongly induced in several cell types (Kinoshita *et al.*, 1992). The differential and modulated expression of the various C/EBP genes and the complexity of the C/EBP network does not allow the clear distinction between unique and redundant functions. In an effort towards the clarification of this issue, we generated mice in which the C/EBPβ gene was inactivated by gene targeting, and describe here the consequences of this mutation.

Results

Generation of C/EBPβ-deficient mice

To generate a null mutation in the C/EBPβ gene, a replacement vector was constructed in which the carboxy-

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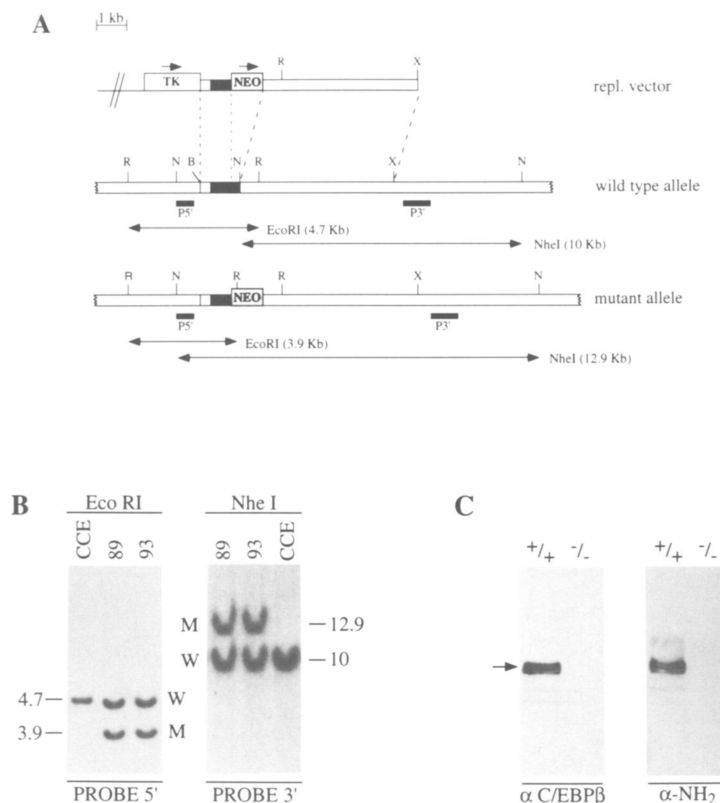

Fig. 1. Homologous recombination strategy and characterization of mutant ES cell lines and mice. (**A**) The replacement vector, the wild-type C/EBPβ allele and the predicted structure of the mutant allele. P5′ and P3′ are the probes used in the Southern blots. (**B**) Genomic DNA from CCE wild-type cells and the homologous recombinant clones was digested with the indicated enzymes and analysed by Southern blotting using the 5′ or 3′ probes indicated in (A). (**C**) Western blot analysis of liver nuclear extracts showing the absence of C/EBPβ in the −/− mice. Antibodies raised either against the full length (α-C/EBPβ) or the amino-terminal part (α-NH_2) of the rat C/EBPβ were used.

terminal part of the gene, coding for the leucine zipper and part of the basic domain, was substituted with an MC1−Neo poly(A)$^+$ cassette (Figure 1A): this mutation will inactivate both the full length protein and its amino-terminal truncated counterpart, LIP. This vector was used to mutate the endogenous C/EBPβ gene in embryonic stem (ES) cells by homologous recombination, and ES cell clones which had undergone the correct recombination event were identified by Southern blotting using two different probes located 5′ and 3′ of the recombination boundaries (Figure 1A and B). Two mutant clones were isolated and microinjected into recipient blastocysts, and clone No. 89 gave origin to chimeric mice able to transmit the mutated allele to the progeny. Heterozygous animals were intercrossed to obtain mice homozygous for the mutant C/EBPβ allele.

To demonstrate that the C/EBPβ mutation introduced into the mouse germ line is a null mutation, we analysed liver nuclear extracts from −/− and +/+ mice by Western blot (Figure 1C), the liver being one of the highest expressing tissues (Descombes *et al.*, 1990). With two kinds of antibodies, one raised against the full length protein and the other against its amino-terminal part, no signal was detectable in the extracts from the −/− mice, demonstrating that indeed no portion of the protein is expressed.

The genotypic analysis of a high number of pups (>400) generated from intercrosses between mice heterozygous for the mutation, showed that the percentage of −/− mice obtained was only 12% instead of the expected Mendelian ratio of 25%, meaning that a certain number of mice could not survive until weaning age. In addition, when the mice were raised in a non-specific pathogen-free facility (SPF), a further 50% mortality was detected in the first weeks after weaning (V.P., unpublished observation). Although the causes of death have not as yet been analysed, the finding of a Mendelian distribution of the different genotypes in embryos up to day 18 of gestation suggests that death is occurring during the first days after birth.

Lymphoproliferative and myeloproliferative alterations in C/EBPβ −/− mice

With age, the C/EBPβ −/− mice developed skin lesions, swellings in the mucosal regions, splenomegaly and lymphoadenopathy. These lesions were more dramatic, more frequent and appeared earlier in life in mice exposed to pathogens than in mice raised in SPF conditions. While morphological and histological analysis of young −/− mice (6–12 weeks) did not show abnormalities, post-mortem examination of mice starting from 16 weeks of age revealed splenomegaly and peripheral lymph node enlargement.

Submandibular, lacrimal, inguinal, peribronchial and mesenteric lymph nodes, draining areas highly exposed to antigen stimulation and/or inflammatory foci, were particularly affected. Lymphoid follicles and large germinal centres containing lymphocytes in varying stages

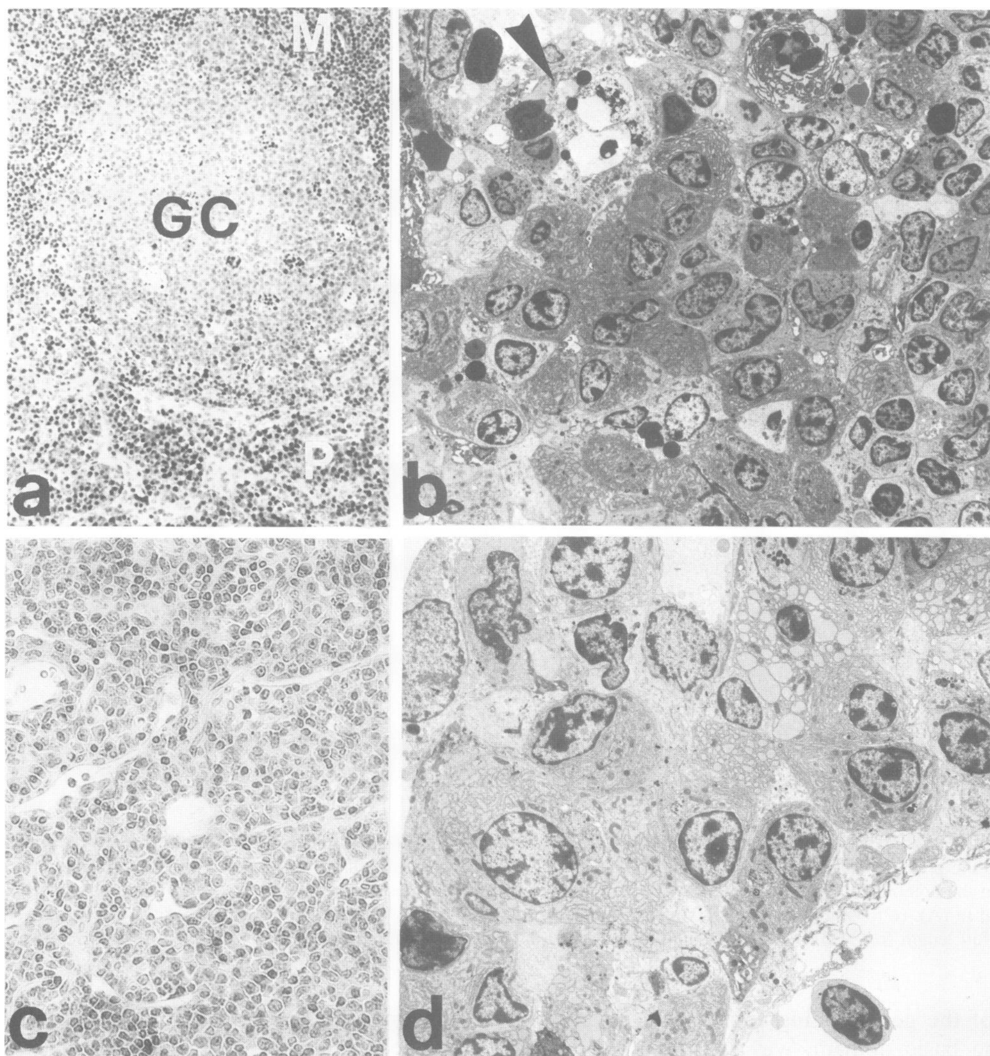

Fig. 2. Histological and ultrastructural features of hyperplastic lymph nodes from C/EBPβ −/− mice. (**a**) Hyperplastic lymph follicle with large germinal centre (GC). A prominent lymphoid cuff is evident in the perifollicular mantle zone (M). Sheets and aggregates of plasma cells are present in the paracortical region (P) (×200). (**b**) Electron micrograph showing aggregates of plasma cells in the paracortical area near the lymph follicle at the edges of which a tingible body macrophage is present (arrowhead) (×1200). (c) Medullary cords packed with plasma cells with pseudotumoral arrangement (×400). (**d**) The medullary cord near a medullary sinus is completely occupied by mature plasma cells (×1450).

of 'blast' transformation could be observed in the cortex, while numerous immunoblasts were present in the compressed paracortical area (Figure 2a and b). Aggregates and masses of mostly mature plasma cells occupied the markedly enlarged medullary portion of the node (Figure 2c and d). Infiltration of plasma cells was also found in the peribronchial region of the lung, in the portal areas of the liver and in the stroma of the kidney (not shown).

The spleens were moderately to markedly enlarged. Both white and red pulp were hyperplastic (Figure 3a and b), and large lymphoid follicles with wide germinal centres were observed in the white pulp, while the red pulp presented many aggregates of plasma cells intermingled with hyperplastic haemopoietic tissue. Megakaryocytes and mature granulocytes were quite numerous. The red pulp of markedly enlarged spleens was engulfed by erythroid cells, and the local excess of iron could be demonstrated by detection of haemosiderin deposits (not shown).

Foci of extramedullary haemopoiesis were also found in the liver (not shown) and lymph nodes (Figure 3c and d) in 6 out of 20 mice examined, whereas they were never observed in control mice. The finding of myeloid cells in the lymph nodes suggests extreme deregulation of haemopoietic proliferation. The bone marrow showed hyperplasia of the haemopoietic tissue with a predominance of myeloblasts, mature granulocytes and megakaryocytes (not shown), indicating that the extramedullary haemopoiesis described above is not due to an intrinsic defect in medullary proliferation and/or differentiation of haemopoietic progenitors.

Histological analysis of the kidney showed glomerular enlargement in 7 out of 20 mice examined, with expansion of the mesangial areas by an increase of mesangial cells and mesangial matrix (not shown).

High circulating IL-6 levels in the C/EBPβ-deficient mice

The pathological alterations found in the C/EBPβ −/− mice were strikingly similar to those observed in mice overexpressing IL-6 (Suematsu *et al.*, 1989; Fattori *et al.*, 1994). We decided therefore to analyse the IL-6 levels in

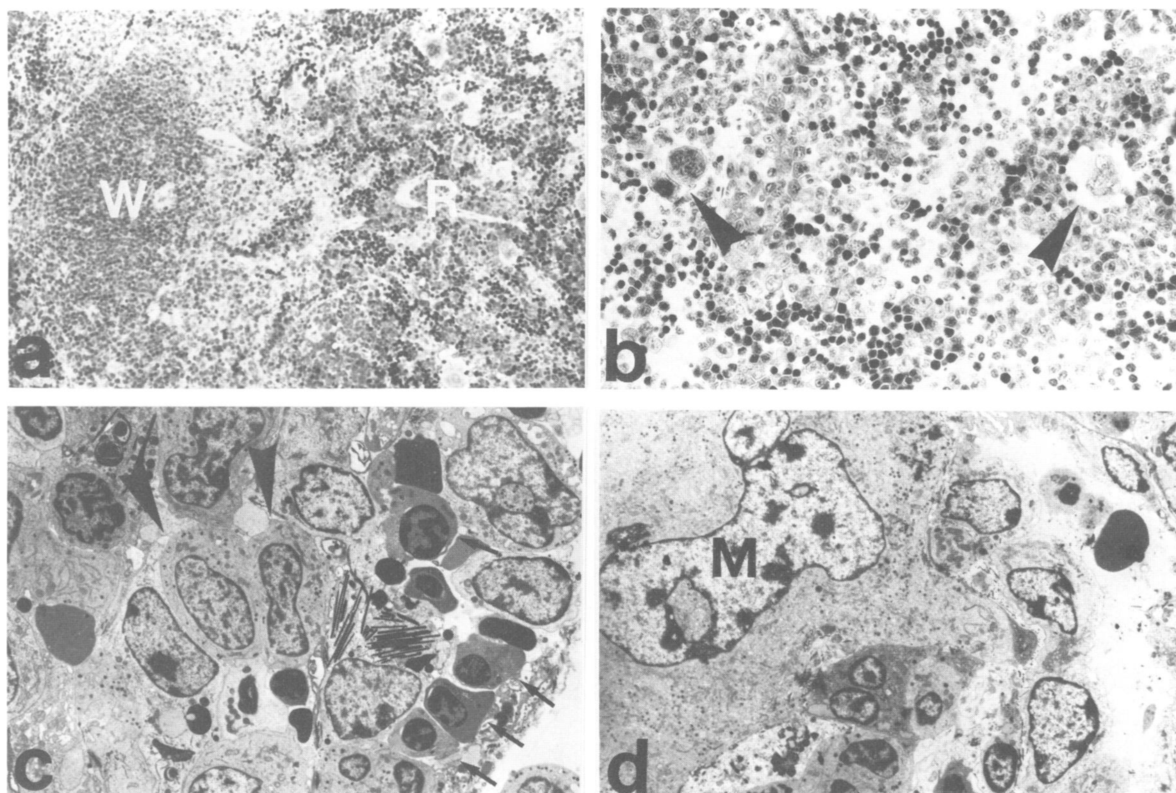

Fig. 3. Plasmacytosis and extra-medullary haemopoiesis in the spleen (a and b) and in the lymph nodes (c and d). (**a**) Many aggregates of plasma cells are intermingled with hyperplastic haemopoietic tissue in the red pulp (R) near to the white pulp (W) in which an evident marginal zone is present (×200). (**b**) Hyperplastic haemopoietic tissue with megakaryocytes (arrowheads) in the red pulp (×400). (**c** and **d**) Ultrastructural examination of the lymph node, showing that several erythroblasts (arrows), myelocytes at varying stages of maturation (arrowheads) (c), and (d) megakaryocytes (M) can be found in the paracortical and medullary regions. Macrophages containing crystalloid bodies originating from the digestion process are also present in (c) (×1900).

a representative sample of the population. Nineteen male −/− mice and 18 +/+ controls of different ages were bled and the serum analysed for IL-6 content by an enzyme-linked immunosorbent assay (ELISA). Almost all −/− mice were found to have significantly higher IL-6 levels than their +/+ controls (Figure 4), with the mean values increasing with age.

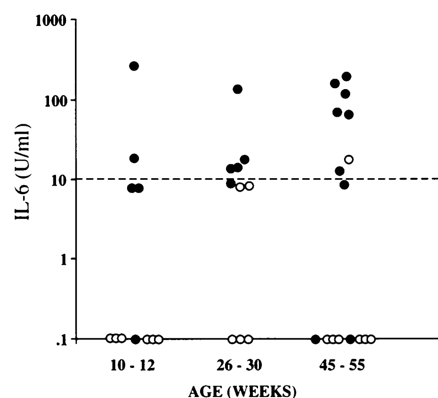

Fig. 4. IL-6 levels in the serum of C/EBPβ-deficient mice and wild-type controls. Mice at different ages were bled through the retro-orbital cavity and IL-6 was measured by ELISA. The difference between −/− and +/+ mice as unique groups was statistically significant ($P<0.015$). C/EBPβ −/− mice: black circles; wild-type mice: white circles.

Expansion of the B-cell compartment in the lymph nodes and spleens of −/− mice

Lymphocyte populations in the thymus, lymph nodes and spleen of −/− and +/+ animals were analysed at different ages. While the total thymic cell recovery and thymocyte subset distribution appeared unchanged in the −/− mice with respect to control animals (not shown), the flow cytometric analysis of lymphocyte subsets in spleen and lymph nodes revealed significant differences between mutant and control mice, mainly evident after 16 weeks of age.

Figure 5A–C show the phenotype of splenic lymphocytes. Total cell recovery was increased in older −/− mice (Figure 5A), in which also the distribution of B and T lymphocyte subsets was clearly altered, with a significant increase in B lymphocytes expressing surface IgGl immunoglobulins (sIgG1, Figure 5B), accompanied by a significant decrease in the percentage of $CD3^+$ T lymphocytes (Figure 5C). This decrease, which involves both $CD4^+$ and $CD8^+$ subsets (not shown), is only apparent, and is a consequence of the higher total cell number due to the increased B-cell population.

The phenotypic analysis of lymph node cells is shown in Figure 5D–F. Total cell recovery confirms the enormous enlargement of lymph nodes observed in C/EBPβ −/− mice compared with +/+ littermates (Figure 5D). Moreover, the same imbalance in the distribution of B and T lymphocytes described in the spleen is evident also

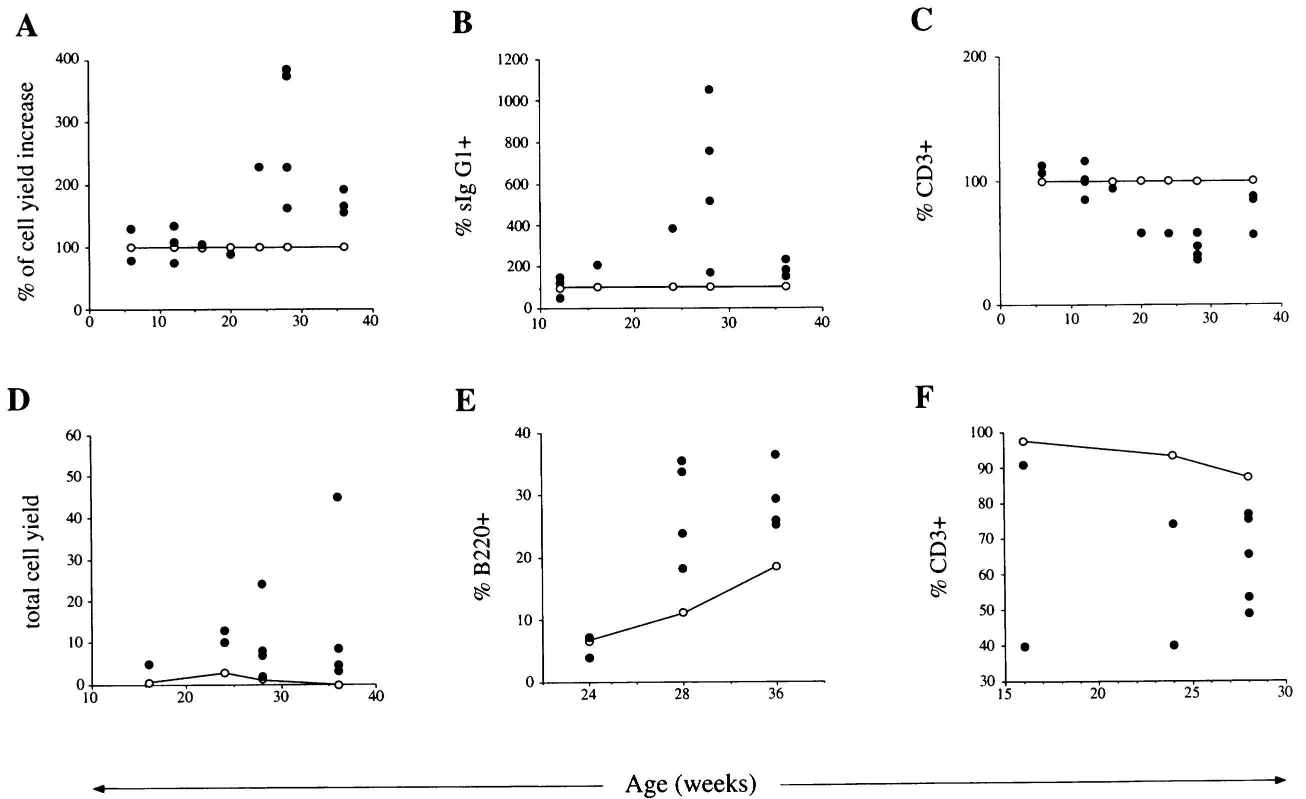

Fig. 5. Phenotypic analysis of splenic (A–C) and lymp node (D–F) cells. C/EBPβ −/− mice: black circles; wild-type mice: white circles. (**A**) Total splenic lymphocytes recovered at different ages. The results are expressed as percentage of increase of C/EBPβ −/− total splenic lymphocytes with respect to the total yield of control splenic lymphocytes for each specific age considered as 100%. (**B**) Percentage of $sIgG^+$ B lymphocytes recovered at each age. (**C**) Percentage of decrease of $CD3^+$ T lymphocytes in C/EBPβ −/− mice, with respect to the percentage of $CD3^+$ cells from control mice, considered as 100% for each respective age. (**D**) Total lymph node cells ($\times 10^6$) recovered at different ages. (**E** and **F**) Percentage of $B220^+$ and $CD3^+$ lymphocytes respectively, recovered from lymph nodes of +/+ and −/− mice at different ages.

in lymph nodes (Figure 5E and F), where it is again due to the net increase in B lymphocyte number rather than to a decrease in total T lymphocytes. The appearance of these phenotypic anomalies showed the same age dependency already observed in the spleen. However, while in the spleen we detected a specific increase in memory $sIgG1^+$ B lymphocytes rather than of surface IgM-bearing cells (not shown), this difference was not observed in the lymph nodes where, therefore, B220 expression is reported as a general marker of B cells.

Several two-colour cytofluorimetric analyses demonstrated, in both spleen and lymph nodes from C/EBPβ −/− mice, the increase of a large-sized cell population that does not express any specific B- or T-cell surface markers, which possibly represents the plasma cells described above. In line with these results, the densitometric profile of cellulose acetate electrophoresis of the serum showed an increased immunoglobulin fraction (mostly of the IgG1 isotype) without a monoclonal spike (not shown).

C/EBPβ −/− mice are more susceptible to systemic Candida albicans infection

Taken together, the alterations detected in the C/EBPβ −/− mice may indicate the existence of an altered immune response and, in particular, that the antibody response may be prevailing over the cell-mediated immunity. As antibody production and cell-mediated immunity are often reciprocal responses associated with the activation of different $CD4^+$ T-helper (Th) subsets (reviewed by Mosmann and Coffman, 1989), we analysed the Th response to infection. For this purpose, we used a murine model of systemic *C.albicans* infection, in which the development of protective immunity is known to be associated with the prevalence of Th1-mediated cellular responses, while a prevailing Th2 cell response is connected to high susceptibility to infection (Romani *et al.*, 1991, 1992, 1993).

Susceptibility to *C.albicans* infection was analysed in C/EBPβ −/− and wild-type mice intravenously injected with either the highly virulent CA-6 or the live vaccine PCA-2 strains of the yeast. Mice were monitored for mortality and survivors for resistance to a subsequent lethal challenge (Table I). Both types of mice were equally susceptible to a high inoculum (10^6 cells) of the CA-6 strain, there being no significant difference in the median survival times between the two groups. However, C/EBPβ −/− mice were found to be much more susceptible to a lower inoculum (10^5 cells), in that seven out of eight mice succumbed to infection in 14 days, while all wild-type mice survived. In contrast, both types of mice survived challenge with 10^6 cells of the low virulence PCA-2 strain. However, when susceptibility to a subsequent lethal CA-6 challenge was evaluated, it was found that wild-type mice survived the secondary infection, while C/EBPβ-deficient mice did not, indicating a defect in the development of protective immunity. Quantification of yeast cells recovered from infected mice revealed the presence of a high

Table I. Course of systemic *C.albicans* infection in C/EBPβ −/− and +/+ mice.

Groups	Infection with PCA-2			CA-6		
	INOC	MST	D/T	INOC	MST	D/T
C/EBPβ −/−	–	–	–	10^5	14	7/8
C/EBPβ +/+	–	–	–	10^5	>60	0/8
C/EBPβ −/−	–	–	–	10^6	7.5	6/6
C/EBPβ +/+	–	–	–	10^6	6.5	6/6
C/EBPβ −/−	10^6	>60	0/8	10^6	5.0	4/4
C/EBPβ +/+	10^6	>60	0/8	10^6	>60	0/4

Mice were injected intravenously with the indicated number of cells (INOC), either of the low virulence PCA-2 strain or of the highly virulent CA-6 strain. Half of the PCA-2-injected mice were infected after 2 weeks with the CA-6 strain. Median survival times (MST, days) and number of dead mice at 60 days over total number of animals tested (D/T) were recorded.

number of *C.albicans* cells in the kidneys of −/− mice succumbing to infection (data not shown).

Both T-helper and humoral anti-candidal immunities are altered in C/EBPβ −/− mice

We then looked for parameters of specific Th immunity in PCA-2-infected C/EBPβ-deficient and wild-type mice, as they were oppositely susceptibile to the subsequent lethal challenge.

Development of delayed-type hypersensitivity (DTH) reactivity, which is typically associated with a Th1-type response, was impaired in C/EBPβ −/− mice at 10 days after infection, at the same time as a strong DTH reaction was detected in +/+ littermates (Figure 6A). Likewise IL-12, a cytokine considered of primary importance for the generation of Th1 cells (Trinchieri, 1993), was strongly increased in the serum of wild-type mice but remained undetectable in the C/EBPβ −/− mice (Figure 6B). In addition, serum levels of *Candida*-specific IgG1 antibodies were elevated in PCA-2 infected C/EBPβ −/− mice and low in the C/EBPβ +/+ controls which, in contrast, showed increasing levels of antigen-specific IgG2a antibodies (Figure 6C). Finally, it was found that the Th2 cytokines IL-4 and IL-6 were increasingly produced by $CD4^+$ cells in susceptible C/EBPβ −/− mice after infection, while progressively disappearing in resistant wild-type animals, which, as expected, predominantly produced IFN-γ and IL-2 (Figure 6D). Taken together, these results indicate that C/EBPβ-deficient mice have an altered response to systemic *C.albicans* infection, with predominance of a non-protective Th2-type response.

Defective nitric oxide production by splenic macrophages from C/EBPβ −/− mice

It is known that cells of the innate immune system are capable of antifungal effector functions, thus shaping the subsequent adaptive specific immunity. We assessed parameters of innate immunity function by evaluating the ability of splenic macrophages from either uninfected or infected mice to secrete nitric oxide (measured as NO_2^-) after activation *in vitro* with IFN-γ and LPS and subsequent challenge with *C.albicans* (Table II). We found that macrophages from C/EBPβ −/− mice failed to release NO_2^- either before or after *C.albicans* infection, while those from the wild-type mice could produce high amounts of NO_2^-. Moreover, we observed that candidacidal activity of splenic macrophages from infected C/EBPβ −/− mice is also significantly reduced (not shown). These results, together with the impaired production of IL-12, indicate that macrophage function is severely compromised in the C/EBPβ-deficient mice, thus suggesting a possible correlation between this defect and the defective Th1 response described above.

Table II. Nitric oxide production of splenic macrophages from C/EBPβ −/− and +/+ mice infected with *C.albicans*

Days post-infection	NO_2^- (μM/10^7 cells)	
	C/EBPβ −/−	C/EBPβ +/+
0	<0.01[a]	4.0 ± 1.0
3	<0.01	61.0 ± 1.6
10	<0.01	23.4 ± 1.3

Mice were intravenously injected with 10^6 PCA-2 cells for 3 or 10 days. Splenic adherent macrophages were cultured overnight with IFN-γ (100 U/ml) and LPS (10 ng/ml) before infection with *C.albicans*. After 4 h incubation, nitrite contents were assayed.
[a] <0.01 μM/10^7 cells, below the detection limit of the assay. Nitrite contents of supernatants from macrophages cultivated without stimuli were below the detection limits for cells from the C/EBPβ −/− mice, and 10 ± 2.4 and 8.7 ± 1.3 for macrophages from the C/EBPβ +/+ mice, at 3 and 10 days post-infection, respectively.

Discussion

C/EBPβ deficiency causes a lymphoproliferative disorder nearly identical to multicentric Castleman's disease

The lymph node histological alterations detected in C/EBPβ −/− mice, showing diffuse plasmacytosis and prominent germinal centres with preservation of nodal architecture, was remarkably similar to that observed in patients affected by multicentric Castleman's disease (CD) (Frizzera *et al.*, 1985). CD systemic alterations such as increased serum IL-6 levels, polyclonal hypergammaglobulinaemia, transient neutrophilia, a high number of cells of the granulocytic series and glomerulonephritis were also found in the C/EBPβ-deficient mice. Interestingly, the pathogenesis of CD has been linked to deregulated IL-6 production: the germinal centres of hyperplastic lymph nodes from CD-affected patients were found to produce large quantities of IL-6 (Yoshizaki *et al.*, 1989), and alterations very similar to those found in CD patients were observed in mice overexpressing IL-6 in the myeloid compartment (Brandt *et al.*, 1990). Furthermore, anti-IL-6

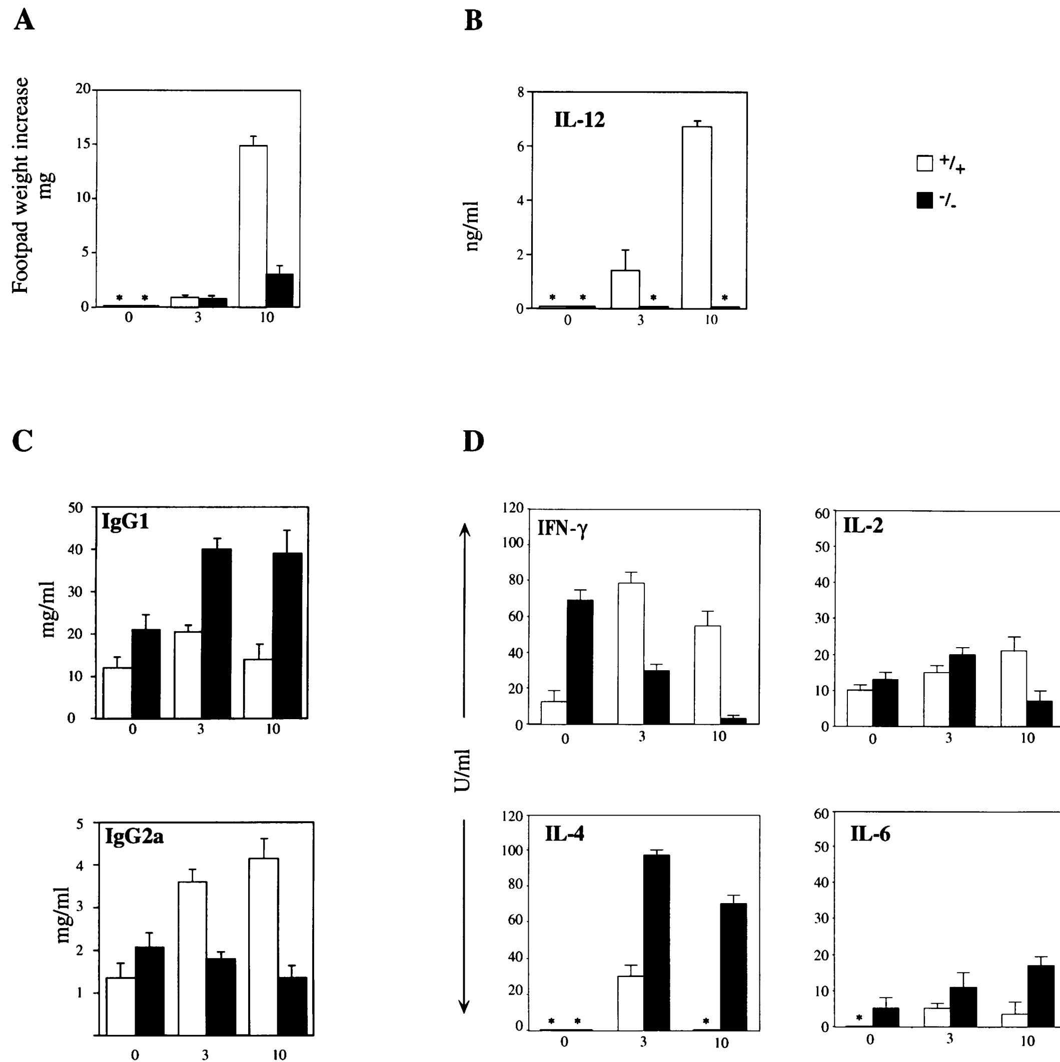

Fig. 6. Responses to systemic candidiasis in C/EBPβ −/− and +/+ mice at 0, 3 and 10 days after PCA-2 infection. (**A**) Development of delayed-type hypersensitivity (DTH) was measured as footpad weight increase (in mg). Values are means ± SEM of six animals per group. (**B**) IL-12 serum levels were measured by a bioassay as described in Materials and methods. (**C**) Anti-*Candida* specific antibody isotypes (IgG1 and IgG2a) from sera were measured by means of a micro-ELISA assay. (**D**) Pattern of cytokine production by $CD4^+$ splenocytes from PCA-2-infected C/EBPβ −/− and +/+ mice before infection (0) and after 3 and 10 days. Mixed $CD4^+$ lymphocyte−yeast cell cultures were established, and supernatants were assayed for IFN-γ, IL-2, IL-4 and IL-6 contents (mean values ± SEM) by ELISA assays.

neutralizing antibodies have been shown recently to exert a therapeutic effect on a CD-affected patient (Beck *et al.*, 1994). In the light of our observation that mice lacking C/EBPβ develop a CD-like disease and have high circulating levels of IL-6, it would be interesting to assess if the IL-6 deregulation observed in CD patients might be somehow linked to altered control of C/EBPβ expression or activity.

Is the C/EBPβ gene a positive or a negative modulator of gene expression?

The finding that C/EBPβ −/− mice can overexpress IL-6 seems to indicate that, in contrast with what was suggested based on tissue culture experiments (Akira *et al.*, 1990), this transcription factor is not essential for IL-6 gene transcription and that it might even act *in vivo* as a negative factor. On the other hand, we found that the induction of AP genes in the liver is impaired, although not dramatically, in the absence of C/EBPβ (V.P. and E.F., manuscript in preparation), suggesting that, in this context, this factor can act as a transcriptional activator. The dual role of C/EBPβ either as transcriptional activator or repressor may be understood in the context of the complex interplay between C/EBP proteins. Depending on the specific nuclear composition in C/EBP factors, products of the C/EBPβ gene might act as repressors, either as the result of a prevalent function of the truncated inhibitory counterpart LIP (Descombes and Schibler, 1991), and/or because the full size C/EBPβ (LAP) is a weak transcriptional activator when compared with other C/EBP proteins (Poli *et al.*, 1990). If the ratio of C/EBPβ isoforms over C/EBPα or δ is high, this might cause down-regulation of promoters able to bind the three proteins independently. Therefore, in the promoter context, the relative levels of C/EBP proteins (which can be differentially modulated according to the cell type and to the differentiative stage), and most probably also the interactions with transcription factors belonging to different families, will determine the activating or repressing role of C/EBPβ on gene transcription.

C/EBPβ deficiency causes altered CD4+ T-helper response and defective macrophage activation

The regulation of the immune response relies on complex interactions between cells of the immune system, and in particular antibody production and cell-mediated immunity are associated with the activation of different $CD4^+$ T-helper cell subsets (reviewed by Mosmann and Coffman, 1989). Moreover, cells mediating innate immunity are known to contribute to the shaping of the Th response, mainly through the production of IL-12, which in turn stimulates the production of IFN-γ and has recently been proposed to be an obligatory factor for Th1 cell generation and proliferation (reviewed by Trinchieri, 1993). Studies in mice have shown that resistance and susceptibility to systemic candidiasis are related to the dissimilar expansion of functionally distinct $CD4^+$ Th cell types during infection (Romani *et al.*, 1991, 1992, 1993). Moreover, Th cell function can be successfully manipulated in murine candidiasis by interfering with the action of selected cytokines, as was shown in the case of non-healer mice made resistant to the infection by treatment with IL-4 or IL-10 neutralizing antibodies (Romani *et al.*, 1992, 1994b), and in the case of healer mice in which development of protective immunity to infection was impaired by treatment with IL-12 neutralizing antibodies (Romani *et al.*, 1994a).

The finding that C/EBPβ-deficient mice possess all the characteristics of strains susceptible to systemic candidiasis, with a reduced Th1 response and a strong Th2 response, is in agreement with the reactive lymphoproliferative disorder displayed by these mice. Indeed, the expansion of the B cell compartment accompanied by high levels of IgG-bearing cells and spontaneously occurring in ageing C/EBPβ −/− mice (shown in Figure 5), as well as the enhanced production of *Candida*-specific antibodies belonging to the IgG1 rather than to the IgG2a isotype (Figure 6C), are all diagnostic of a Th2-biased repertoire in the C/EBPβ −/− mice. At present, we cannot distinguish whether this imbalance is mainly caused by the abnormally high IL-6 production (as detected both in the serum and in $CD4^+$ cells of *Candida*-infected and uninfected mice, Figures 4 and 6), or by direct interference with the production of some other cytokine like IL-12 or by a combination of both mechanisms. The influence of a deregulated IL-6 expression on Th cell development has not been studied in detail previously, although it is known that burn patients, who typically present high levels of IL-6, manifest impaired DTH responses and increased immunoglobulin synthesis (Warden and Ninnemann, 1981).

On the other hand, the finding that splenic macrophages from the C/EBPβ −/− mice are defective in nitric oxide production (Table II) is very suggestive, since abundant evidence indicates that NO can mediate several macrophagic functions (reviewed by Nathan, 1992), and we have shown that up-regulating early NO production in highly susceptible mice enhances resistance to systemic candidiasis, and is associated with the activation of the Th1 pathway (Romani *et al.*, 1994b). Interestingly, it was recently shown that the macrophage NO synthase promoter is activated by C/EBPβ (Lowenstein *et al.*, 1993), thus suggesting a direct link between the absence of this factor and defective NO production. Macrophage activation is believed also to play an important role in priming the Th response, in that macrophages are known to be the main source of IL-12. While the defective IL-12 production shown in the C/EBPβ −/− mice (Figure 6B) might be a consequence of defective activation of macrophages, a direct effect of C/EBPβ on IL-12 transcription cannot be excluded.

An altered Th response has been previously reported to occur in mice defective for the production of cytokines involved in the selective activation of Th1 or Th2 cell subsets (Kanagawa *et al.*, 1993; Kühn *et al.*, 1993). We show here that this activation pathway can also be altered by acting on intracellular pathways connected with cytokine activity, through interference with a class of transcription factors. Although not all the specific molecular and cellular targets of C/EBPβ disruption have been identified, we believe that our results represent a step towards a wider understanding of the molecular mechanisms leading to the control of natural and acquired immunity. The overall consequences of C/EBPβ deficiency appear to be highly deregulated myeloid and B-cell activities associated with increased IL-6 levels. A primary myeloid defect may lead to defective Th1 priming which, by default, may promote Th-2 driven B-cell expansion and IL-6 overproduction. The striking resemblance of the described phenotype to CD suggests that C/EBPβ-deficient mice may provide a useful animal model to dissect some of the molecular events underlying the progression to lymphoproliferative disorders.

Materials and methods

Vector construction

A C/EBPβ genomic clone was isolated by screening a CBA genomic library with a rat cDNA probe (Poli *et al.*, 1990) and used as a source of genomic sequences. The 5′ homology, a 1.12 kb *Bam*HI−*Rsa*I fragment containing 450 bp of 5′ untranslated region and the first 673 bp of the coding region (up to amino acid 225), was cloned upstream of an MC1−Neo poly(A) cassette from Stratagene (Thomas and Capecchi, 1987). An MC1 herpes simplex thymidine kinase (TK) cassette was inserted upstream of the 5′ homology as described (Poli *et al.*, 1994). The 3′ homology (a *Nhe*I−*Xba*I fragment of 5.4 kb carrying the 3′ untranslated region of the gene starting from the stop codon) was inserted downstream of the Neo cassette. The construct resulted in the deletion of the last 72 amino acids of the protein.

Generation and analysis of ES cell recombinant clones and of C/EBPβ-deficient mice

The replacement vector was linearized with *Xba*I and used to electroporate 1×10^7 ES cells from the CCE cell line (Robertson *et al.*, 1986). The cells were plated on mitomycin-treated G418-resistant STO feeder cells, and G418 and Gancyclovir selection was applied as described (Poli *et al.*, 1994). Two clones that had undergone homologous recombination were identified out of 120 doubly resistant clones analysed. Genomic DNA from the clones and from wild-type CCE cells was digested with the indicated restriction enzymes and analysed by Southern blot. Both 5′ and 3′ probes were fragments derived from regions of the genomic clones which were not contained in the vector.

ES cell clones carrying the mutation were injected into C57BL6 blastocysts and transplanted into the uteri of F1 (CBA×C57BL6) foster mothers. Male chimeras were mated to MF1 females, and offspring heterozygous for the mutant allele were intercrossed to obtain homozygous mice.

For Western blots, liver nuclear extracts were prepared according to Gorsky *et al.* (1986) from mice either untreated or treated for 4 h with 1 mg/kg body weight of LPS (i.p.). Equal amounts of nuclear proteins were fractionated on a 12.5% SDS−PAGE gel and transferred to nitrocellulose filters. The anti-NH_2 antibody (gift from U.Schibler) and the antibody against the full length protein were both rabbit polyclonal raised against the rat protein expressed in bacteria, and they were

revealed with a polyclonal anti-rabbit IgG antibody conjugated with alkaline phosphatase (Promega)

Histology, immunohistochemistry and histochemistry

Animals were sacrificed by cervical dislocation. For histologic evaluation, tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm and stained with haematoxylin–eosin and Giemsa. For electron microscopy, specimens were fixed in cacodylate-buffered 2.5% glutaraldehyde, post-fixed in osmium tetroxide and then embedded in Epon 812; ultra-thin sections were stained with uranyl acetate/lead hydroxide.

Phenotypic analysis of lymphoid organs

Cell suspensions obtained from the thymuses, spleens and lymph nodes were single- or double-stained with different monoclonal antibodies (mAbs). The following mAbs were used in the flow cytometric analysis: fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (clone 145-2C11 from Boehringer Mannheim), PE-conjugated anti-CD3 (clone 145-2C11), PE-conjugated anti-B22O (clone RA3-6B2), FITC-conjugated anti-IgG1 and anti-IgM (clones G1-6.5 and R6-60.2, respectively), PE- and FITC-conjugated rat and hamster IgG immunoglobulin standard antibody, used as a control for immunofluorescence (from Pharmingen, San Diego, CA)

For staining, $3\times10^5-10^6$ cells were incubated for 20 min on ice with saturating amounts of antibody, then washed and suspended in 0.3 ml of ice-cold phosphate-buffered saline (PBS) and analysed on FACScan (Becton Dickinson, Mountain View, CA), with at least 1×10^4 events scored. Dead cells were excluded from the analysis by propidium iodide staining. Fluorescence data were analysed by FACScan or Consort 30 programs.

Yeasts, infections and DTH reaction

The origin, characteristics and culturing procedures of the *C.albicans* highly virulent CA-6 strain and the live vaccine strain PCA-2 used in this study have already been described in detail (Romani *et al.*, 1991, 1992, 1993). For infection, cells were washed twice in saline and diluted to the desired density to be injected i.v. into mice. Mice succumbing to yeast challenge were routinely necropsied for histopatological confirmation of disseminated candidiasis.

The direct assay for measuring the mouse footpad response to yeast antigens was as described (Romani *et al.*, 1991, 1992, 1993) and results (i.e. increase in footpad weight over that of saline-injected counterpart) are the means ± SEM of four mice per group.

Selection of CD4$^+$ cells and measurement of cytokines

CD4$^+$ lymphocytes were positively selected from pools of spleen cells by means of a panning procedure using anti-murine CD4 mAb GK1.5, which resulted in a >95% pure population on FACS analysis. Supernatants from mixed lymphocyte–*Candida* cultures were obtained by culturing CD4$^+$ lymphocytes in the presence of yeast cells and irradiated splenocytes as accessory cells. Source and characteristics of the antibody reagents used in interferon (IFN)-γ, IL-2 and IL-4 ELISA were as described (Romani *et al.*, 1991, 1992, 1993; Puccetti *et al.*, 1994). In IL-6 measurement, two-site ELISA involved the use of mAb MP5720F3 in combination with biotinylated monoclonal MP5-32c11 (American Type Culture Collection, ATCC, Rockville, MD, USA). Levels of circulating IL-12 were determined as described (Romani *et al.*, 1994a), by evaluating the IFN-γ production induced by serum dilutions on naive Balb/c splenocytes. The specificity of the assay was determined by incubating serum samples with neutralizing polyclonal rabbit anti-murine IL-12 antibodies (Genetics Institute, Cambridge, MA).

Antibody assay

A micro-ELISA procedure was used to quantitate yeast-specific antibodies in the sera of mice (Romani *et al.*, 1994a). The assay involved coating the microtitre plate wells with *Candida* antigen, addition of appropriate dilutions of test antisera and a further reaction with alkaline phosphatase-conjugated rabbit anti-mouse IgG1 and IgG2a (Zymed Laboratories). After addition of the substrate solution, the OD of triplicate samples was read with an ELISA reader, using a 405 nm filter.

Macrophage cultures and nitrite determination

Macrophages were obtained from spleen cell suspensions and nitrite concentration, a measure of NO synthesis, was determined as described (Cenci *et al.*, 1993). Briefly, adherent splenic cells were incubated overnight in the presence of 100 U/ml of rIFN-γ (Genzyme) and of 10 ng/ml of LPS (Sigma). Viable yeast cells (1:5 ratio) were then added, and supernatants were removed after 4 h and assessed for nitrite content.

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