Inactivation of the IL-6 Gene Prevents Development of Multicentric Castleman’s Disease in C/EBPβ-deficient Mice

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Summary

Castleman’s disease is a lymphoproliferative disorder thought to be related to deregulated production of IL-6. We have previously shown that mice lacking the trans-acting factor C/EBPβ, a transcriptional regulator of IL-6 and a mediator of IL-6 intracellular signaling, develop a pathology nearly identical to multicentric Castleman’s disease, together with increasingly high levels of circulating IL-6. We describe here how the simultaneous inactivation of both IL-6 and C/EBPβ genes prevents the development of pathological traits of Castleman’s disease observed in C/EBPβ-/- mice. Histological and phenotypic analysis of lymph nodes and spleen of double mutant mice did not show either the lymphadenopathy and splenomegaly or the abnormal expansion of myeloid, B and plasma cell compartments observed in C/EBPβ-/- mice, while B cell development, although delayed, was normal. Our data demonstrate that IL-6 is essential for the development of multicentric Castleman’s disease in C/EBPβ-/- mice.
both the IL-6 and the C/EBPβ genes were inactivated, and describe here the consequences of this double mutation. Our results indicate that IL-6 is responsible for the development of the multicentric CD-like disease in the C/EBPβ−/− mice.

Materials and Methods

Generation of IL-6−/−, C/EBPβ−/− Double Mutant Mice.

The generation of IL-6- and C/EBPβ-deficient single mutant mice has been recently described (11, 12). Mice heterozygous for both mutations were obtained by intercrossing IL-6−/−
and C/EBPβ−/− mice. The double heterozygous mice were then mated to obtain mice homozygous for both mutations. The genotypes of the mice obtained from the intercrosses were determined by Southern blot as previously described (10, 11). Procedures involving animals and their care were conducted in conformity with national and international laws and policies.

**Histology and Immunohistochemistry.** Animals of different age groups (from 16 to 32 wk of age) were killed by cervical dislocation. For histologic evaluation, tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm and stained with hematoxylin-eosin and Giemsa. For immunohistochemistry, paraffin-embedded sections were digested with Proteinase K (Dako, Glostrup, Denmark) in phosphate-buffered saline, pH 7.6, for 5 min, incubated with anti-mouse kappa light chain monoclonal antibodies (PharMingen, San Diego, CA), washed and overlaid with biotinilated rabbit anti-rat Ig (Vector Laboratories, Burlingame, CA). Unbound antibody was removed by washing, and the slides were incubated with Strep-AB complex/HRP (Dako).

**Phenotypic Analysis of Lymphoid Organs.** Cell suspensions were single- or double-stained with different monoclonal antibodies (mAbs) for flow cytometric analysis: phycoerytrin (PE)-conjugated anti-CD4 (clone H129.19), FITC-conjugated anti-CD8 (clone 53-6.7) and FITC-conjugated anti-CD3 (clone 145-2C11), from Boehringer Mannheim (Mannheim, Germany). PE-conjugated anti-CD3 (clone 145-2C11), PE-conjugated anti-B220 (clone RA3-6B2), FITC-conjugated anti-IgG1 and anti-IgM (clones G1-6.5 and R6-60.2, respectively), FITC-conjugated anti-CD23 (clone B3B4) and PE- and FITC-conjugated rat and hamster IgG immunoglobulin standard antibody, used as control of immunofluorescence, from PharMingen.

For staining, 3 × 10^5–10^6 cells were incubated on ice with saturating amounts of antibody, washed in ice-cold PBS, suspended in 0.3 ml of ice-cold PBS and analyzed 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 analyzed by FACScan® or Consort 30 programs.

**Results**

**Histological Features of Lymphoid Organs from IL-6−/−, C/EBPβ−/− Double Mutant Mice.** We previously reported that post-mortem examination of C/EBPβ−/− mice (single mutant, SM) revealed splenomegaly and peripheral lymph node enlargement from 16 wk of age (11). Lymph nodes draining areas highly exposed to antigen stimulation and/or inflammatory foci showed prominent germinal centers and a diffused plasmacytosis in the paracortical and medullary area (Fig. 1 A), with plasma cells so numerous and crowded that they confer a pseudotumoral appearance to the nodes involved. This observation was confirmed by immunohistochemical staining with anti-mouse kappa light chain (Fig. 2 A), as well as by electron microscopy (data not shown). The enlarged spleens showed white and red pulp hyperplasia due to the expansion of both lymphoid and myeloid compartments (Fig. 1 B). The diffuse plasmacytosis in lymph nodes and spleen and the splenic hyperplastic hemopoietic tissue were observed in all but one of the 20 C/EBPβ SM mice studied from 16 through 32 wk of age. In contrast, DM mice failed to show any major alterations
in the lymphoid organs. Moreover, the histological alterations observed in the lymph nodes and spleens of the C/EBPβ SM mice were not detected in either the IL-6, C/EBPβ double mutant (DM) (Fig. 1, C and D and Fig. 2 C) or the IL-6 SM mice (not shown). In these animals, as in the wild-type mice, well developed germinal centers were present in the cortical areas of lymph nodes and in the white pulp of spleen (Fig. 1, C−F). Mature plasma cells were observed in lymph node medullary cords (Fig. 1, C and E) as well as in the intestinal mucosa and the peribronchial region of the lung (not shown). Remarkably, the DM mice did not show any sign of the dramatic plasmacytosis detected in the lymph nodes of the C/EBPβ SM mice as shown by the histological analysis (Fig. 1, compare A with C), and failed to stain with anti-κ light chain antibodies (Fig. 2, compare A with C). DM mice did not show either any spleen hyperplasia or expansion of lymphoid and myeloid compartments (Fig. 1, B and D). These data indicate that IL-6 plays an essential role in the development of the lymphoproliferative and myeloproliferative disease developed by the C/EBPβ SM mice.

**Inactivation of the IL-6 Gene Reverses the Abnormal B and Plasma Cell Expansion of the C/EBPβ−/− Mice.** As previously shown, total cell recovery from both the spleen and the lymph nodes of C/EBPβ SM mice strongly increased from 21 wk of age onwards, matching the enlargement of both organs observed in post-mortem examination (Fig. 3, A and C). This increase involved a dramatic expansion of the B cell compartment, as shown by the abnormally high number of cells expressing surface B220 antigen (Fig. 3, B and D). In contrast, total cell recovery never increased in either spleen or lymph nodes from the DM mice (Fig. 3, A and C). Moreover, the flow cytometric analysis of lymphocyte subsets in spleen and lymph nodes clearly showed that the number of B220+ lymphocytes was analyzed in IL-6 SM mice, and proved to be normal from 27 wk onwards (see below) (Fig. 3, A–D). In most of the DM mice, the expression of IgM, IgG1, and CD23 by lymphocytes from spleen and lymph nodes were also analyzed and always came within the normal ranges (not shown).
Development of Lymphoid Compartment in IL-6−/−, C/EBPβ−/− Double Mutant Mice. It has been previously shown that young (6-12-wk-old) IL-6−/− mice present a reduction in the total number of thymocytes and peripheral T cells, but maintain a normal subset distribution for what concern the expression of CD4, CD8, and T cell receptor repertoire (9). We have found that also older IL-6−/− mice (from 16 wk of age) have a decreased number of thymocytes (Fig. 4A). Although we never observed any alteration with respect to normal mice in phenotype or cell number in the thymus of the C/EBPβ SM mice (11), the DM mice showed, at all ages analyzed, a decrease in total thymocyte number comparable to that found in the IL-6 SM mice: this decrease was slightly higher at younger ages in both kinds of mice (Fig. 4A). The subset distribution did not present any significant alteration in either SM or DM mice with respect to the wild-type controls (Fig. 4B). These data suggest that while IL-6 is involved in thymocyte proliferation mainly at young ages, its absence is less critical when involution of the thymus has started; it would also appear that C/EBPβ does not play an important role in thymocyte proliferation and/or differentiation, at least in the age groups studied.

The total mononucleated cell number also significantly decreased in the spleen of IL-6 SM mice at 16 and 21 wk of age, but became normal by the 27th wk (Fig. 3A). This decrease involved both CD3+ T cells and B220+ B lymphocytes (not shown and Fig. 3B). Interestingly, not only was the decrease in total cell yield and percentage of B220+ lymphocytes also detected in the spleens of the DM mice (Fig. 3), but it was significantly more pronounced than in the IL-6 SM mice, suggesting that IL-6 and C/EBPβ act synergistically in splenic lymphocyte development.

Discussion

IL-6 is a potent growth factor for myeloma and plasma-cytoma cells (13), being required for in vitro growth of both primary cell explants and established cell lines. In parallel with its growth promoting activity, IL-6 is thought to exert an anti-apoptotic effect on myeloma cells in vitro and possibly in vivo, thus playing an important role in the expansion of the tumor population (6). On the other hand, IL-6 overproduction has also been linked to non-malignant lymphoproliferative disorders such as CD (2), where again it may inhibit the naturally occurring apoptotic suppression of B and plasma cell populations which have undergone expansion after antigenic stimuli. We have previously shown that mice lacking the transcription factor C/EBPβ develop an age-related lymphoproliferative and myeloproliferative disorder reminiscent of multicentric CD and possibly linked to IL-6 overproduction (11). Here we show that this disorder can be completely blocked by inactivating the IL-6 gene, indicating that indeed deregulation of IL-6 production may represent one of the triggering events. It is tempting to speculate that interference with the normal activity of C/EBPβ and the consequent IL-6 deregulation, might be among the causes of CD, a disease whose aethiology is still unknown. In this light, the localized or multicentric CD forms might arise from inactivation of C/EBPβ occurring either in very early myeloid/B lymphoid precursors, or in B cells at later differentiation stages. Moreover, providing a model of multicentric CD development in which the abnormal lymphoproliferation can be prevented by IL-6 gene inactivation supports the hypothesis that IL-6 plays a critical role in multicentric CD pathogenesis.

Interestingly, a deletion of human chromosome 20q involving the region in which the C/EBPβ gene maps has been reported in several cases of myeloproliferative disorders and myelodisplastic syndromes (14), and in 16 out of 68 patients (24%) affected by myeloid leukemia (15). It has also been shown that such a deletion in myelodysplasia can arise in a multipotent precursor of both myeloid and B cells, suggesting that this mutation can cause a disease arising in a cell with both myeloid and lymphoid potential (16). These reports together with our observation of abnormal proliferation in both myeloid and B cell compartments in C/EBPβ−/− mice, suggest that inactivation of the C/EBPβ gene could represent a link between myeloid- and lympho-proliferative disorders. If this is indeed the case, blockage of IL-6 production could help interfere with myeloid- and/or lympho-proliferative disorders involving C/EBPβ gene inactivation.

Although IL-6 is an important B cell growth factor and C/EBPβ has been suggested to play a role in B cell development, our data also show that both IL-6 SM and IL-6, C/EBPβ DM mice have well-developed germinal centers in both lymph nodes and spleen, and mature plasma cells in several tissues, thus indicating that neither gene product is essential for B cell proliferation and final maturation. However, it is interesting to note that younger IL-6 SM mice showed a significant reduction in total cellularity and B220+ lymphocytes in the spleen up to 27 wk of age, suggesting that IL-6 does play a role in regulating B lymphocyte proliferation at young ages, although it can be functionally compensated for by other cytokines thereafter. Moreover, the B cell population dropped even further when the C/EBPβ gene was also inactivated, suggesting that the two genes play partly independent roles, C/EBPβ probably being involved in the signaling of B cell growth factors other than IL-6. One of such factors may be IL-7, since it was recently shown that bone marrow stromal cells from C/EBPβ SM mice have a reduced potential for B cell differentiation, which could be related to a defect in responsiveness to IL-7 (Chen, X., W. Liu, C. Ambrosino, M.R. Ruocco, V. Poli, L. Romani, I. Quinto, S. Venuta, and G. Scala, manuscript submitted for publication).

We have previously suggested that the deregulation of IL-6 expression in the C/EBPβ−/− mice may either be a consequence of defects in their immune system, which cause a chronic preferential expansion of CD4+ T helper type 2 cell population, producing IL-6 among other cytokines, or it may be due to C/EBPβ playing a direct role in regulating IL-6 gene transcription, or both (11). Although
this transcription factor was believed to be an activator of the IL-6 gene (7), our data imply that it may instead act as an inhibitor. Indeed, Kishimoto and colleagues have shown that peritoneal macrophages from C/EBPβ (NF-IL6)-deficient mice constantly produce IL-6 (12), suggesting that peritoneal macrophages from C/EBPβ (NF-IL6)-deficient mice shown a significant enhancement of IL-6 induction in response to IL-6 treatment (Screpanti, l., and M. Maroder, unpublished observation), again indicating deregulation of IL-6 gene transcription and possibly increased sensitivity to the IL-6-induced signaling pathway. Only more detailed studies performed on cell lines of different kinds derived from the mutant mice will allow the precise definition of the molecular basis for IL-6 deregulation in the C/EBPβ−/− mice.

We wish to thank Dr. Gennaro Ciliberto and Mrs. Janet Clench for critically reading the manuscript.

This work was partially supported by the Associazione Italiana per la Ricerca sul Cancro (AIRC) and the National Research Council (CNR) ACRO Project.

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Received for publication 25 April 1996 and in revised form 9 August 1996.

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