Th2 cell-specific cytokine expression and allergen-induced airway inflammation depend on JunB

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Naïve CD4⁺ T cells differentiate into effector T helper 1 (Th1) or Th2 cells, which are classified by their specific set of cytokines. Here we demonstrate that loss of JunB in *in vitro* polarized Th2 cells led to a dysregulated expression of the Th2-specific cytokines IL-4 and IL-5. These cells produce IFN- γ and express T-bet, the key regulator of Th1 cells. In line with the essential role of Th2 cells in the pathogenesis of allergic asthma, mice with JunB-deficient CD4⁺ T cells exhibited an impaired allergen-induced airway inflammation. This study demonstrates novel functions of JunB in the development of Th2 effector cells, for a normal Th2 cytokine expression pattern and for a complete Th2-dependent immune response in mice.

Keywords: asthma/IL-4/IL-5/immune response/T-bet

Introduction

Antigen-stimulated naïve CD4⁺ T helper (Th) cells differentiate into two distinct subsets of effector cells, Th1 and Th2, that are defined by their distinct cytokine profiles and their immune regulatory functions (Paul and Seder, 1994). Th1 cells provide protection against intracellular pathogens and viruses, and produce IFN- γ , IL-2 and lymphotoxin α (TNF- β) predominantly. Th2 cells mainly produce IL-4, IL-5, IL-9, IL-10 and IL-13, and are involved in humoral immunity, immunity to parasites and the allergic response in the presence of antigen. IL-12 drives CD4⁺ T cell differentiation to the Th1 lineage through the Stat4 signalling pathway (for reviews, see Glimcher and Murphy, 2000; Ho and Glimcher, 2002; O'Shea *et al.*, 2002). The critical Th2-inducing cytokine is IL-4, which mediates its effects through the Stat6 signal transduction pathway (Wurster *et al.*, 2000; O'Shea *et al.*, 2002). The development of immunopathologies may be explained by an imbalance of both regulatory routes. For example, overproduction of Th1 cytokines has been implicated in auto-immune diseases, and dysregulation of the Th2-type response mediates development and maintenance of many allergic diseases such as allergic airway inflammation (allergic asthma) (Gleich and Kita, 1997; Ray and Cohn, 1999; O'Garra and Arai, 2000) or atopic dermatitis (Akdis *et al.*, 2000; Yamazaki *et al.*, 2002).

The hallmarks of allergic asthma are infiltration of eosinophils into the bronchial wall and lumen, mucus production in the airways, airway hyperresponsiveness (AHR) and elevated serum IgE levels (Kon and Kay, 1999; Wills-Karp, 1999). The Th2 cytokines IL-4, IL-5 and IL-13 have distinct roles in the pathogenesis of allergic asthma. IL-4 is responsible for induction and maintenance of the Th2 lineage (Paul and Seder, 1994) and therefore is essential for the development of allergic airway inflammation (Brusselle *et al.*, 1994). Together with IL-13, it is of critical importance to trigger B cell immunoglobulin class switching to IgE (Vercelli, 2001). While mucus production by goblet cells was shown to be dependent on IL-13 (Grunig *et al.*, 1998; Wills-Karp *et al.*, 1998; Zhu *et al.*, 1999), IL-5 specifically regulates growth, differentiation, activation and mobilization of eosinophils from the bone marrow (O'Byrne *et al.*, 2001).

Major efforts have been made to elucidate the molecular basis of Th2 versus Th1 cell subset differentiation and to identify transcription factors controlling Th2 and Th1 cytokine expression. The T-box transcription factor T-bet is involved in the commitment of Th1 cells (Szabo *et al.*, 2000, 2002; Mullen *et al.*, 2001) by inducing IFN- γ synthesis and repressing IL-4 and IL-5 production, even in fully polarized Th2 cells (Szabo *et al.*, 2000). Mice lacking T-bet develop spontaneous airway inflammation (Finotto *et al.*, 2002) and fail to generate a functional Th1 response (Szabo *et al.*, 2002). The transcription factors found to regulate IL-4 expression and the commitment of Th2 cells include GATA-3 (Zhang *et al.*, 1997, 1998, 1999; Zheng and Flavell, 1997), NFAT (Chuvpilo *et al.*, 1993; Szabo *et al.*, 1993), c-Maf (Ho *et al.*, 1996; Kim *et al.*, 1999), NF κ B (Yang *et al.*, 1998; Ferreira *et al.*, 1999; Das *et al.*, 2001) and JunB (Rincon *et al.*, 1997; Li *et al.*, 1999). NFAT and NF κ B are present in both Th1 and Th2 cells. In contrast, c-Maf (Ho *et al.*, 1996), GATA-3 (Zheng and Flavell, 1997) and JunB (Rincon *et al.*, 1997; Li *et al.*, 1999), a member of the activator protein-1 (AP-1) transcription factor family (Angel and Karin, 1991), are upregulated in the Th2 lineage. While the *in vivo* functions of GATA-3 and NF κ B subunit p50 in transcriptional control of cytokines and regulation of Th2-dependent allergic airway inflammation have been studied in great detail (Zhang *et al.*, 1997, 1999; Zheng and Flavell, 1997; Ouyang *et al.*, 1998, 2000; Yang *et al.*, 1998; Das *et al.*, 2001; Finotto *et al.*, 2001), the contribution of JunB and JunB target genes to these processes have not been defined. Upon ectopic expression in a B-cell lymphoma line, JunB synergizes with c-Maf to activate the murine IL-4 promoter, and in Th1 cells, it induces the Th2-specific cytokines IL-4 and IL-5 (Li *et al.*, 1999). In line with these data, in Th2 cells of *itch*^{-/-} mice exhibiting enhanced level of JunB, IL-4 and IL-5 production was augmented (Fang *et al.*, 2002).

By a loss-of-function approach, we investigated the regulatory function of JunB in Th2 cell differentiation, cytokine expression and type 2 immune responses *in vivo*. We generated two different mouse lines harbouring a *junB* transgene on the background of mutated *junB* loci. In both lines, transgene expression rescued embryonic lethality (Schorpp-Kistner *et al.*, 1999). Yet transgene expression was extremely low with only traces of JunB protein in CD4+ T cells. JunB-deficient CD4+ T cells, polarized towards the Th2 direction *in vitro*, displayed normal IL-13 production, but were severely reduced in IL-4 and IL-5 synthesis and showed an aberrant expression of GATA-3. Mutant cells synthesized IFN- γ and expressed the Th1-specific transcription factor T-bet. Moreover, the manifestation of the antigen-induced inflammatory response in the airways, which critically depends on functional Th2 cells, was attenuated in the JunB mutant mice. Thus, our studies have unraveled an essential role of JunB for Th2 cell development and effector functions, which cannot be substituted by other AP-1 members.

Results

Generation of JunB-deficient thymocytes and fully polarized T helper cells

To understand the role of JunB in T helper cell differentiation, we generated mice with a loss of JunB expression in the T cell population. Two JunB transgenic mouse lines (288K, 311K) were generated expressing *junB* under the control of the human ubiquitin C promoter (Schorpp *et al.*, 1996) and their transgenic loci were introduced into the *junB*^{-/-} background (Schorpp-Kistner *et al.*, 1999). The *rescue* mice 311R and 288R harbouring an ubi-JunB/*junB*^{-/-} genotype overcame embryonic lethality and were born in the expected Mendelian frequency. RNase protection analysis on RNA from different tissues of 311R and 288R mice revealed high levels of transgene expression in the brain but low levels in the thymus (Figure 1A). This result becomes evident when the amount of transgenic *junB* transcripts (t) is compared with the level of the non-functional endogenous *junB*-neo-fusion transcript (e) derived from the targeted *junB* locus (Schorpp-Kistner *et al.*, 1999). To monitor *junB* expression in CD4+ T cells of 311R and 288R mice, cells were polarized *in vitro* towards the Th2 direction or kept under non-polarizing conditions (Th0). After four days of cultivation, mRNA was prepared and subjected to RNase protection analysis. In 288R and 311R CD4+ T cells, the level of transgenic JunB transcripts were ~10% and <1% of the amount of non-functional endogenous transcript, respectively (Figure 1B). On the protein level, loss of functional JunB was even more obvious (Figure 1C). Western blot analysis on nuclear extracts of 288R cells directed towards Th2 for four days revealed only traces of JunB when compared with control cells. In nuclei of 311R cells, JunB levels were not detectable. These data show that both 288R and 311R mice provide an excellent tool to investigate the role of JunB in T helper effector cell differentiation and function. Since JunB expression in Th2 cells from transgenic (288K, 311K) and non-transgenic (wild type) *junB*^{+/+} mice did not differ (data not shown), transgenic lines were used as controls.

Fig. 1. Reduced JunB expression in thymus and differentiated CD4+ T cells of 288R and 311R mice. (A) JunB-specific RNase protection assay with 3 μ g total RNA prepared from the thymus of 288R and 311R mice. The protected fragments indicate RNA transcripts from transgene (t) and endogenous (e) loci. As a control, 3 μ g of RNA isolated from brain of 288R, 311R and wild-type mice was used. (B) JunB-specific RNase protection assay using 2 μ g RNA prepared from splenic CD4+ T cells isolated from 311R and 288R mice, stimulated with ConA (5 μ g/ml) and cultured for 4 days under Th2- or non- (Th0) polarizing conditions. As a control, 2 μ g of RNA from the brains of wild-type and 311R mice was used. (C) Nuclear extracts isolated from splenic CD4+ T cells of 311R and 288R mice that were stimulated with ConA (5 μ g/ml) and cultured for 4 days under Th2-polarizing conditions were analysed for JunB expression by western blotting using a JunB-specific antiserum. Expression of p58 primase was used as an internal control for quality and quantity of the protein extracts. All experiments were repeated at least twice, giving essentially identical results.

Generation of fully polarized splenic CD4+ cells from control and rescue mice

To determine the B to T cell and CD4+ to CD8+ cell ratio in 311R and 288R mice, FACS analysis on lymphocytes isolated from spleens of 6- to 8-week-old mice was performed. As shown in Figure 2A, the percentage of CD19-expressing B cells and CD3-expressing T cells in 288R and 311R was not significantly different from 288K, 311K (Figure 2A) and wild-type (data not shown) control mice. Similarly, in spleen of the *rescue* mice the CD4+ to CD8+ T cell ratio was almost indistinguishable from that of control mice (Figure 2B; data not shown), indicating that the distribution of lymphoid cell populations was not altered in the *rescue* mice.

In response to specific stimuli, CD4+ T cells are activated expressing different surface activation markers and have the ability to rapidly expand. Therefore, we investigated whether loss of JunB in splenic CD4+ T cells of 6- to 8-week-old mice affected the activation and proliferation potential in response to *in vitro* stimuli such as ConA (Figure 3) and anti-CD3/anti-CD28 (data not

Fig. 2. Normal lymphocyte populations in JunB mutant mice. (A) Splenic lymphocytes from 6- to 8-week-old mice were stained with PE-anti-CD19 and APC-anti-CD3 and analysed by FACS. Statistical analyses from independent measurements of control (311K) and *rescue* (288R, 311R) mice are shown ($n = 8$). (B) Splenic lymphocytes from 6- to 8-week-old mice were stained with PE-anti-CD4 and APC-anti-CD8 and analysed by FACS. Statistical analyses from independent measurements of different mice are shown (control, $n = 18$; 288R, $n = 6$; 311R, $n = 14$).

shown) when cultured in the presence of IL-2. The degree of activation was measured by the expression profile of the surface activation marker CD25 (Figure 3A), directly after isolation of CD4⁺ T cells from spleen (0 h) and 48 h post-*in vitro* stimulation and cultivation under Th1- and Th2-polarizing conditions. In addition, the surface expression profile of CD69 was determined after isolation and 18 h after stimulation, respectively (data not shown). Under these optimal conditions of exogenously added IL-2, the *rescue* cells showed no significant differences in activation compared with the control cells.

We subsequently determined the proliferation potential of the JunB-deficient CD4⁺ cells in response to ConA. For that purpose, CD4⁺ T cells were isolated from 6- to 8-week-old mice, stimulated with ConA and cultured under Th1 or Th2 polarizing conditions in the presence of IL-2. Cell proliferation assays were performed in a 24 h-rhythm for three days post-ConA stimulation (Figure 3B). During three days of cultivation for both polarizing conditions, the proliferation rate of 311R or 288R CD4⁺ cells did not significantly differ from that of the control.

Altered cytokine production by polarized *rescue* CD4⁺ T cells

To investigate the effect of JunB loss on Th2 cell differentiation and function, the expression levels of the

Fig. 3. Normal surface activation marker expression and proliferation response of ConA-stimulated 311R and 288R CD4⁺ T cells. Splenic CD4⁺ T cells prepared from 6- to 8-week-old mice were stimulated with ConA (5 μ g/ml) and cultured under Th1- and Th2-polarizing conditions (see methods), respectively. (A) Cells were stained for CD25 directly after isolation (0 h) and 48 h after ConA stimulation. The experiment was repeated three times with similar results. (B) To determine the proliferation response after ConA stimulation cells were measured in a 24 h-rhythm by the ViaLight[®] assay. RLUs were measured at indicated time points post-stimulation and normalized to RLUs obtained prior stimulation. The fold amplification rate is plotted. Results shown are representative of three independent experiments.

Th2 cytokines IL-4, IL-5 and IL-13 produced by CD4⁺ T cells under Th2 conditions were determined by ELISA. For different stimuli (ConA and anti-CD3/anti-CD28), the IL-4 production of *rescue* cells was reduced to ~50% in 288R and to almost 25% in 311R, when compared with control cells (Figure 4A). Furthermore, IL-5 production was strongly reduced in 288R and nearly absent in 311R cells (Figure 4B), whereas the production of IL-13 was not significantly altered (Figure 4C).

The dysregulated Th2 cytokine profile of the *rescue* CD4⁺ cells cultured under Th2 polarizing conditions suggested that the cells were not able to differentiate into

Fig. 4. Altered Th2 cytokine expression profile in 288R and 311R cells. Purified splenic CD4⁺ T cells from 6- to 8-week-old control 311R and 288R mice were stimulated with ConA (5 μ g/ml), cultured under Th2-polarizing conditions for 4–7 days, washed and restimulated with ConA (5 μ g/ml) for 24 h. IL-4 (A), IL-5 (B) and IL-13 (C) concentrations were determined by ELISA. Cytokine concentration of control cells was set to one. Mean values and standard deviations of at least three experiments are shown. The experiment was repeated with coated anti-CD3 (2 μ g/ml)/anti-CD28 (2 μ g/ml) stimulation with similar results and cytokine measurements performed 6 h after restimulation yielded comparable data (not shown).

Fig. 5. 288R and 311R Th2 cells exhibit reduced IL-4 and increased IFN- γ levels. CD4⁺ T cells from spleens and peripheral lymph nodes of 6- to 8-week-old control 288R and 311R mice were stimulated with ConA (5 μ g/ml), cultured under Th2-polarizing conditions for 3 days and restimulated with PMA (10 ng/ml)/ionomycin (250 ng/ml) for 4.5 h, adding brefeldin A (10 μ g/ml) for the last 2.5 h. After intracellular staining with PE-anti-IL-4 (A) and FITC-anti-IFN- γ (B), the cells were analysed by FACS (311K control in black, 288R in green, 311R in red). The bars in the overlay histogram indicate the cells positive for the respective intracellular cytokine staining. Results are representative of three separate experiments.

proper Th2 cells. To examine whether the JunB-deficient cells, despite being kept under Th2 conditions, displayed Th1 characteristics, we determined IFN- γ expression by intracellular staining (ICS, Figure 5B). To validate the obtained IL-4 ELISA data, we also performed ICS for IL-4 in parallel (Figure 5A). IL-4-producing cells were detected in both control and *rescue* cells, but, concomitant with the degree of JunB inactivation in 288R and 311R cells, IL-4 production was decreased, supporting the data obtained on IL-4 measurement by ELISA. Intracellular staining for IFN- γ revealed no considerable IFN- γ expression in control cells (Figure 5B). In contrast, ~30% of the 288R cells and almost 70% of the 311R cells expressed the Th1-specific cytokine. This increase in IFN- γ -expressing cells was obtained regardless of the treatment used for restimulation (P/I, Figure 5B; ConA, data not shown). Thus, under Th2 conditions, a minority of the JunB-deficient cells expresses IL-4 whereas the majority produces IFN- γ .

Enhanced levels of T-bet in JunB-deficient Th2 cells

The T-box transcription factor T-bet has been identified as an important regulator for Th1 lineage commitment and as a direct activator of the IFN- γ promoter (Szabo *et al.*, 2000, 2002; Mullen *et al.*, 2001; Finotto *et al.*, 2002). In contrast, GATA-3 is essential for Th2 lineage commitment, as it plays a critical role for IL-4, IL-5 and IL-13 expression (Siegel *et al.*, 1995; Zhang *et al.*, 1997, 1999; Zheng and Flavell, 1997; Lee *et al.*, 1998). To elucidate the consequences of JunB deficiency in T helper cells for the expression of these transcription factors, the level of T-bet and GATA-3 in Th1- and Th2-polarized CD4⁺ T cells was determined by northern blot analysis. In control cells, strong T-bet expression was found under Th1 but not under Th2 conditions (Figure 6). In contrast, 288R and 311R cells expressed T-bet both under Th1 conditions and in cells that were skewed towards the Th2 lineage. The level of T-bet expression in the *rescue* cells directly

correlated with the extent of JunB reduction. On the other hand, GATA-3 transcripts were found only in the Th2 polarized subset independent of the genotype of the cells analysed. However, the expression level was slightly reduced in both *rescue* lines. According to the degree of JunB deficiency, a moderate downregulation of GATA-3, as well as a strong upregulation of T-bet expression, was more pronounced in 311R than in 288R. These data suggest that JunB is involved in the regulation of two of the key transcription factors that control Th1 versus Th2 subset differentiation.

Fig. 6. JunB-deficient Th2 cells express T-bet. Splenic CD4⁺ T cells purified from 6- to 8-week-old 311 and 288 control (K) mice and 311 and 288 rescue (R) mice were stimulated with coated anti-CD3 (2 µg/ml) + anti-CD28 (2 µg/ml) and cultured under Th1- and Th2-polarizing conditions for 5 days. Total RNA was prepared from the cells and 8 µg were analysed by northern blotting using T-bet- and GATA-3-specific probes. Quality and quantity of the RNA was confirmed by visualization of 18S ribosomal RNA.

Attenuated manifestation of allergen-induced airway inflammation in 288R and 311R mice

Previous studies showed that an orchestrated Th2 immune response is critical for the development and maintenance of allergic airway inflammation in humans as well as in the murine model (Yang *et al.*, 1998; Cohn *et al.*, 1999; Zhang *et al.*, 1999; Das *et al.*, 2001; Finotto *et al.*, 2001). Based on our observation that polarized *rescue* CD4⁺ T cells displayed strongly impaired Th2 characteristics *in vitro*, we analysed the ability of the 311R mice to perform a proper Th2 immune response *in vivo* by induction of an allergic airway inflammation. For this purpose, 311R and control (311K, wild type) littermates were immunized twice with chicken egg ovalbumin (OVA) in Alum and subsequently challenged with OVA aerosol inhalation (OVA/OVA) for a period of three days. As a negative control, mice were sham (phosphate-buffered saline, PBS)-immunized and challenged with OVA (PBS/OVA). Forty hours post the last aerosol challenge, mice were sacrificed and the lungs were examined histologically by haematoxylin–eosin (H/E) staining for eosinophilic infiltration (Figure 7A, C and E) and by periodic acid-Schiff (PAS) staining for mucus (Figure 7B, D and F). As shown in Figure 7A and B, sham-immunized mice showed no sign of inflammation. On the other hand, OVA/OVA 311K (Figure 7) and wild-type (data not shown) control mice displayed a prominent inflammatory response with massive perivascular and peribronchiolar eosinophilic infiltration (Figure 7C), numerous mucus-producing goblet cells and impressive amounts of mucus blocking the bronchial lumen (Figure 7D). In contrast, in 311R mice, the inflammatory response within the airways was notably attenuated. Peribronchiolar eosinophilic infiltrates

Fig. 7. Reduced airway inflammation in 311R mice. Control (311K) and 311R mice were sensitized twice with OVA in Alum and exposed three times to 1% aerosolized OVA (OVA/OVA). As a negative control, 311K mice were sham-immunized and challenged with OVA aerosol (PBS/OVA). Forty hours after the last aerosol exposure, lungs were prepared for histology and stained with H/E for eosinophilia and PAS for mucus. Data shown here are representative for three individuals of each genotype tested. Induction of airway inflammation in wild-type mice was similar to 311K (data not shown). The bar shown represents 55 µm.

were completely absent (Figure 7E). Perivascularly, only very few eosinophils were noted. In the majority of the bronchial tubes the number of mucus-producing goblet cells was low, and mucus detected by PAS staining (Figure 7F) was much less than in OVA/OVA control littermates. Thus, loss of JunB in T helper cells was accompanied by a reduction of the symptoms of allergen-induced airway inflammation in mice.

Discussion

The characterization of signalling pathways and transcription factors regulating Th cell differentiation and lineage commitment will be a major step towards the understanding of the pathogenesis of chronic inflammatory diseases caused by an imbalanced Th1 to Th2 cell content. A key player in this regulatory network determining Th2 differentiation is IL-4, which is selectively expressed by Th2 cells. *In vitro* binding studies as well as overexpression of the AP-1 member JunB in transgenic mice have provided evidence that expression of this gene is regulated by the concerted action of c-Maf and JunB (Rincon *et al.*, 1997; Li *et al.*, 1999). Here we have used a loss-of-function approach to demonstrate the requirement of JunB for IL-4 expression and the development of proper Th2 cells. When we generated mice expressing only traces of JunB in CD4⁺ T cells and analysed the cytokine profile of *in vitro* polarized mutant Th2 cells, a complete loss of IL-5 expression and severely impaired IL-4 synthesis was observed. Previously, independent studies have shown that ectopically expressed GATA-3, c-Maf and JunB can induce production of IL-4 in non-Th2 cells (Ho *et al.*, 1996; Zhang *et al.*, 1997; Zheng and Flavell, 1997; Ouyang *et al.*, 1998, 2000; Li *et al.*, 1999; Kishikawa *et al.*, 2001). Apparently, c-Maf, together with either GATA-3 or JunB, synergistically transactivates the IL-4 promoter, at least under tissue culture conditions (Li *et al.*, 1999; Kishikawa *et al.*, 2001). Vice versa, loss of JunB protein (this study), c-Maf deficiency (Ho *et al.*, 1996), expression of a dominant negative mutant of GATA-3 (Zhang *et al.*, 1999) or antisense-induced blockade of GATA-3 (Finotto *et al.*, 2001) led to a severe impairment of IL-4 production in Th2 cells. In the light of these data, we propose that full induction of IL-4 gene expression in Th2 cells requires the concerted action of all three transcription factors, JunB, GATA-3 and c-Maf. The remaining IL-4 production found in the JunB-deficient Th2 cells may be due to the activity of c-Maf and residual GATA-3 expression. On the other hand, we cannot exclude that other Jun family members may substitute for the loss of JunB, at least to some extent. In the future, it will be interesting to see whether simultaneous ectopic supply of GATA-3, c-Maf and JunB results in a full redirection of polarized Th1 cells into the Th2 lineage.

In addition to GATA-3, an intact AP-1 site within a conserved lymphokine element 0 (CLE0) is required for the proper expression of IL-5 (Siegel *et al.*, 1995; Zhang *et al.*, 1997). JunD, Fos family members and JunB have been identified within the complex binding to the CLE0 element in the mouse thymoma line EL-4 (Siegel *et al.*, 1995). In addition, ectopic expression of JunB in Th1 cells was found to induce not only IL-4 but also IL-5 production (Li *et al.*, 1999). In the loss-of-function approach

described here, we demonstrated that JunB is not only sufficient but is absolutely required for IL-5 expression in Th2 cells. The precise mechanism by which JunB controls IL-5 production in Th2 cells remains to be clarified, but it is feasible to hypothesize that JunB directly binds to the AP-1 site of the IL-5 gene promoter activating IL-5 transcription. If this is true, IL-5 would be another example of a potentially growing list showing that in addition to the proposed function as a transcriptional repressor, JunB also positively regulates expression of AP-1 target genes (Li *et al.*, 1999; Schorpp-Kistner *et al.*, 1999; Passegue and Wagner, 2000; Passegue *et al.*, 2001; Andrecht *et al.*, 2002).

Production of the Th2 cytokine IL-13 was normal in JunB-deficient Th2 cells. Sequence analysis of the IL-13 gene promoter revealed potential binding sites for NF-AT, GATA-3 and AP-1 (Buitkamp *et al.*, 1999), but so far functional significance has only been demonstrated for GATA-3 (Kishikawa *et al.*, 2001). In line with these data, we could find expression of GATA-3 and consequently of IL-13 in the JunB-deficient Th2 cells excluding a requirement for JunB. Our data suggest that, in contrast to IL-4 and IL-5, JunB is either not involved in IL-13 gene expression or it can be replaced by other AP-1 components. The requirement of JunB for IL-4 and IL-5 expression and JunB-independent IL-13 production support previous suggestions (Ho *et al.*, 1996; Zhang *et al.*, 1998; Kim *et al.*, 1999; Kishikawa *et al.*, 2001) that despite their close chromosomal linkage, the mechanisms regulating the expression of these Th2 cytokines are distinct from each other. In the future, it will be interesting to examine whether JunB is also involved in positive or negative regulation of other Th2 specific cytokine genes, such as IL-10, IL-9 and IL-6, or of other Th2 characteristic features such as CCR3 and CCR4 chemokine receptor expression (Bonecchi *et al.*, 1998; D'Ambrosio *et al.*, 1998).

JunB-deficient Th2 cells do not only display an impaired expression of the Th2 cytokines IL-4 and IL-5 but also produce high levels of the Th1-specific cytokine IFN- γ . This phenotype may be explained by JunB acting as a repressor of IFN- γ transcription, possibly through a proximal regulatory element in the IFN- γ promoter, which is capable of binding CREB, Oct-1 and AP-1 proteins (Penix *et al.*, 1996). Alternatively, c-Jun may be a positive regulator of IFN- γ promoter activity and antagonistic action of JunB on c-Jun may be required to properly regulate IFN- γ transcription, as previously shown for GM-CSF and KGF expression in the skin (Szabowski *et al.*, 2000). Enhanced levels of IFN- γ may explain the aberrant expression of T-bet in JunB-deficient Th2 cells, as it has recently been shown that IFN- γ is a potent inducer of T-bet expression (Lighvani *et al.*, 2001).

Yet, JunB may also serve as a negative regulator of T-bet gene expression in Th2 cells, in a direct or indirect manner. This is supported by the fact that the level of JunB protein is selectively induced in Th2, but not in Th1, wild-type cells (Li *et al.*, 1999). Since T-bet has been reported to induce IFN- γ expression and to repress IL-4 and IL-5 in fully differentiated Th2 cells (Szabo *et al.*, 2000), loss of a supposed JunB-mediated T-bet repression could explain the dysregulated cytokine profile observed in JunB-deficient cells. However, as only a little is known about

regulation of T-bet expression, further investigation will require cloning and characterization of T-bet promoter regions, which are currently not available.

During the priming of wild-type Th cells, T-bet and GATA-3 are expressed in a mutually exclusive manner (Zhang *et al.*, 1997; Zheng and Flavell, 1997; Szabo *et al.*, 2000). Interestingly, the JunB-deficient CD4⁺ T cell population that was strictly kept under Th2 polarizing conditions expressed both GATA-3 and T-bet. The population of JunB-deficient Th2 cells may consist either of (i) two different subtypes, one expressing GATA-3 and the other expressing T-bet, or (ii) of one homogenous cell group expressing both transcription factors simultaneously. Preliminary data of intracellular IFN- γ /IL-4 double-staining experiments showed a major group of cells producing large amounts of IFN- γ , but lacking IL-4, and a minor group expressing reduced levels of IL-4 and no IFN- γ (data not shown), suggesting the existence of two subgroups in the JunB-deficient Th2 population.

In agreement with our *in vitro* data, the mice with JunB-deficient T cells were affected in developing symptoms of allergen-induced airway inflammation. As numerous studies have demonstrated the critical role of Th2 cells in allergic diseases such as allergic asthma (Robinson *et al.*, 1992; Gleich and Kita, 1997; Romagnani, 1997; Umetsu and DeKruyff, 1997; Ray and Cohn, 1999; O'Garra and Arai, 2000), the murine model of allergen-induced airway inflammation provides an excellent tool to study Th2 responses *in vivo* (Yang *et al.*, 1998; Cohn *et al.*, 1999; Zhang *et al.*, 1999; Das *et al.*, 2001; Finotto *et al.*, 2001). In addition to Th2 cells, other players of the immune response, such as B cells, mast cells and eosinophils, have an important function in this process (for reviews, see Holgate, 1999; Hamelmann and Gelfand, 2001). At present, we cannot exclude that JunB-dependent defects in these cell types occur in the JunB mutant mice described here. Nevertheless, our loss-of-function approach complements and functionally supports the result of the reciprocal transgenic approach, showing that mice with an enhanced JunB activity in the CD4⁺ T cells display an augmented Th2 cytokine expression and a shift towards Th2 characteristics (Li *et al.*, 1999; Fang *et al.*, 2002).

Loss of JunB *in vivo* has dramatic consequences in the animal, e.g. during placentation (Schorpp-Kistner *et al.*, 1999) and transformation of myeloid cells (Passegue *et al.*, 2001). In contrast, apart from the CD4⁺ T cell population, previously analysed cells from JunB-overexpressing mice did not yield an obvious phenotype (Schorpp *et al.*, 1996; Schorpp-Kistner *et al.*, 1999; Passegue *et al.*, 2002). In the light of previous data (Li *et al.*, 1999; Fang *et al.*, 2002), our results have identified CD4⁺ T cells as the first example where JunB expression is not only essential, but has to be tightly adjusted to ensure a proper cell function, such as a Th2 immune response. Obviously, dysregulated JunB expression in either direction will result in a shift of Th1- and Th2-specific genetic programs, which dominantly determine the fate of the CD4⁺ T cell. The JunB *rescue* mouse lines described may be an informative tool to define more precisely the process and factors of T helper cell differentiation and may provide new insights into the pathogenesis of immunological diseases caused by an imbalance in Th1 and Th2 cell populations.

Materials and methods

Mice

JunB^{-/-} mice have been described previously (Schorpp-Kistner *et al.*, 1999). Transgenic *junB*^{-/-} (288R, 311R), transgenic *junB*^{+/+} (288K, 311K) and non-transgenic *junB*^{+/+} (wild type) littermates were all on a C57BL/6 background. Genotyping was performed by PCR using genomic tail DNA as described previously (Schorpp-Kistner *et al.*, 1999). Mice aged between 6 and 8 weeks were used. All mice were housed in a specific-pathogen-free facility at the DKFZ, Heidelberg, under light, temperature and humidity controlled conditions. The procedures for performing animal experiments were in accordance with the principles and guidelines of the ATBW (officials for animal welfare) and were approved by the Regierungspräsidium Karlsruhe, Germany.

Flow cytometry analysis

Single-cell suspensions of spleens of 311R, 288R and control mice or *in vitro* differentiated T helper cells were stained with FITC-, PE- or APC-conjugated antibodies to surface markers (CD4, CD8, CD3, CD19, CD69, CD25) from PharMingen (San Diego, CA). The cells were pre-incubated with anti-FC γ R for 10 min, stained with the antibodies listed above for 15 min and, after washing, cells were analysed on a FACScan flow cytometer (Beckton Dickinson, Los Angeles, CA) and CellQuest software.

In vitro differentiation, T cell activation and proliferation

CD4⁺ cells were isolated from spleens of 6- to 8-week-old mice by positive selection using monoclonal antibodies to CD4 coupled to magnetic beads (Dynabeads, Dynal, Oslo, Norway) according to manufacturer's instructions. CD4⁺ cells (1×10^6 cells/ml) were stimulated under Th1- or Th2-differentiating conditions *in vitro* either with ConA (5 μ g/ml) or with plate-bound anti-CD3 (2 μ g/ml) and anti-CD28 (2 μ g/ml) from PharMingen (San Diego, CA). Cells were cultured in TCM (DMEM, 10% FCS, 5 mM β -mercaptoethanol, 50 U/ml IL-2) along with either IL-12 (4 ng/ml) for Th1 differentiation or IL-4 (1000 U/ml) and anti-IL-12 (5 μ g/ml) for Th2 differentiation. All murine recombinant interleukins were from PharMingen and the anti-IL-12 mAb was from R&D Systems (Minneapolis, MN). To determine T cell activation potential, 1×10^5 cells were stained for the surface activation markers CD69 and CD25 directly after isolation or 18 and 48 h after stimulation, respectively. Stained cells were analysed by flow cytometry. To determine cell proliferation response to ConA, CD4⁺ T cells (1×10^6 cells/ml) were stimulated and cultured under Th1 and Th2 polarizing conditions, respectively. Proliferation was determined by measuring the increase in ATP content by Vialight[®] HS (LumiTech, Nottingham, UK) according to manufacturer's instructions directly after stimulation (0 h), 24, 48 and 72 h later. To obtain the amplification rate, relative light units (RLUs) observed at 24, 48 and 72 h were referred to RLUs observed at 0 h.

Cytokine measurements

For measurement of cytokine production by ELISA, we used CD4⁺ T cells cultivated for 4–7 days under Th2-differentiating conditions. Cells were washed extensively, resuspended in TCM at a concentration of 1×10^6 cells/ml and restimulated with ConA (5 μ g/ml) or with plate-bound anti-CD3 (2 μ g/ml) and anti-CD28 (2 μ g/ml), respectively. For IL-4 and IL-13, 6 h and 24 h, and for IL-5, 24 h post-restimulation, protein levels in cell culture supernatants were assayed using ELISA kits as recommended by the manufacturer (R&D Systems, Minneapolis, MN).

For intracellular staining, CD4⁺CD62L^{high} cells were isolated from spleen and lymph node of mice by positive selection with antibodies to CD4 and CD62L in succession (MACS, Myleni Biotech, Bergisch Gladbach, Germany) according to manufacturer's instructions. Cells were cultivated for 4 days under Th2-differentiating conditions, were restimulated with ConA (5 μ g/ml) or phorbol-12-myristate-13-acetate (PMA; 10 ng/ml)/ionomycin (250 ng/ml) for 4 h and brefeldin A (10 μ g/ml) was added to the dish in the last 2.5 h. Cells were then fixed in 4% paraformaldehyde (PFA) for 30 min at 4°C and after permeabilization with saponin (0.5% in PBS) cells were stained with PE-coupled anti-IL-4 or FITC-coupled anti-IFN- γ for 30 mins. After washing, cells were analysed on a FACScan flow cytometer (Beckton Dickinson) and CellQuest software.

RNA preparation, RNase protection and northern blotting

Total RNA from cells or tissues were prepared using peqGold RNAPure[™] kit (PeqLab, Erlangen, Germany) according to manufacturer's protocol.

RNAse protection mapping was performed with total RNA as described previously (Schorpp *et al.*, 1996). For northern blot analyses, total RNA were electrophoresed on a 1.4% agarose gel, blotted onto Hybond N+ membrane (Amersham Pharmacia Biotechnology) and hybridized at 65°C overnight with Church-Gilbert hybridization buffer (1 mM EDTA, 0.25 M NaPi, 7% SDS). The T-bet cDNA probe was generated by PCR using the primers 5'-CGCTGGGGCCCTTCTCCTTTTG-3' (sense) and 5'-CCCAGTCCGCCCGCAGTCACC-3' (antisense). The GATA-3 cDNA probe was generated by PCR using the primers 5'-GGGCGCGAG-CACAGC-3' (sense) and 5'-ATACTGCTCCTGCGAAAAAC-3' (antisense). A probe for 18S rRNA was used as an internal control for RNA quality and quantity. Probe labelling with [³²P]dCTP was performed using RediPrime™ Labelling Kit (Amersham Pharmacia Biotechnology).

Preparation of nuclear extracts

Cells were collected by centrifugation at 1800 g for 15 s at 4°C and gently resuspended in 400 µl buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2.5 mM DTT, proteinase inhibitor cocktail). Suspension was incubated on ice for 15 min and 25 µl of 10% Nonidet P-40 was added. Next, the suspension was vortexed for 10 s and centrifuged at 6000 g for 1 min at 4°C. The nuclear pellet was washed once in 500 µl buffer A. Pelleted nuclei were floated in buffer C (20 mM HEPES pH 7.9, 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 2.5 mM DTT, proteinase inhibitor cocktail), samples were put on shaker for 15 min at 4°C and centrifuged at 6000 g for 5 min at 4°C. The supernatant was used for determination of protein content and immunoblot analysis.

Immunoblot analysis

Thirty micrograms of protein from nuclear extracts was subjected to 10% SDS-PAGE and after electrophoresis, proteins were transferred onto a PVDF membrane (Millipore, Bedford, MA). After blocking with 10% non-fat dry milk in TBS-T (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Tween 20) for 2 h at room temperature, the membrane was incubated with the specific antibody in 3% non-fat dry milk rotating overnight at 4°C. The antibody to JunB was from Santa Cruz Biotechnology (Santa Cruz, CA) and the one to primase p58 was from NeoMarkers (Fremont, CA). The membrane was washed with TBS-T and incubated for another hour with HRP-conjugated secondary antibody (DAKO Diagnostika GmbH, Hamburg, Germany). After washing, bands were visualized by using a chemoluminescence kit (Renaissance™, NEN Lifescience, Boston, MA).

Sensitization and challenge of mice

Mice were sensitized and challenged as described previously (Hamelmann *et al.*, 1997). 311R and control littermates received i.p. injections of 20 µg OVA (Sigma, Taufkirchen, Germany) in 2 mg alum (Pierce, Rockford, IL). After 14 days, mice received a second i.p. injection of 20 µg OVA in alum. Sham-immunized controls always received injection of 2 mg alum in PBS. Twenty-eight days after the first immunization, mice were challenged with inhaled 1% OVA in PBS for 20 min daily over a period of three days. For inhalation, mice were placed in a plastic chamber (28 × 22 × 10 cm) fitted with an attachment to allow entry of the aerosol from an ultrasonic nebulizer (DeVILBISS, Mode II Ultra-Neb 99, Dietzenbach, Germany). Two small holes on the opposite end of the chamber ensured continuous airflow. Forty hours after the final inhalation procedure, mice were sacrificed and the lungs were analysed histologically.

Lung histology

The lungs were perfused via the right ventricle with PBS to remove residual blood, dissected, fixed with 4% PFA over night and embedded in paraffin. Sagittal sections from the left lobe were stained with hematoxylin/eosin for determination of eosinophilic infiltrates or with PAS for evaluation of mucus (Romeis, 1989).

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