The EMBO Journal Vol.18 No.10 pp.2803–2811, 1999

Rel/NF-κB can trigger the Notch signaling pathway by inducing the expression of Jagged1, a ligand for Notch receptors

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Jagged1 belongs to the DSL family of ligands for Notch receptors that control the proliferation and differentiation of various cell lineages. However, little is known about the transcription factors that regulate its expression. Here, we show that Jagged1 is a Rel/NF-κB-responsive gene. Both c-Rel and RelA induced *jagged1* gene expression, whereas a mutant defective for transactivation did not. Importantly, *jagged1* transcripts were also upregulated by endogenous NF-κB activation and this effect was inhibited by a dominant mutant of IκBα, a physiological inhibitor of NF-κB. Cell surface expression of Jagged1 in c-Rel-expressing cell monolayers led to a functional interaction with lymphocytes expressing the Notch1/TAN-1 receptor. This correlated with the initiation of signaling downstream of Notch, as evidenced by increased levels of *HES-1* transcripts in co-cultivated T cells and of *CD23* transcripts in co-cultivated B cells. Consistent with its Rel/NF-κB-dependent induction, Jagged1 was found to be highly expressed in splenic B cells where c-Rel is expressed constitutively. These results demonstrate that c-Rel can trigger the Notch signaling pathway in neighboring cells by inducing *jagged1* gene expression, and suggest a role for Jagged1 in B-cell activation, differentiation or function. These findings also highlight the potential for an interplay between the Notch and NF-κB signaling pathways in the immune system.

Keywords: Jagged1/NF-κB/Notch/Rel/TAN-1

Introduction

The Rel/NF-κB transcription factors play fundamental roles in the immune system. These structurally related proteins share common pathways of activation that involve their release from inhibitory IκB factors in response to stimuli (reviewed in Whiteside and Israel, 1997). The degradation of IκB results in the nuclear translocation of active Rel dimers, their binding to κB DNA sites and the activation of cellular gene expression (reviewed in Baldwin, 1996). NF-κB regulates the expression of various cytokines, chemokines, immunoreceptors, growth factors, acute phase proteins and adhesion molecules (reviewed in Kopp and Ghosh, 1995; May and Ghosh, 1997). It is therefore not surprising to find its implication in the immune, inflammatory and acute phase responses (reviewed in Wulczyn *et al*., 1996; Barnes and Karin, 1997).

The *Notch* gene family encodes transmembrane receptors that control cell proliferation and differentiation in response to extracellular ligands expressed on neighboring cells (reviewed in Robey, 1997; Weinmaster, 1997). Among the four mammalian Notch receptors identified to date, three of them are expressed in hematopoietic cells. Human *Notch1/TAN-1* and its murine counterpart are found in spleen, $CD34^+$ hematopoietic stems cells and early progenitor cells, thymic T cells, peripheral blood lymphocytes and a B-lymphoma cell line (Ellisen *et al*., 1991; Milner *et al*., 1994; Hasserjian *et al*., 1996). Similarly, *Notch2* is highly expressed in spleen, and both *Notch2* and *Notch3* are found in hematopoietic and myeloid progenitor cell lines (Weinmaster *et al*., 1992; Milner *et al*., 1996; Bigas *et al*., 1998). *Notch1* was isolated originally as a translocation in a human acute T-cell lymphoblastic leukemia/lymphoma, and its constitutively active form produces T-cell neoplasms in mice (Ellisen *et al*., 1991; Pear *et al*., 1996). Two other *Notch* genes also map to chromosomal locations associated with human immune system malignancies (Larsson *et al*., 1994; reviewed in Gridley, 1997).

Jagged1, Jagged2, Delta1 and Delta2 belong to the DSL (Delta/Serrate/LAG-2) family of ligands for mammalian Notch receptors (reviewed in Weinmaster, 1997). All are transmembrane proteins with an extracellular domain important for receptor binding. Ligand-mediated activation of Notch induces the proteolytic release of the intracellular domain of Notch, activation of cellular gene expression and suppression of differentiation (Schroeter *et al*., 1998; reviewed in Robey, 1997; Weinmaster, 1997; Bray, 1998; Chan and Jan, 1998). Studies point to the CSL [CBF1/Su(H)/Lag-1/RBP-Jκ/KBF2] family of DNA-binding proteins as downstream effectors in this pathway (Brou *et al*., 1994; Dou *et al*., 1994; Fortini and Artavanis-Tsakonas, 1994; Jarriault *et al*., 1995; Tamura *et al*., 1995; Hsieh *et al*., 1996). In the nucleus, CSL factors serve as docking proteins that direct the intracellular domain of activated Notch to the promoter of target genes (Jarriault *et al*., 1995; Hsieh *et al*., 1996; Kopan *et al*., 1996; Schroeter *et al*., 1998; reviewed in Bray, 1998; Chan and Jan, 1998). The mammalian basic helix–loop–helix (bHLH) transcription factor Hairy Enhancer of Split

(HES-1) and the B-cell activation marker CD23 are two cellular target genes directly regulated by this pathway (Wang *et al*., 1987, 1991; Cordier *et al*., 1990; Sasai *et al*., 1992; Ling *et al*., 1994; Bailey and Posakony, 1995; Jarriault *et al*., 1995, 1998; Lecourtois and Schweisguth, 1995; de la Pompa *et al*., 1997). Little is known of the factors that trigger ligand-mediated signaling upstream of Notch.

In this report, we identified *jagged1* as a novel Rel/NF-κB-responsive gene. We show that *jagged1* gene expression can be induced specifically by transcriptionally competent NF-κB subunits and that it is suppressed by a super-IκBα inhibitor (IκBαΔN). Our finding that NF-κB-mediated induction of Jagged1 can initiate signaling downstream of Notch correlates with the expression of Jagged1 in peripheral lymphoid tissues. These results suggest a direct interplay of the Notch and NF-κB signaling pathways in the immune system, both of which have been implicated in lymphoid cell proliferation and function.

Results

Ectopic Rel protein expression induces jagged1 transcripts

The CCR43 cell line conditionally expresses c-Rel under the control of a tetracycline-regulated transactivator (tTA), resulting in inducible transactivation of Rel target genes such as *IκBα* (Bash *et al*., 1997). An mRNA differential display analysis identified a 106 bp cDNA fragment that was induced specifically in CCR43 cells expressing c-Rel. When used as a probe in Northern blots, this fragment hybridized to a 5.8 kb transcript induced in response to c-Rel expression in CCR43 cells (data not shown). A complete cDNA was isolated from a human HeLa cell library (clone 14-6; 5457 bp). This clone showed 90% homology to rat *jagged1* and was virtually identical to two human *jagged1* cDNA clones that were isolated recently (Lindsell *et al*., 1995; Oda *et al*., 1997; Li *et al*., 1998). Clone 14-6 contained a 3657 bp open reading frame encoding a 1218 amino acid human Jagged1 protein, along with 263 bp of 5′-untranslated sequence and 1537 bp of 3′-untranslated sequence (DDBJ/EMBL/GenBank accession No. AF028593). Two amino acid differences were found between the Jagged1 protein sequence reported by Oda *et al*. (1997) and the sequence described by us and by Li *et al*. (1998).

The differential expression of *jagged1* was characterized further by Northern blot analysis of CCR43 cells during a time course of c-Rel induction, using the *jagged1* cDNA as a probe (Figure 1A). *jagged1* transcripts were induced with kinetics that paralleled those for c-*rel* and for the Rel/NF-κB target gene encoding IκBα (lanes 10–18). No induction was seen in the parental HtTA-1 cells that expressed the tTA activator alone (lanes 1–9), indicating that *jagged1* is under c-Rel control. The transactivation of *jagged1* was specific, as endogenous transcripts for the ligand Delta1 and for the Notch1/TAN-1 receptor were unaffected (Figure 1B, lanes 1 and 2). Importantly, a cell line expressing a mutant c-Rel protein deleted of its transactivation domain (denoted CCR-H5) failed to upregulate *jagged1* transcripts (Figure 1B, lanes 3 and 4). Similar results were observed with cells expressing p50/NF-κB1, a subunit of NF-κB that fails to activate gene expression from most promoters containing a κB DNA site (data not shown). In contrast, *jagged1* transcripts were induced in HtTA-RelA cells that conditionally expressed the RelA transactivating subunit of NF-κB, although some leaky expression of *jagged1* was observed in these cells in the presence of tetracycline (Figure 1C, lanes 1 and 2). Together, these results indicated that the transcriptional activity of c-Rel and RelA was necessary to activate *jagged1* gene expression.

jagged1 gene expression is dependent upon endogenous NF-κB activation

To investigate further the functional linkage between *jagged1* and Rel/NF-κB factors, we monitored the effect of endogenous NF-κB activation on *jagged1* gene expression. Human Jurkat T cells were stimulated with phorbol 12-myristate 13-acetate (PMA) plus ionomycin to activate endogenous NF-κB factors. This treatment led to a 4-fold increase in *jagged1* mRNA levels in comparison with unstimulated cells (Figure 1D, compare lanes 1 and 2). Similar results were obtained in human CEM T cells (data not shown). Importantly, the constitutive repression of endogenous NF-κB by a dominant mutant of IκBα in Jurkat T cells interfered strongly with the induction of *jagged1* in response to PMA plus ionomycin (Figure 1D, lanes 3 and 4; Boothby *et al*., 1997; Chu *et al*., 1997). Rehybridization to an interleukin-2 receptor alpha (IL-2Rα) probe confirmed the activation of NF-κB in Jurkat T cells treated with PMA plus ionomycin, and the reduction in NF-κB activation in Jurkat-IκBαΔN cells (Figure 1D, lanes 2 and 4). These results agreed with our findings in the CCR43 and HtTA-RelA cell lines and indicated that the expression of *jagged1* was dependent on Rel/NF-κB activity.

Cell surface expression of Jagged1 promotes cell–cell interactions

The expression of c-Rel led to increased levels of Jagged1 protein in CCR43 cells. Immunoblot analyses using a monoclonal antibody specific for the unique intracellular domain of human Jagged1 (Ab #TS1) showed the accumulation of Jagged1 in response to c-Rel induction (Figure 2A, lanes 1–5). Immunofluorescence analysis of live non-permeabilized cells using an antibody directed against the extracellular domain of Jagged1 verified the localization of the protein at the surface of CCR43 cells expressing c-Rel (Figure 2B, panel c). In contrast, only background staining was detected in CCR43 cells cultured in the presence of tetracycline to prevent c-Rel expression (panel b). Taken together, these results indicated the accumulation of the Jagged1 protein at the cell surface in response to c-Rel expression.

The Notch signaling pathway is triggered by the interaction of cells expressing Notch receptors with cells that express Notch ligands at their surface. We tested whether the Rel-dependent induction of Jagged1 in CCR43 cells could promote a functional interaction with cells expressing endogenous Notch1/TAN-1 receptors in co-cultivation assays. As shown in Figure 3A, non-adherent human Jurkat T cells that expressed a wild-type *TAN-1* gene aggregated onto a monolayer of CCR43 cells induced to express c-Rel (panel b). This interaction was observed

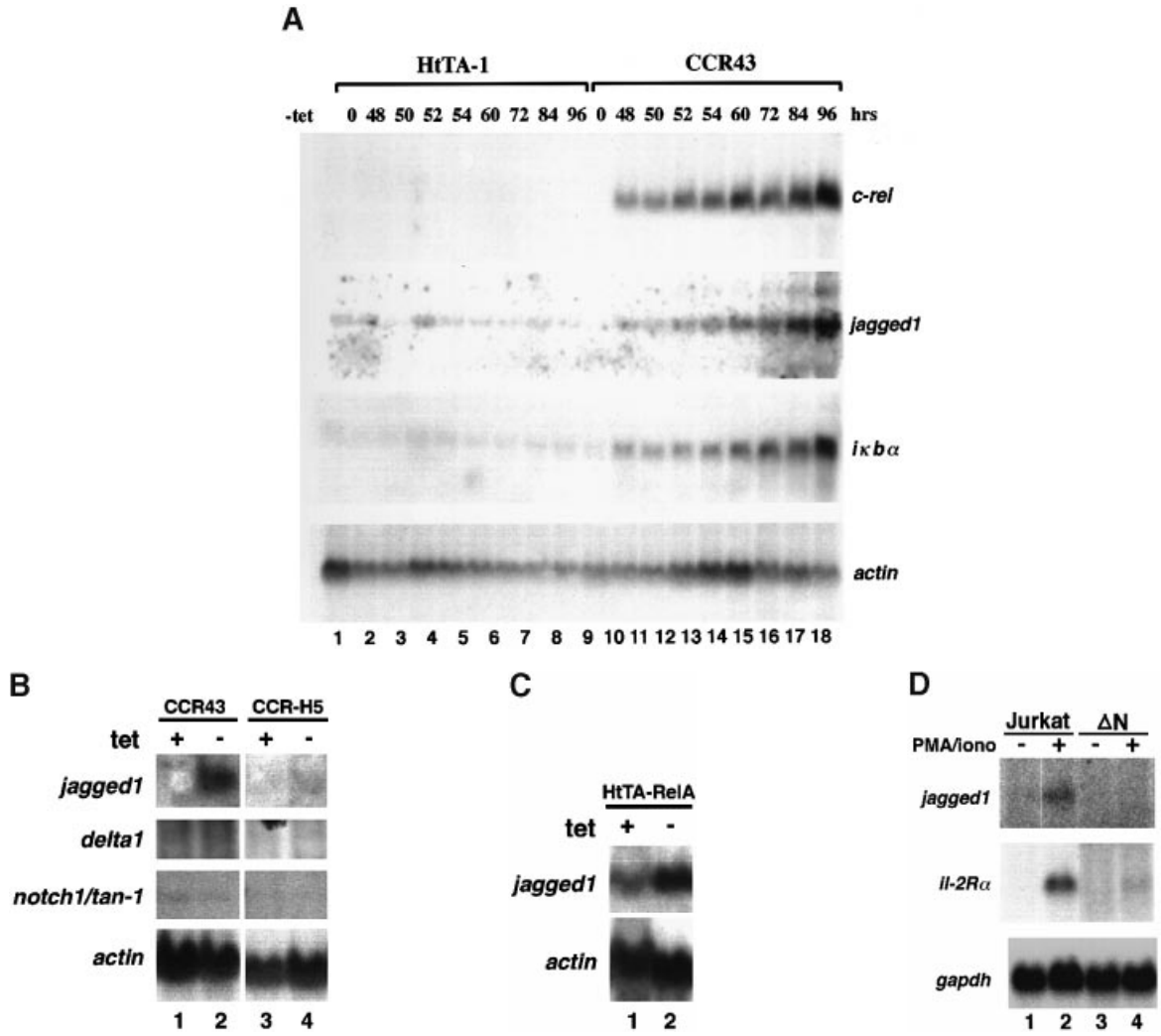

Fig. 1. Rel/NF-κB-dependent induction of *jagged1* expression. (**A**) Northern blot analysis of *jagged1* expression in CCR43 cells, during a time course of c-Rel induction. RNA from control HtTA-1 cells (lanes 1–9) and CCR43 cells (lanes 10–18) maintained in the presence (lanes 1 and 10) or absence (lanes 2–9 and 11–18) of tetracycline, was analyzed using human *jagged1* cDNA clone λ2-3 as a probe. The blot was rehybridized successively to control c-*rel*, *IκBα* and actin gene probes. (**B**) Comparative Northern blot analysis of *jagged1*, *delta1* and *Notch1/TAN-1* transcripts in cells expressing wild-type c-Rel or a mutant c-Rel protein deleted of its transactivation domain. CCR43 (lanes 1 and 2) and CCR-H5 cells (lanes 3 and 4) were cultured in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of tetracycline. Expression was analyzed with probes derived from the human *jagged1* cDNA clone λ2-3, a rat *delta1* cDNA and a human *Notch1/TAN-1* cDNA fragment. Rehybridization to actin was used as a control. (**C**) Northern blot analysis of *jagged1* expression in HtTA-RelA cells expressing the RelA protein. HtTA-RelA cells were cultured in the presence (lane 1) or absence of tetracycline to induce RelA expression (lane 2). Expression was analyzed using the human *jagged1* cDNA clone λ2-3 as a probe. Rehybridization to actin was used as a control. (**D**) Induction of *jagged1* gene expression in response to endogenous NF-κB activation in T cells. Jurkat T cells and a Jurkat T-cell clone that expressed a dominant IκBαΔN inhibitor of Rel/NF-κB factors were left untreated (lanes 1 and 3), or were stimulated with PMA plus ionomycin for 2 h to activate endogenous NF-κB factors (lanes 2 and 4). Total cellular RNA (20 μg) was analyzed for the expression of *jagged1* transcripts in Northern blots. The blot was rehybridized successively to Il-2Rα and *gapdh* probes as controls.

within 2 h of co-cultivation. Whereas gentle washings failed to disrupt the cell aggregates that formed on c-Rel-expressing cell monolayers, few T cells remained attached to CCR43 cells that were not induced to express c-Rel (Figure 3A, compare panels a and b). No aggregation was observed when Jurkat T cells were cultivated with cell monolayers expressing a mutant c-Rel protein deleted of its transactivation domain (CCR-H5 cells) or with the parental HtTA-1 cells (panels c and d; data not shown). These results agreed with our finding that the transcriptional activity of c-Rel was necessary to induce the expression of *jagged1*.

Although the data suggested the interaction of Jagged1 and Notch proteins expressed at the surface of CCR43 and Jurkat T cells, respectively, it remained possible that cytokines or cell adhesion molecules induced by c-Rel could be responsible for the observed cell interactions. We therefore investigated whether an intact Notch receptor was required for cell aggregation, by testing the ability of CCR43 cells to promote the aggregation of human Sup-T1 T cells. Both Jurkat T cells and Sup-T1 T cells are derived from a human T-lymphoblastic leukemia. While Jurkat T cells lack any known abnormality of chromosome 9 and express an intact Notch1/TAN-1 receptor, Sup-T1 T cells contain two copies of a t(7;9)(q34;q34.3) translocation and have no normal *Notch/*

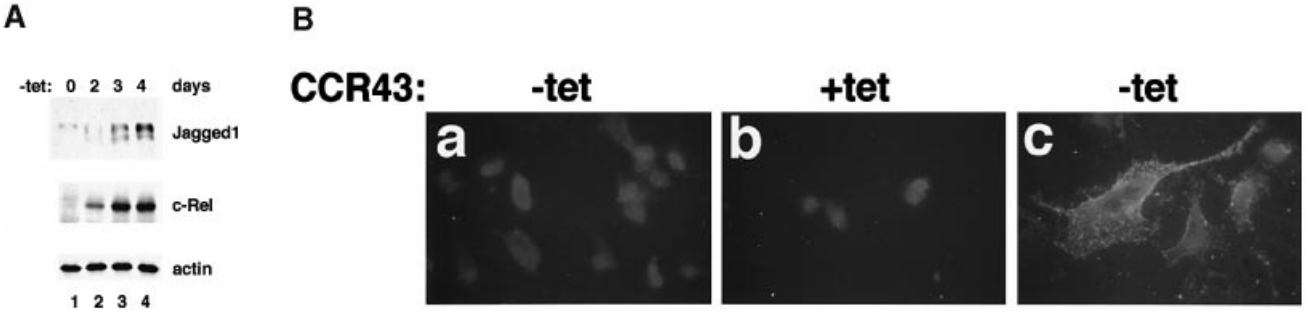

Fig. 2. Jagged1 protein expression in CCR43 cells. (**A**) Immunoblot analysis of the Jagged1 protein in CCR43 cells induced to express c-Rel. CCR43 cells were left uninduced (lane 1) or were induced to express c-Rel (lanes 2–4). Cell extracts (20 μg) were resolved by SDS–5% PAGE and analyzed by ECL immunoblotting using monoclonal anti-human Jagged1 antibody #TS1. Immunoblotting for c-Rel and actin were used as controls. (**B**) Immunofluorescence analysis of Jagged1 protein expression at the surface of CCR43 cells induced to express c-Rel. Live non-permeabilized CCR43 cells were left uninduced (panel b) or were induced to express c-Rel (panels a and c), and stained with polyclonal anti-Jagged1 antibody #PCR8 (panels b and c). CCR43 cells induced to express c-Rel were stained with a pre-immune antibody as a control (panel a).

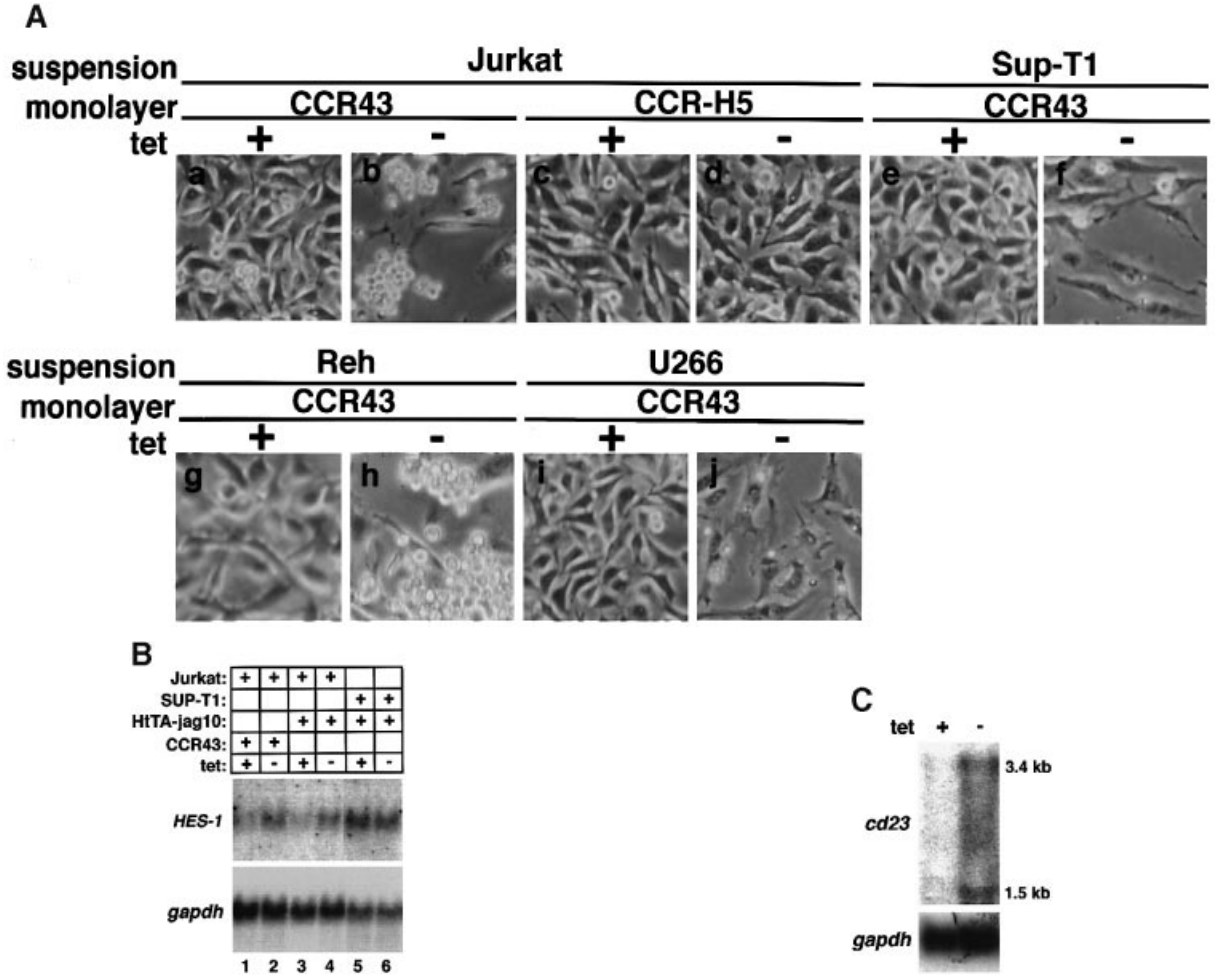

Fig. 3. Functional interaction between c-Rel-expressing cells and cells expressing endogenous Notch1/TAN-1 receptors. (**A**) Co-cultivation assay. Non-adherent human Jurkat T cells (a–d), Sup-T1 T cells (e and f), Reh pre-B cells (g and h) or U266 B cells (i and j) were co-cultivated onto adherent monolayers of uninduced (a, c, e, g and i) or induced (b, d, f, h and j) CCR43 cells (a, b and e–j), or CCR-H5 cells (c and d) for 48 h. After co-cultivation, the non-adherent cells were gently washed away and the cells that remained attached onto the cell monolayers were photographed. In agreement with our previous studies showing a c-Rel-mediated growth arrest in CCR43 cells, the density of the induced CCR43 cell monolayers in panels b, f, h and j is lower than that in panels a, c, d, e, g and i. (**B**) Northern blot analysis of endogenous *HES-1* gene expression in Jurkat T cells (lanes 1–4) or Sup-T1 T cells (lanes 5 and 6) co-cultivated onto cell monolayers of uninduced or induced CCR43 cells (lanes 1 and 2), and uninduced or induced HtTA-jag10 cells (lanes 3–6). The blot was rehybridized to a *gapdh* probe as a control. (**C**) Northern blot analysis of endogenous *CD23* gene expression in human Reh pre-B cells cultivated onto a CCR43 cell monolayer uninduced (lane 1) or induced to express c-Rel (lane 2). The blot was rehybridized to a *gapdh* probe as a control.

TAN-1 gene (Smith *et al*., 1988; Aster *et al*., 1994). The mutant TAN-1 protein in Sup-T1 T cells therefore lacks the extracellular domain necessary for ligand interaction (Ellisen *et al*., 1991; Aster *et al*., 1994). Consistent with this observation, the induction of c-Rel in CCR43 cells failed to induce the aggregation of Sup-T1 T cells

(Figure 3A, panel f). This is in sharp contrast to the cell aggregation that was observed with Jurkat T cells (Figure 3A, panel b). Similarly, human Reh pre-B cells that expressed high levels of endogenous *Notch1/TAN-1* transcripts aggregated onto the CCR43 cell monolayer induced to express c-Rel. In contrast, human U266 mature B cells that did not express any *TAN-1* failed to do so (compare panels h and j; Guan *et al*., 1996; data not shown). While it is possible that other factors may also contribute to these interactions, the results indicated that an intact Notch extracellular domain was necessary for the observed cell–cell interactions and suggested that c-Rel enabled a functional interaction between Jagged1 and Notch.

c-Rel leads to activation of the Notch signaling pathway

Ligand binding to the extracellular domain of Notch initiates signaling downstream of the receptor. The genes encoding the mammalian transcription factor HES-1 and the B-cell activation marker CD23 are two direct downstream targets of this signaling pathway (Wang *et al*., 1987, 1991; Cordier *et al*., 1990; Ling *et al*., 1994; Bailey and Posakony, 1995; Jarriault *et al*., 1995, 1998; Lecourtois and Schweisguth, 1995). We examined whether the c-Rel-dependent induction of Jagged1 could trigger Notch signaling in neighboring cells by monitoring its effect on endogenous *HES-1* and *CD23* gene expression in co-cultivated T cells and B cells, respectively. *HES-1* transcripts reproducibly were increased 2- to 3-fold in Jurkat T cells cultivated onto a monolayer of CCR43 cells expressing c-Rel in comparison with those cultivated with an uninduced CCR43 cell monolayer (Figure 3B, lanes 1 and 2). Importantly, *HES-1* transcripts were also induced in Jurkat T cells cultivated onto a HtTA-1-derived cell monolayer that expressed Jagged1 alone (HtTA-Jag10 cells; compare lanes 3 and 4). Rehybridization to a control *gapdh* probe confirmed the specificity of this induction. As anticipated, high levels of *HES-1* transcripts were observed in the control Sup-T1 T cells that express a truncated and constitutively active Notch-1/TAN-1 receptor (lanes 5 and 6). In agreement with these findings, the co-cultivation of human Reh pre-B cells with a CCR43 cell monolayer expressing c-Rel resulted in the induction of *CD23* gene expression, in comparison with Reh cells cultivated onto an uninduced CCR43 cell monolayer (Figure 3C). Together, these data support a model whereby c-Rel can trigger Notch signaling in neighboring cells by inducing the expression of Jagged1.

jagged1 is highly expressed in peripheral lymphoid organs and in splenic B cells

The induction of *jagged1* gene expression in response to endogenous Rel/NF-κB activation led us to examine its expression in immune tissues by Northern blot analysis. High levels of *jagged1* transcripts were found in human spleen and lymph nodes, in agreement with the high levels of c-*rel* transcripts in these tissues (Figure 4A, lanes 1 and 2). Lower levels of *jagged1* mRNAs were seen in human thymus, bone marrow and fetal liver (lanes 3, 5 and 6). No expression was detected in peripheral blood leukocytes (PBL; lane 4).

Immunohistochemistry analysis using a monoclonal antibody specific for the intracellular domain of the Jagged1 protein was used to investigate its expression in adult mouse spleen. Strong Jagged1 staining was confined to the B-cell areas of the spleen (Figure 4B, panel a). Positive cells were located mainly in the marginal zone. A weaker signal was detected in the perifollicular zone, the periarterial sheath and the red pulp. Jagged1 was found to co-localize with the B-cell antigen CD20 (compare panels a and c). In contrast, only background staining was observed with normal rat serum (panel b) and pre-immune mouse IgG (panel d), which were used as negative controls. Importantly, the distribution of Jagged1 in mouse spleen coincided with that previously reported for c-Rel (Carrasco *et al*., 1994).

Immunofluorescence assays were performed on purified mouse splenic B cells to examine further the correlation between the expression of Jagged1 and c-Rel. Flow cytometry analysis demonstrated that spleen cells expressing the B-cell marker CD45R-B220 were also positive for c-Rel and for Jagged1 protein expression (Figure 4C). These results are consistent with our finding of a correlation between the expression of Rel/NF-κB factors and that of Jagged1. These data suggest that Jagged1 may play a role in B-cell activation, differentiation or function.

Discussion

The important role of the Rel and NF-κB factors in the immune system has elicited a fervent search for Rel-regulated genes that may participate in the control of cell proliferation, differentiation and malignant transformation (reviewed in Luque and Gelinas, 1997). Here, we report that the gene encoding the Notch ligand *jagged1* is a novel NF-κB-responsive gene. The induction of *jagged1* by endogenous NF-κB factors and its inhibition by a dominant IκBαΔN transgene demonstrate its dependence on nuclear NF-κB transcriptional activity. This study is also shows that c-Rel can trigger the Notch signaling pathway in neighboring cells. The endogenous expression of Jagged1 in splenic B cells is consistent with its Rel-dependent induction and points to a possible role for Jagged1 in B-cell activation, differentiation or function.

Interplay between the Rel/NF-κB and Notch signaling pathways

This study is the first to demonstrate that Rel/NF-κB can function upstream of Notch. In our assays, c-Rel, RelA and endogenous NF-κB factors elicited specific activation of the Notch ligand Jagged1. In turn, the expression of *jagged1* correlated with the aggregation of non-adherent Jurkat T cells and Reh pre-B cells onto CCR43 cell monolayers. This effect was dependent on the transcriptional activity of c-Rel, as well as on the expression of an intact Notch receptor at the surface of co-cultivated lymphoid cells. Sup-T1 T cells that lacked a normal *Notch1/TAN-1* allele and U266 B cells that did not express *Notch1* failed to adhere to the cell monolayers and remained in suspension. We do not rule out the possibility that cytokines or adhesion molecules induced by c-Rel may also contribute to these interactions. However, the observed requirement for Notch receptor expression in these assays supports a model whereby c-Rel can promote

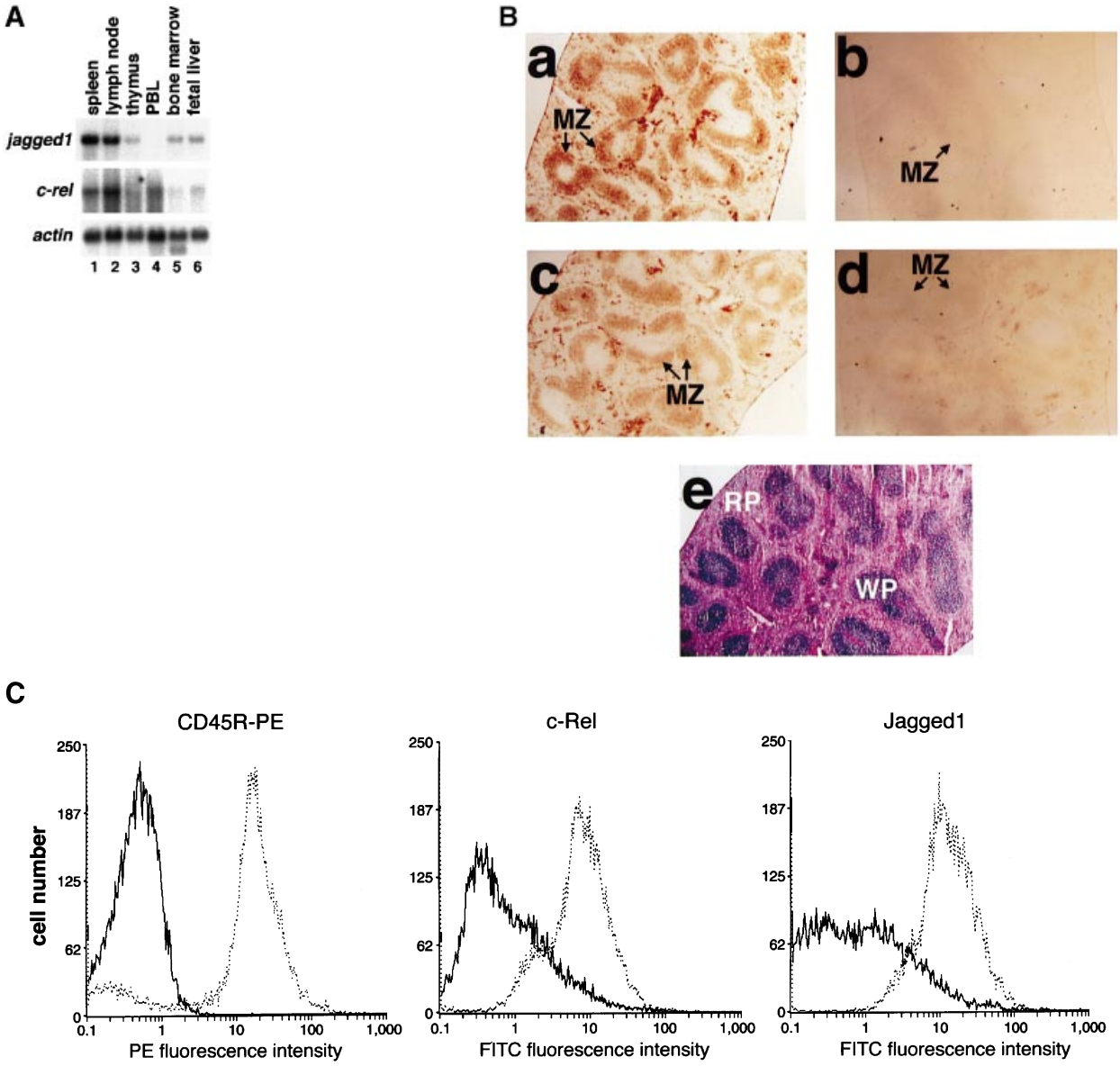

Fig. 4. Expression of *jagged1* in immune cells and tissues. (**A**) Northern blot analysis of endogenous *jagged1* gene expression in human immune tissues. A human immune system II Northern blot (Clontech) was hybridized to a human *jagged1* cDNA probe. The blot was rehybridized successively to c-*rel* and actin probes. (**B**) Immunohistochemical localization of Jagged1 to the B-cell areas of the spleen. Cross-sections from adult mouse spleen were analyzed by immunoperoxidase staining of a monoclonal anti-Jagged1 antibody (a) or a monoclonal anti-CD20 antibody (c). Normal rat serum (b) and pre-immune mouse IgG (d) were used as negative controls. The marginal zone (MZ) showed strong staining for Jagged1. Hematoxylin–eosin staining is shown (e). RP, red pulp; WP, white pulp. (**C**) Flow cytometry analysis of c-Rel and Jagged1 protein expression in mouse splenic B cells. Spleen cell populations were enriched for B cells by antibody plus complement-mediated killing of T cells. Cells were stained for expression of the B-cell marker CD45R-B220 with a PE-conjugated monoclonal antibody (left panel). This resulted in a population of >80% B cells. Cells were analyzed for expression of endogenous c-Rel (middle panel) or Jagged1 proteins (right panel) with polyclonal antibodies specific for c-Rel (sc-71) or Jagged1 (#PCR8), and a fluorescein isothiocyanate (FITC)-labeled secondary goat anti-rabbit antibody. Staining with the FITC-labeled secondary antibody alone was used a negative control (middle and right panels).

the functional interaction of Jagged1, with Notch receptors expressed at the surface of neighboring cells.

The Rel-dependent induction of *jagged1* in cell monolayers also coincided with the initiation of signaling downstream of Notch, as shown by the activation of two different CBF1/Su(H)/RBP-Jκ-regulated genes in co-cultivated lymphoid cells. Endogenous *HES-1* transcripts were upregulated reproducibly in Jurkat T cells cultured with CCR43 cell monolayers expressing c-Rel and Jagged1, or with HtTA-Jag10 cells expressing Jagged1 alone. This agrees with a recent report showing that ectopic expression of the vertebrate Notch ligand Delta-1 can transactivate endogenous *HES-1* gene expression in Notch1-expressing cells (Jarriault *et al.*, 1998). Likewise, the cultivation of Reh pre-B cells with a CCR43 cell monolayer led to the specific induction of mRNAs for the B-cell activation marker CD23. These results indicate that c-Rel can promote activation of the Notch signaling pathway and suggest that c-Rel-mediated activation of Jagged1 is responsible for this effect. Our findings do not

exclude the possibility that other transcription factors contribute to the regulation of *jagged1* gene expression in different cell lineages and at different stages of differentiation. It also remains to be determined whether Rel/NF-κB-mediated activation of *jagged1* gene expression is direct or indirect. Further work is required to isolate the *jagged1* promoter and establish whether its expression is controlled directly by Rel/NF-κB factors.

Relevance to the immune system

In addition to its role in neurogenesis, myogenesis, angiogenesis and retinal cell development, the Notch signaling pathway has also been implicated in hematopoiesis and in immune cell malignancies (reviewed in Robey, 1997). Consistent with this notion, the expression of Jagged1 was shown to promote the development of primitive hematopoietic precursor cells, whereas activated forms of Notch1 and Notch2 influenced the differentiation of myeloid progenitors in response to different cytokines (Bigas *et al*., 1998; Jones *et al*., 1998; Varnum-Finney *et al*., 1998). In independent studies, overexpression of an activated form of Notch1 influenced T-cell differentiation during thymic development (Robey *et al*., 1996; Washburn *et al*., 1997). This process recently was proposed to involve the silencing of *CD4* gene expression by HES-1 (Kim and Siu, 1998).

The mapping of three human *Notch* genes to chromosomal locations associated with leukemia, lymphoma and myeloproliferative disorders has also suggested a role in immune cell proliferation and malignancy (Ellisen *et al*., 1991; Larsson *et al*., 1994; Milner *et al*., 1994; Hasserjian *et al*., 1996; Gridley, 1997). The demonstration that constitutively active forms of Notch1 induced T-cell leukemia/lymphoma in mice confirmed this prediction (Pear *et al*., 1996). In experiments not shown here, we mapped the *jagged1* locus to human chromosome 20p12 by fluorescence *in situ* hybridization (FISH) in order to assess its possible implication in immune cell malignancies. While this study was in progress, mutations at this locus were shown to be associated with Alagille syndrome (Li *et al*., 1997; Oda *et al*., 1997). Although alterations in immune or hematopoietic function were not reported, it remains to be determined whether the disease results from haploinsufficiency or from a dominant-negative effect exerted by the mutant protein. The ability of the Notch signaling pathway to influence the differentiation and proliferation of different cell lineages may also depend on different inducing signals and on the cellular microenvironment.

Our immunohistochemistry analyses revealed that Jagged1 is highly expressed in the B-cell areas of the spleen, particularly in the splenic marginal zone that is rich in plasma and memory B cells. Consistent with these results, flow cytometry analyses further demonstrated a correlation between the expression of Jagged1 and c-Rel in purified mouse splenic B cells. Although the function of Jagged1 in secondary lymphoid organs remains to be determined, both Notch1 and Notch2 are also expressed in the spleen (Hsieh *et al*., 1997; data not shown). This raises the possibility of a role for Jagged1-mediated signaling through Notch in the pathways that control the later stages of B-lymphocyte activation, differentiation and/or immune function. The ability of the EBNA-2 protein of Epstein–Barr virus to induce expression of the B-cell activation marker CD23 through its association with the Notch effector CBF1 in transformed B lymphocytes agrees with a role for Notch signaling in B cells (Ling *et al*., 1994; Hsieh *et al*., 1997). Our co-cultivation assays demonstrating that Jagged1-expressing cells induced *CD23* gene expression in neighboring B cells is consistent with this hypothesis. Future studies will help to define the role of Jagged1 in the splenic microenvironment, and to clarify whether it signals through Notch in the context of a heterotypic cell–cell interaction or in a cell-autonomous fashion. Recent work suggesting that soluble forms of Notch ligands can trigger signaling through Notch *in vivo* would be compatible with either possibility (Qi *et al*., 1999).

c-*rel* knock-out mice are impaired for B-cell activation and antibody production (Kontgen *et al*., 1995; Carrasco *et al*., 1998). The observation that all known B-cell growth factors failed to rescue the proliferative defect of these cells suggests that c-Rel may regulate the expression of genes, other than those for cytokines and growth factors, which are crucial for the activation and proliferation of B lymphocytes (Kontgen *et al*., 1995). Our finding that c-Rel can trigger the expression of Jagged1 raises the possibility of a connection between the Rel/NF-κB and Notch signaling pathways in secondary lymphoid organs. Thus, in addition to controlling the expression of cytokines, immunoregulatory and adhesion molecules, Rel/NF-κB factors may also trigger a Notch signaling cascade important for lymphocyte activation and immune function.

Materials and methods

Plasmids and cell culture

pUHD10-3 contained seven tetracycline operator sites upstream of a minimal cytomegalovirus (CMV) promoter (Gossen and Bujard, 1992). An *Xho*I–*Eco*RI fragment containing the human *jagged1* cDNA in pBluescript was subcloned into pUHD10-3, after addition of an *Xba*I linker to its *Xho*I end (pUHD10-3-*jagged1*). pHMR272 was used to confer resistance to the drug hygromycin B (Bernard *et al*., 1985).

The parental HeLa-derived HtTA-1 cells stably expressed a fusion protein comprised of the *Escherichia coli* tetracycline repressor fused to the activation domain of the herpes simplex virus VP16 protein (tTA; Gossen and Bujard, 1992). HtTA-1-derived cell clones that conditionally expressed c-*rel* (CCR43 cells), *relA* (HtTA-RelA cells) or a truncated c-Rel protein lacking the c-*rel* transactivation domain were also described (CCR-H5 cells; Bash *et al*., 1997; Zong *et al*., 1998). The tetracycline-regulated HtTA-jag10 cell clone that conditionally expressed *jagged1* was generated by co-transfection of HtTA-1 cells with pUHD10-3-*jagged1* and pHMR272 using a modified calcium phosphate procedure, followed by selection for resistance to hygromycin B (225 U/ml, Calbiochem; Chen and Okayama, 1987). Drug-resistant colonies were screened for the inducible expression of Jagged1 using monoclonal antibody #TS1, specific for the intracellular domain of the human Jagged1 protein (a gift of Dr Artavanis-Tsakonas). All HtTA-1-derived cell clones were maintained in the presence of tetracycline (1 μg/ml).

Human Jurkat and Sup-T1 T-lymphoblastic leukemia cells, Reh pre-B cells and U266 B-myeloma cells were obtained from ATCC. The Jurkat T-cell clone constitutively expressing the IκBαΔN transdominant inhibitor of Rel/NF-κB was cultured in the presence of hygromycin B, as described previously (300 μg/ml; Chu *et al*., 1997). Where indicated, Jurkat T cells (5×10^5/ml) were treated with PMA (50 ng/ml) plus ionomycin (1 μM) for 2 h to induce endogenous NF-κB/Rel activity. Cells were treated with dimethylsulfoxide (DMSO; 0.1%) as a control.

Cloning of a human jagged1 cDNA

Differential display PCR (DD-PCR) analysis was performed as described previously (Liang and Pardee, 1992) using RNA from uninduced and induced CCR43 cells. RNA samples (2 μg) were treated with DNase and reverse transcribed with Superscript reverse transcriptase (Gibco-

BRL) and a 5′-T_{11}CA primer. Partial cDNA libraries were PCR amplified with 5′-T_{11}CA and 5′-AGACGTCTGT-3′ primers in the presence of [α-^{35}S]dATP and resolved on 6% sequencing gels. cDNA fragments differentially expressed in response to c-Rel were introduced into pCRII (Invitrogen) by TA cloning and screened by dot-blot analysis (Callard *et al*., 1994). One of these cDNA fragments (clone 1-2, 106 bp) was used to screen a human spleen cDNA library in λgt11 (Clontech). Human clone λ2-3 contained a 1778 bp insert homologous to nucleotides 1059–2837 of the rat *jagged1* cDNA (DDBJ/EMBL/GenBank accession No. L38483). A full-length cDNA for human *jagged1* was isolated from a HeLa Uni-ZAP™ XR cDNA library (Stratagene) with probes derived from clone λ2-3 and from human expressed sequence tag (EST) clone 117734, homologous to the 3′ end of rat *jagged1* (Research Genetics). Phages positive with both probes were excised in pBluescript phagemids. Human cDNA clone 14-6 contained a 5457 bp insert comprised of the complete coding region for *jagged1* (3657 bp) together with 263 bp of 5′-untranslated sequence and 1537 bp of 3′-untranslated sequence (DDBJ/EMBL/GenBank accession No. AF028593). The sequence of human *jagged1* cDNA clone 14-6 was analyzed using the EditSeq and MegAlign programs of DNASTAR.

Northern blot analysis

Total RNA (20 μg) extracted with RNAzol B (TEL-TEST) was analyzed in 1% agarose–formaldehyde gels and transferred onto Hybond-N or Hybond-NX membranes (Amersham). Membranes were baked for 10 min at 80°C and UV cross-linked with a Stratalinker (Stratagene). A multiple human immune system II Northern blot was purchased from Clontech. Probes were generated by random priming with Klenow DNA polymerase in the presence of ^{32}P-labeled dCTP and dGTP. Membranes were hybridized to probes specific for c-*rel*, *jagged1*, *delta1* (a gift of G.Weinmaster, UCLA, CA), *IκBα* (a gift of A.Baldwin, University of North Carolina, NC), *Notch1/TAN-1*, *HES-1* (a gift of R.Kageyama, Kyoto University, Japan), *CD23* (a gift of E.Kieff, Harvard Medical School, MA) or the actin gene. Where indicated, blots were hybridized to human Il-2Rα- and *gapdh*-specific probes.

Immunoblotting

Cell extracts were prepared and quantitated as described (Bash *et al*., 1997). Proteins (20 μg) were resolved by SDS–PAGE and transferred to nitrocellulose membranes. Membranes were incubated with immune serum, followed by donkey anti-rabbit (Amersham) or goat anti-rat horseradish peroxidase (HRP)-conjugated antibodies (Jackson Immuno-Research Labs, PA) and detected by enhanced chemiluminescence (Amersham). Rat monoclonal antibody #TS1 (a gift of S.Artavanis-Tsakonas, Yale University, CT) was specific for the intracellular domain of human Jagged1. Polyclonal anti-Rel antibody Ab #1801 specific for the unique C-terminus of c-Rel (Kumar and Gelinas, 1993) and an antibody specific for actin (Sigma) were also used.

Immunofluorescence and immunohistochemistry analyses

Cell surface expression of the human Jagged1 protein was detected by immunofluorescence of live non-permeabilized cells, with all steps prior to fixation carried out at 4°C. Cells were washed in phosphate-buffered saline (PBS), blocked in PBS plus 5% goat serum and incubated with polyclonal antibody #PCR8 raised against the extracellular domain of rat Jagged1 overnight (a gift of G.Weinmaster, UCLA, CA). Cells were washed in PBS, incubated with a biotinylated goat anti-rabbit antibody (Vector Labs) and then fixed in 4% paraformaldehyde. Cells were washed in PBS, stained with FITC-labeled streptavidin (Jackson Immuno-Research Labs) and mounted with 0.2% *p*-phenylenediamine (Sigma). Spleen cells from C57BL/6 mice were enriched by antibody plus complement-mediated cell killing with rat anti-mouse Thy-1.2 antibody J1j to deplete T cells. This resulted in a population of >80% B cells. Cells were analyzed by double-immunofluorescence staining for expression of the B220 B-cell marker with a phycoerythrin (PE)-conjugated rat monoclonal anti-mouse CD45R-B220 antibody (PharMingen), and for the expression of c-Rel or Jagged1 using rabbit polyclonal antibody sc-71 (c-Rel; Santa Cruz Biotechnology) or #PCR8 and a fluorescein-labeled goat anti-rabbit secondary antibody (Jackson ImmunoResearch Labs, PA). Immunohistochemistry analysis of cross-sections from adult mouse spleen was carried out by the Immunohisto-chemistry Core Facility (Cancer Institute of New Jersey, New Brunswick, NJ). Primary antibodies were rat monoclonal anti-Jagged1 antibody #TS1 and mouse monoclonal antibody #L26 that recognized the B-cell antigen CD20 (Dako Corporation). Normal rat serum and pre-immune mouse IgG were used as negative controls. Reactivity was revealed by immunoperoxidase staining.

Co-cultivation assays

HtTA-1, CCR43 or CCR-H5 cells were plated in duplicate in the presence of tetracycline. After 24 h, one dish from each series was induced in medium lacking tetracycline. After 48 h, the cells were washed three times with Dulbecco's modified Eagle's medium (DMEM) with or without tetracycline, and overlaid with Jurkat, Sup-T1, Reh or U266 non-adherent cells in suspension (2.5×10^6 cells/dish). The co-cultures were incubated for 48 h. Photographs were taken after the cells remaining in suspension were washed away. For Northern blot analyses, firm tapping was used to dislodge the lymphoid cells that aggregated onto the adherent cell monolayer, prior to RNA extraction from the co-cultivated lymphoid cells.

Acknowledgements

We are very grateful to S.Artavanis-Tsakonas, A.Baldwin, H.Bujard, R.Kageyama, E.Kieff, N.Rice, G.Weinmaster and E.White for the gifts of reagents. We thank R.Donnelly for DNA sequencing, and P.Amenta and E.Smirnova for immunohistochemistry analysis. We thank F.Agnes for assistance with the EST database search, and F.Agnes, C.Chen, I.Luque and A.B.Rabson for fruitful discussions during the course of this work. We also thank C.Chen, L.C.Edelstein, B.Rayet and R.Steward for helpful comments on the manuscript. This work was supported by grants from the National Institutes of Health (CA54999), The Council for Tobacco Research USA, Inc. (4175) and the New Jersey Commission on Science and Technology (C.G.). The work was also supported partially by National Institutes of Health grants AI33839 (D.W.B.) and AG04821 (S.B.). J.B. was a pre-doctoral fellow of the New Jersey Commission on Cancer Research. A.R. was supported by an Individual National Research Service Award from the National Institutes of Health (GM19331).

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Received January 15, 1999; revised and accepted March 29, 1999