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Direct Regulation of *Gata3* Expression Determines the T Helper Differentiation Potential of Notch

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SUMMARY

CD4$^+$ T helper cells differentiate into T helper 1 (Th1) or Th2 effector lineages, which orchestrate immunity to different types of microbes. Both Th1 and Th2 differentiation can be induced by Notch, but what dictates which of these programs is activated in response to Notch is not known. By using T cell-specific gene ablation of the Notch effector RBP-J or the Notch1 and 2 receptors, we showed here that Notch was required on CD4$^+$ T cells for physiological Th2 responses to parasite antigens. GATA-3 was necessary for Notch-induced Th2 differentiation, and we identified an upstream *Gata3* promoter as a direct target for Notch signaling. Moreover, absence of GATA-3 turned Notch from a Th2 inducer into a powerful inducer of Th1 differentiation. Therefore, *Gata3* is a critical element determining inductive Th2 differentiation and limiting Th1 differentiation by Notch.

INTRODUCTION

Immunity against different classes of microorganisms is directed by specialized effector CD4$^+$ T helper subsets, of which the best characterized are called T helper 1 (Th1) and Th2 cells (Mosmann and Coffman, 1989). Th1 cells are characterized by production of interferon-γ (IFN-γ) and are responsible for generating immunity against intracellular pathogens. In contrast, Th2 cells produce interleukin-4 (IL-4), IL-5, and IL-13; are necessary for protection against helminth worms; and are involved in allergic reactions (Abbas et al., 1996).

The different types of effector T helper cells are derived from a common precursor, the mature naive CD4$^+$ T cell. Skewing of CD4$^+$ T cells into different lineages depends on signals provided by antigen-presenting cells (APCs) (Moser and Murphy, 2000). One APC-derived differentiation signal is IL-12, which promotes differentiation of naive CD4$^+$ T cells into the Th1 lineage (Kapsenberg, 2003; Moser and Murphy, 2000). Several APC-derived Th2 promoting signals have been described, which include OX40 ligand, IL-6, and the Notch ligand Jagged (Amsen et al., 2004; Flynn et al., 1998; Ito et al., 2005; Mowen and Glimcher, 2004; Rincon et al., 1997; So et al., 2006).

Tbet and GATA-3 are transcription factors that regulate the differentiation of naive CD4$^+$ T cells into the Th1 and Th2 lineages, respectively (Lee et al., 2000; Mullen et al., 2001; Ouyang et al., 1998; Szabo et al., 2000; Zhang et al., 1997; Zheng and Flavell, 1997). These factors not only promote differentiation into their respective lineages, but also limit differentiation into the other lineage (Hwang et al., 2005; Usui et al., 2003, 2006). GATA-3 is both necessary and sufficient for Th2 differentiation (Lee et al., 2000; Murphy and Reiner, 2002; Ouyang et al., 2000; Pai et al., 2004; Yamashita et al., 2004; Zhu et al., 2004). Little is known about the signals regulating expression of the *Gata3* gene. Expression of GATA-3 can be induced by IL-4 receptor signaling, in a signal transducer- and activator of transcription 6 (STAT6)-dependent manner (Kurata et al., 1999; Murphy and Reiner, 2002; Ouyang et al., 2000). How this pathway connects with the *Gata3* gene remains to be determined. Importantly, signals other than IL-4 must be able to drive Th2 differentiation and expression of *Gata3*. A source of IL-4 for initiation of Th2 differentiation in vivo has not been identified (Ansel et al., 2006), and Th2 responses can be generated when only T cells

can make IL-4, arguing against a requisite role for an external source of IL-4 in Th2 responses (Schmitz et al., 1994). Finally, Th2 responses do occur under conditions where IL-4 receptor signaling is prevented (Ansel et al., 2006; Finkelman et al., 2000; Jankovic et al., 2000; Noben-Trauth et al., 1997) and STAT6-independent expression of GATA-3 has been described (Ouyang et al., 2000).

Notch is a cell-surface receptor known for its role in binary cell fate decisions (Bray, 2006). In some settings, Notch regulates such decisions by a lateral inhibition mechanism, in which adoption of a primary fate is inhibited by Notch signaling, allowing cells to differentiate into a secondary fate by default. In other settings, Notch acts through inductive signaling by actively promoting expression of lineage-differentiation genes (Bray, 2006).

Notch consists of an extracellular ligand-binding chain noncovalently associated with a transmembrane polypeptide with a long intracellular tail (Bray, 2006). Mammals have four different *Notch* genes (Maillard et al., 2005). Two conserved families of ligands for Notch exist, called Jagged and Delta (Maillard et al., 2005). Upon binding its ligand, Notch undergoes proteolytic cleavages, including one catalyzed by a gamma secretase complex, which result in the release of the intracellular domain (NICD) from the membrane (Artavanis-Tsakonas et al., 1999). In the canonical signaling pathway used by all four Notch receptors, this NICD translocates to the nucleus where it associates with the DNA-binding factor RBP-J (also known as CBF1 or CSL) believed to be prebound to its target site on DNA (Artavanis-Tsakonas et al., 1999). Binding of the NICD to RBP-J results in displacement of transcriptional corepressors from RBP-J (Maillard et al., 2005) and recruitment of the Mastermind protein, thereby converting RBP-J from a transcriptional repressor into a transactivator.

No consensus exists about the role of Notch in T helper differentiation. Gain of Notch function promoted either Th1 or Th2 differentiation (Amsen et al., 2004; Maekawa et al., 2003; Minter et al., 2005). Only Th2 responses were blocked in RBP-J-deficient mice or in mice expressing a dominant-negative Mastermind transgene, however (Amsen et al., 2004; Tanigaki et al., 2004; Tu et al., 2005). In sharp contrast, inhibition of activation of Notch itself, with chemical inhibitors of the gamma secretase or a soluble Delta-Fc fusion protein, resulted in inhibition of Th1 but not Th2 responses (Minter et al., 2005; Maekawa et al., 2003). These apparent discrepancies might be a consequence of Notch-independent effects of some of the experimental approaches used. All of these (including ours) have relied on inhibition of Notch activity by indirect methods. Gamma secretase cleaves membrane molecules other than Notch (Wolfe and Kopan, 2004), and prominent Notch-independent functions exist for Mastermind (Katada et al., 2006; Shen et al., 2006) and RBP-J (Barolo et al., 2000). To resolve the controversy, it is necessary, therefore, to use direct loss-of-function approaches of the essential components of the pathway, RBPJ and the Notch genes themselves.

In the present study, we examined Th2 differentiation by using mice carrying T cell-specific deletions of RBP-J or the *Notch1* and *Notch2* genes themselves. We established that Notch is required for Th2 responses to parasite antigens under physiological conditions, and we reveal that Notch drives Th2 differentiation by an inductive mechanism through direct *trans* activation of the *Gata3* gene. Furthermore, we report that GATA-3 acts as a switch factor, as shown by the fact that its absence converts Notch from a Th2 inducer into a powerful inducer of Th1 differentiation.

RESULTS

RBP-J Is Required for Th2 Responses In Vivo

The requirement for the Notch pathway in Th2 responses is unclear. A Th2 defect was previously found in mice with a T cell-specific deletion of the *Rbpj* gene under weak Th2-inducing conditions (Tanigaki et al., 2004). It has not been tested whether this factor is required under strong and physiologically important Th2-inducing conditions, such as those elicited by parasite antigens. Therefore, we immunized *Rbpj*-deficient mice with extract of the eggs obtained from *Schistosoma mansoni*, which induce pronounced Th2-type immunity during normal infection (Pearce et al., 2004; Fallon et al., 2000). Extracts from such eggs contain potent adjuvant activity and have been used extensively to study Th2 responses in vivo (Pearce et al., 2004). Indeed, immunization with these extracts elicited strong Th2 responses as witnessed by the production of Th2-dependent antibody isotypes IgG1 and IgE, as well as by the presence in spleens of these mice of antigen-specific $CD4^+$ T cells secreting the signature Th2 cytokine IL-4 upon in vitro rechallenge (Figure 1). Strikingly, both Th2-dependent antibody production and secretion of IL-4 were abrogated in mice lacking RBP-J in T cells (Figure 1), documenting the in vivo requirement for the Notch pathway in Th2 responses. In contrast, production of IgM, which is partially dependent on $CD4^+$ T cell help, but not on a particular effector type, was not significantly affected by *Rbpjk* deficiency. Although SEA does not elicit strong Th1 responses, some Th1-dependent antibodies (IgG2b and IgG2c) and cytokine (IFN-γ) could be measured in these immunized mice (Figure 1). We did not, however, find a consistent effect from RBP-J deficiency on this type of response. Thus, although these data do not exclude a role for the Notch pathway in Th1 induction (see Discussion), they do reinforce the hypothesis that this pathway is required for Th2 responses.

Th2 Responses Depend on Expression of Notch 1 and 2

It is conceivable that the results presented above are a reflection of a Notch-independent function of RBP-J (Barolo et al., 2000). Consistent with this idea, inhibition of activation of Notch itself by gamma secretase inhibitors did not perturb Th2 responses in vitro (Minter et al., 2005). We therefore determined whether deficiency for Notch affects Th2 differentiation.

Of the four mammalian Notch proteins, Notch 1 and 2 are phylogenetically closest to one another, whereas

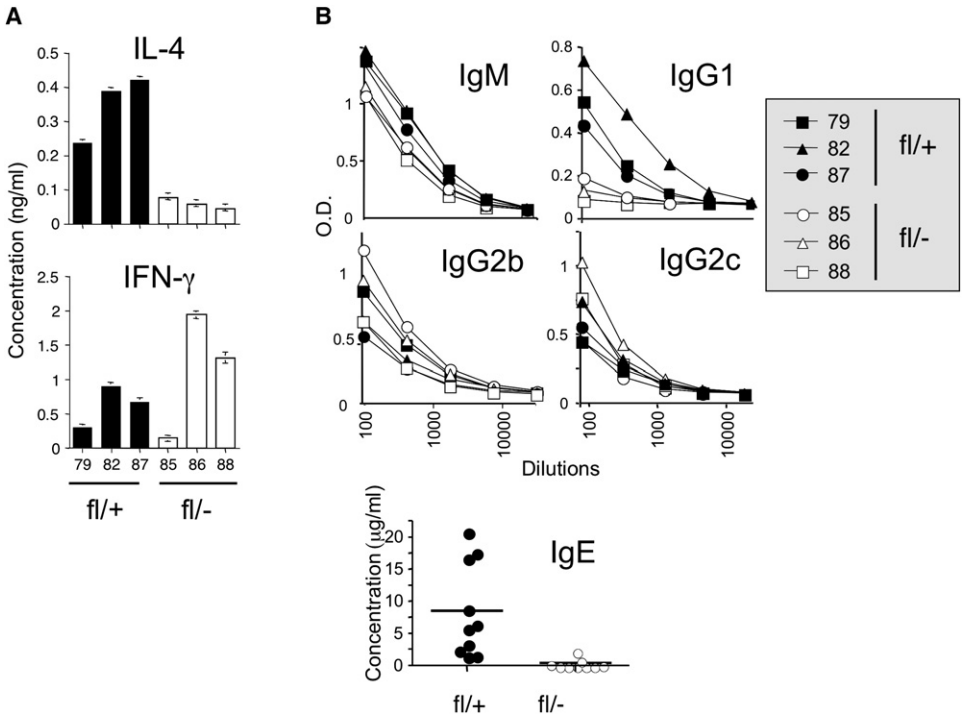

Figure 1. Generation of Th2-Mediated Immunity In Vivo Is Dependent on RBP-J

(A) RBP-J-deficient ($RBP^{fl/-}$-CD4Cre) (white bars and symbols) or heterozygous ($RBP^{fl/+}$-CD4Cre) littermates (black bars and symbols) were immunized with extract from *Schistosoma mansoni* eggs (SEA). Cytokine responses of purified splenic $CD4^+$ T cells were measured upon in vitro restimulation with SEA. Each bar represents the response of $CD4^+$ T cells from an individual mouse. Error bars represent standard error of the mean for duplicate measurements.

(B) In addition, sera from individual heterozygous control (closed symbols) or RBP-J-deficient mice (open symbols) were tested for the presence of various isotypes of antibodies against SEA. Data in (A) and (B) are representative of five separate experiments. For IgE, values obtained from several experiments are shown combined, with each dot representing a single mouse.

Notch 3 and 4 are more divergent (Maillard et al., 2005). Mice lacking individual Notch genes have no defect in T helper differentiation (Tacchini-Cottier et al., 2004; and our unpublished observations). We therefore examined whether double deficiency in *Notch1* and *Notch2*, the Notch genes expressed in naive $CD4^+$ T cells (Amsen et al., 2004), affects T helper responses in the presence of SEA as a strong Th2 adjuvant. Deletion of *Notch1* and *Notch2* by CD4 promoter-driven Cre does not affect thymic development (data not shown), allowing the study of peripheral T helper responses. To this end, we cultured AND TCR transgenic naive $CD4^+$ T cells, derived from mice carrying T cell-specific deletions of both *Notch1* and *Notch2* genes, in vitro with SEA-treated APC and pigeon cytochrome C. After 5 days, we assayed T helper differentiation by intracellular cytokine staining for IL-4 and IFN-γ. Strikingly, Notch1 and Notch2 double-deficient $CD4^+$ T cells failed to make IL-4, whereas no consistent effect was found on production of IFN-γ (Figure 2A). Thus, Notch1 and 2 are essential for induction of Th2 differentiation under strong Th2-inducing conditions, such as those created by SEA-treated APC. In contrast, no effect from *Notch1* and *Notch2* double deficiency was found in standard in vitro Th1 and Th2 differentiation paradigms, in which differentiation is induced by the addition of cytokines (IL-12 or IL-4) and neutralizing antibodies (anti-IL-4 or anti-IFN-γ) (Figure 2B). Collectively, these data reveal that the Notch pathway is required for induction of Th2 responses under physiological conditions. This role is obscured, however, in standard in vitro T helper cultures, where exogenous cytokines likely override the physiological mechanisms, presumably explaining the previously reported inability of gamma secretase inhibitors to block Th2 differentiation (Minter et al., 2005).

Promoter-Specific Regulation of the *Gata3* Gene by Notch

In mice lacking RBP-J, decreased Th2 responses were accompanied by increased Th1 responses (Tanigaki et al., 2004). Thus, Notch might promote Th2-mediated immunity as a default consequence of interfering with Th1 induction (Tu et al., 2005). Alternatively, Notch may actively promote Th2 differentiation in an inductive manner, in which case the increased Th1 responses in RBP-J-deficient mice (Tanigaki et al., 2004) result from a lack of cross inhibition by Th2 factors.

The most conclusive evidence for an inductive mechanism would be to establish that key Th2 differentiation

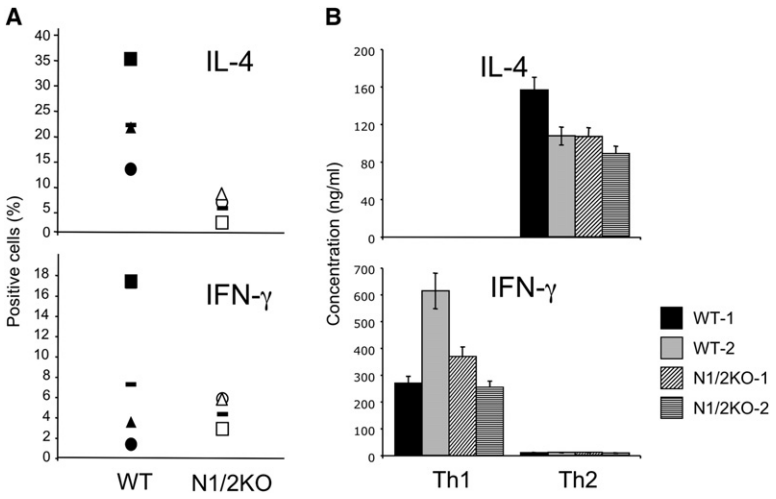

Figure 2. Notch1 and 2 Are Required for Physiological Th2 Responses

(A) Naive CD4$^+$ T cells were isolated from AND TCR transgenic Notch1 and Notch2 double-deficient mice (Notch1$^{fl/fl}$Notch2$^{fl/fl}$ CD4Cre) (open symbols) or wild-type (Notch1$^{fl/+}$Notch2$^{fl/+}$ CD4Cre) (closed symbols) littermates and stimulated in vitro with collagenase-treated splenic APC in the presence of SEA (20 μg/ml) and pigeon cytochrome C (10 μg/ml). After 5 days, viable effector cells were restimulated with PMA and ionomycin, and IL-4 and IFN-γ production was measured by intracellular cytokine staining. Each dot represents percentage IL-4$^+$ or IFN-γ$^+$ cells in cells obtained from an individual mouse. Data represent cumulative results from three independent experiments.

(B) Naive CD4$^+$ T cells were isolated from Notch1 and Notch2 double-deficient (hatched bars) or wild-type (filled bars) littermates (all positive for the CD4 Cre transgene) and activated in vitro with soluble anti-CD3 and anti-CD28 and splenic APC in the presence of Th1 (IL-12 and anti-IL-4) or Th2 (IL-4 and anti-IFN-γ) polarizing conditions. After 5 days, viable effector cells were harvested and restimulated with plate-bound anti-CD3 (10 μg/ml), and 48 hr supernatants were assayed for IL-4 and IFN-γ. Cytokine concentrations from cells obtained from individual mice are shown. These results are representative of three independent experiments. Error bars represent standard error of the mean for duplicate measurements.

genes are regulated directly by Notch. The central factor for the differentiation of Th2 cells is GATA-3 (Lee et al., 2000; Ouyang et al., 1998, 2000; Pai et al., 2004; Yamashita et al., 2004; Zhang et al., 1997; Zheng and Flavell, 1997; Zhu et al., 2004). As shown in Figure 3A, under physiological conditions, expression of this factor is dependent on Notch: it is abrogated in AND TCR transgenic Notch1/2 double-deficient T cells activated with antigen in the presence of SEA. To examine whether Notch regulates expression of the *Gata3* gene in a direct fashion, we introduced an activated Notch allele (NICD) in CD4$^+$ T cells. Expression of this allele, consisting of the intracellular domain of Notch, results in Th2 differentiation (Amsen et al., 2004). Although this result was not obtained in another study (Maekawa et al., 2003), this effect was likely obscured because of the use of a mixed T cell population (including memory and effector cells) from naturally Th2-prone Balb/c T cells. In fact, NICD

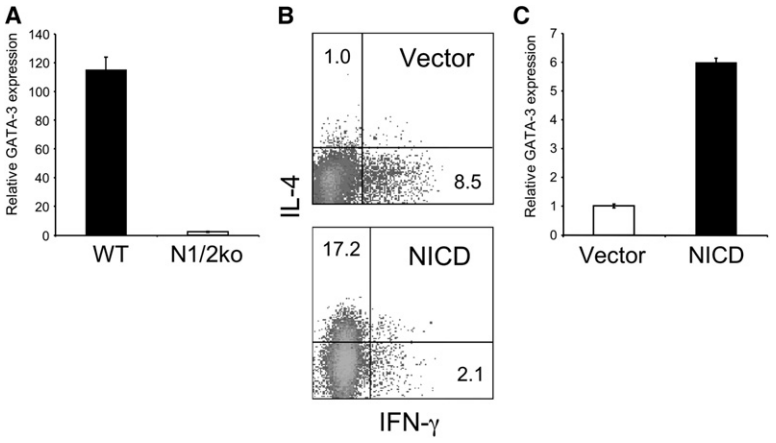

Figure 3. Notch Regulates Expression of *Gata3* and Th2 Differentiation Independently of IL-4

(A) Notch is required for GATA-3 expression. Naive CD4$^+$ T cells from wild-type (closed bar) or Notch1 and Notch2 double-deficient mice (open bar) were isolated and stimulated as in Figure 2A. After 5 days in culture, cDNA was prepared and tested by quantitative real-time PCR for the abundance of *Gata3* transcripts. Values were normalized against those obtained for HPRT. Bars represent relative GATA-3 mRNA abundance with the value for Notch1 and Notch2 double-deficient mice arbitrarily set to 1. Shown is the mean ± standard deviations from triplicate measurements. Results are representative of two experiments.

(B) Notch ICD induces IL-4-independent Th2 differentiation. Naive *Stat6*$^{-/-}$ CD4$^+$ T cells were activated with splenic APC and soluble antibodies to CD3 and CD28 and transduced with a retrovirus encoding NICD linked to GFP through an IRES sequence (bottom) or with control virus (top). 3 days after transduction, viable effector cells were restimulated with PMA and ionomycin, and IL-4 and IFN-γ production by GFP$^+$ cells was measured by intracellular cytokine staining. Numbers represent percentages in each quadrant. Results are representative of more than five experiments.

(C) Notch ICD induces IL-4-independent GATA-3 expression. Naive *Stat6*$^{-/-}$ CD4$^+$ T cells were activated and transduced as in (B). 3 days after transduction, GFP$^+$ cells were isolated by FACSorting, cDNA was prepared, and the abundance of *Gata3* transcripts was determined by quantitative real-time PCR. Bars represent relative mean GATA-3 mRNA levels ± standard deviations from triplicate measurements of vector-transduced (open bars) and NICD-transduced (closed bars) cells normalized for HPRT expression. The value obtained from vector-transduced cells was arbitrarily set to 1 and values are relative to this. Results are representative of four independent experiments.

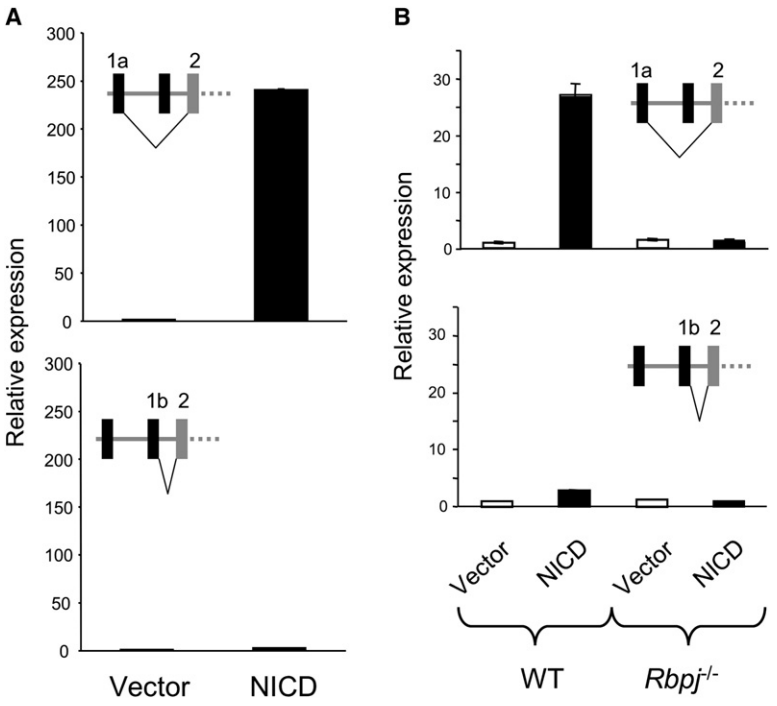

Figure 4. Notch Activates the Upstream *Gata3* Promoter through RBP-J

(A) Notch stimulates transcriptional activity of the upstream but not the downstream *Gata3* promoter. Naive *Stat6*$^{-/-}$ CD4^{+} T cells were transduced and sorted for GFP^{+} cells, and cDNA was prepared as in Figure 3C. The abundance of exon 1a or exon 1b-containing transcripts was determined by real-time PCR via specific primers annealing in exon 1a and exon 2 (top) or exon 1b and exon 2 (bottom). Relative mean transcript levels are shown ± standard deviations from triplicate measurements normalized for HPRT expression. Values are relative to those from vector-transduced cells, which were arbitrarily set to 1. This result is representative of three independent experiments.

(B) Notch-induced activity of the upstream *Gata3* promoter is dependent on RBP-J. Naive CD4^{+} T cells from wild-type or RBP-J-deficient mice were transduced and analyzed as in (A) for the relative abundance of exon 1a (top) or exon 1b (bottom)-containing transcripts. Values are relative to those from vector-transduced wild-type cells, which were arbitrarily set to 1. Neutralizing antibody to IL-4 (10 μg/ml) was included in the cultures. Results are representative of two independent experiments.

induces Th2 differentiation even in STAT6-deficient T cells (Figure 3B). NICD also induced expression of the *Gata3* gene in STAT6-deficient T cells (Figure 3C). STAT6 deficiency excludes auto and paracrine effects from elevated IL-4 production induced by NICD, so these results are consistent with a direct link between Notch and GATA-3.

We then studied whether Notch regulates global activity of this gene or the activity of a specific promoter. Expression of the *Gata3* gene is controlled by two different promoters, separated by approximately 10 kB (Asnagli et al., 2002). Each of these drives expression of a transcript containing a unique first exon, 1a or 1b, respectively, which splices to a common exon 2. Both these exons 1 contain only 5′ UTR sequence: the translational start site is present in the common second exon. Thus, both transcripts encode for the same GATA-3 protein. The usage of distinct first exons by the different promoters allows specific measurement of the activities of these promoters separately in their native chromatin contexts. We therefore measured the induction of both transcripts by NICD, again by using STAT6-deficient CD4^{+} T cells. Strikingly, although Notch did not markedly affect expression of exon 1b, expression of exon 1a was strongly induced (Figure 4A). Notch responsiveness of exon 1a was abrogated in RBP-J-deficient CD4^{+} T cells (Figure 4B), consistent with the requirement for this Notch effector in Th2 differentiation (Figure 1). Thus, Notch does not affect global activity of the *Gata3* gene but specifically activates the upstream *Gata3* promoter in an RBP-J-dependent manner.

A Physical Link between the Notch Pathway and the *Gata3* Gene

To determine whether the upstream *Gata3* promoter is a direct target of Notch, we scanned it for RBP-J-binding sites. A potential RBP-J-binding element was found (Figure 5A). This site is conserved between mice and humans, suggesting that it may be important. No RBP-J-binding sequence was found in the downstream promoter. The putative RBP-J element in the upstream *Gata3* promoter (hereafter referred to as R^{G3P1a}) corresponds to one published RBP-J consensus binding sequence (Bailey and Posakony, 1995) but not to another (Tun et al., 1994). To determine whether R^{G3P1a} is a bona fide RBP-J-binding element, we used double-strand DNA oligonucleotides containing R^{G3P1a} in EMSA with extracts from cells transfected with RBP-J. Indeed, a shifted complex was obtained when radiolabeled R^{G3P1a} was incubated with extracts from cells transfected with RBP-J but not with extracts from cells transfected with control vector (Figure 5B). No complex was formed either with extracts from cells transfected with RBP-J R218H, which contains a single amino acid substitution perturbing its ability to bind DNA (Kato et al., 1997). Furthermore, no complex was formed upon incubation of RBP-J-containing lysate with radiolabeled oligos containing a point mutation that precludes binding of RBP-J (mR^{G3P1a}) (Figure 5B). Formation of the radioactive RBP-J-R^{G3P1a} complex could be inhibited by addition of excess unlabeled R^{G3P1a}, or oligos encompassing a previously characterized RBP-J-binding element (RE) (Tun et al., 1994), but not by addition of mR^{G3P1a}. Finally, binding activity

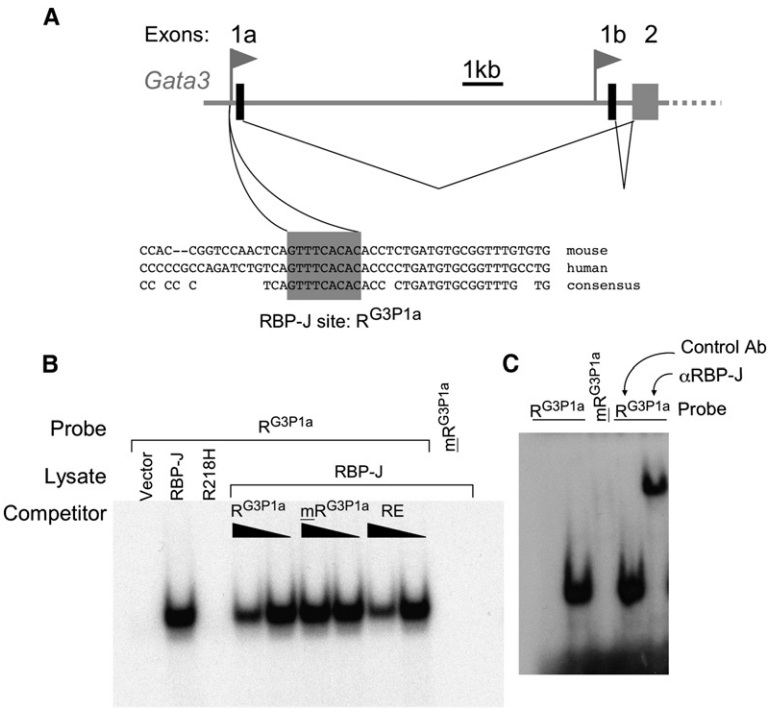

Figure 5. The Upstream *Gata3* Promoter Contains a Bona Fide Conserved RBP-J-Binding Element

(A) Diagram of the *Gata3* locus showing upstream and downstream *Gata3* promoters each driving expression of a unique exon 1, which is spliced to the common exon 2. Underneath, the consensus RBP-J-binding element is shown and its conservation between mouse and human.

(B) EMSA via radiolabeled double strands probe containing the RBP^{G3P1a} and lysates from CHO cells transfected with vector alone (lane 1), RBP-J (lanes 2 and 4–10), or RBP-J R218H (lane 3). A probe carrying a point mutation, $\underline{m}RBP^{G3P1a}$, was used as specificity control (lane 10). The specific RBP-J-RBPG3P1a complex was competed away by addition of (10-fold or 3-fold) excess unlabeled RBP^{G3P1a} (lanes 4 and 5) or the previously described RBP-J-binding element RE (lanes 8 and 9) but not by the mutant probe $\underline{m}RBP^{G3P1a}$ (lanes 6 and 7). Results are representative of two independent experiments.

(C) EMSA with lysates from $CD4^+$ T cells (lanes 2–5) and radiolabeled RBP^{G3P1a} (lanes 1, 2, 4, and 5) or $\underline{m}RBP^{G3P1a}$ (lane 3). Antibody to RBP-J (lane 5) or control mouse Ig (lane 4) was added to the reaction to supershift the RBP-J- RBP^{G3P1a} complex. Results are representative of two independent experiments.

to R^{G3P1a} was found in extracts from $CD4^+$ T cells and identified as RBP-J by supershift via an antibody to RBP-J (Figure 5C). These data establish R^{G3P1a} as a bona fide RBP-J-binding element.

To determine whether RBP-J binds to the (R^{G3P1a}-containing) upstream GATA-3 promoter in vivo, we performed chromatin precipitation. An antibody to RBP-J specifically precipitated the upstream promoter region from wild-type $CD4^+$ T cell-derived chromatin, but not from chromatin obtained from RBP-J-deficient $CD4^+$ T cells (Figures 6A and 6B). The downstream *Gata3* promoter region did not precipitate with this antibody, consistent with the absence of RBP-J consensus sites in this region and its lack of responsiveness to Notch.

GATA-3 Is Required for Induction of Th2 Differentiation by Notch

Although GATA-3 is an important factor in differentiation of Th2 cells in response to IL-4, its requirement in Notch-induced Th2 differentiation has not been established. To test this, we studied Notch-mediated Th2 differentiation in GATA-3-deficient $CD4^+$ T cells. To circumvent effects from GATA-3 deficiency on thymic development (Pai et al., 2003), we isolated mature $CD4^+$ T cells carrying floxed alleles of the *Gata3* gene and extinguished GATA-3 expression by retroviral introduction of the Cre recombinase (Figure 7A). Expression of the Cre recombinase abrogated IL-4-induced Th2 differentiation, documenting the efficacy of the deletion of the *Gata3* gene (Zhu et al., 2004; and data not shown). To determine whether GATA-3 is required for Notch-induced Th2 differentiation, we carried out double infections of $Gata3^{fl/-}$ $CD4^+$ T cells with Cre- and NICD-encoding retrovirus. To prevent potential induction of GATA-3 expression by NICD before complete deletion of the *Gata3* gene, we performed these infections sequentially. Thus, cells were infected with Cre retrovirus first, and 6 hr later underwent a second round of infection with NICD. We minimized spontaneous effector differentiation by adding blocking antibodies to IL-4 and IFN-γ. Indeed, these antibodies worked effectively, because production of IL-4 and IFN-γ by cells infected with control retroviruses was undetectable (Figure 7B). Importantly, NICD elicited marked IL-4 production in control cells, but not in cells lacking GATA-3 expression (Figure 7B). Thus, GATA-3 is instrumental in Notch-mediated Th2 responses. Strikingly, instead of eliciting production of IL-4, in the absence of GATA-3, NICD strongly induced production of IFN-γ (Figure 7B). This was actively induced by Notch because vector control cells lacking GATA-3 expression did not default to Th1 differentiation.

DISCUSSION

The role of Notch in T helper cell differentiation has been controversial, with different groups showing apparently contradictory results. By using indirect approaches to interfere with Notch signaling, some studies supported a role in Th1 and others a role in the opposite Th2 responses (Amsen et al., 2004; Maekawa et al., 2003; Minter et al., 2005; Tanigaki et al., 2004; Tu et al., 2005). Here, by

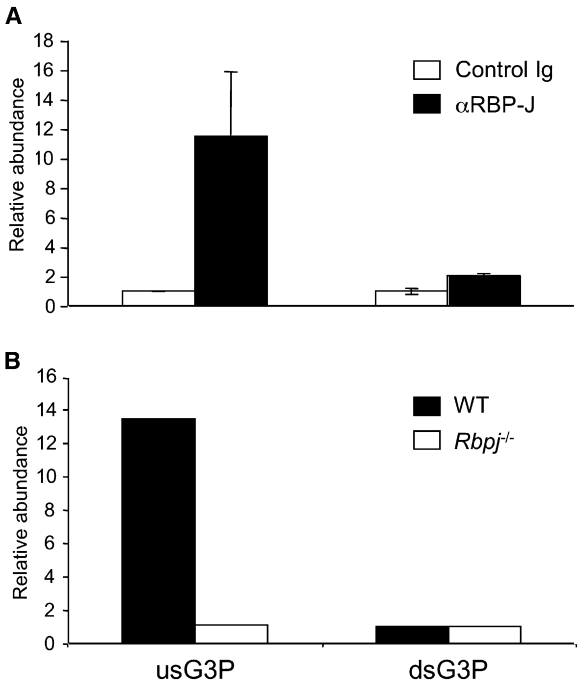

Figure 6. RBP-J Binds the Upstream *Gata3* Promoter In Vivo

(A) ChIP was performed on chromatin from CD4$^+$ T cells with control antibody (white bars) or antibody to RBP-J (black bars). Precipitation of the upstream *Gata3* promoter (USG3P) or the downstream *Gata3* promoter (DSG3P) was determined by quantitative real-time PCR with promoter-specific primers. Samples were normalized to values obtained from input material for each sample without immunoprecipitation. Relative mean values ± standard deviation from triplicate measurements are shown. The normalized value for the usG3P precipitated with control Ig was arbitrarily set to 1. Results are representative of three experiments.

(B) ChIP was performed as in (A) with chromatin from wild-type (black bars) or RBP-J-deficient (white bars) CD4$^+$ T cells. Shown values are the means ± standard deviation from triplicate measurements normalized to input material without precipitation. Values are relative to the value for usG3P precipitated from RBP-J-deficient T cells, which was arbitrarily set to 1. Results are representative of two independent experiments.

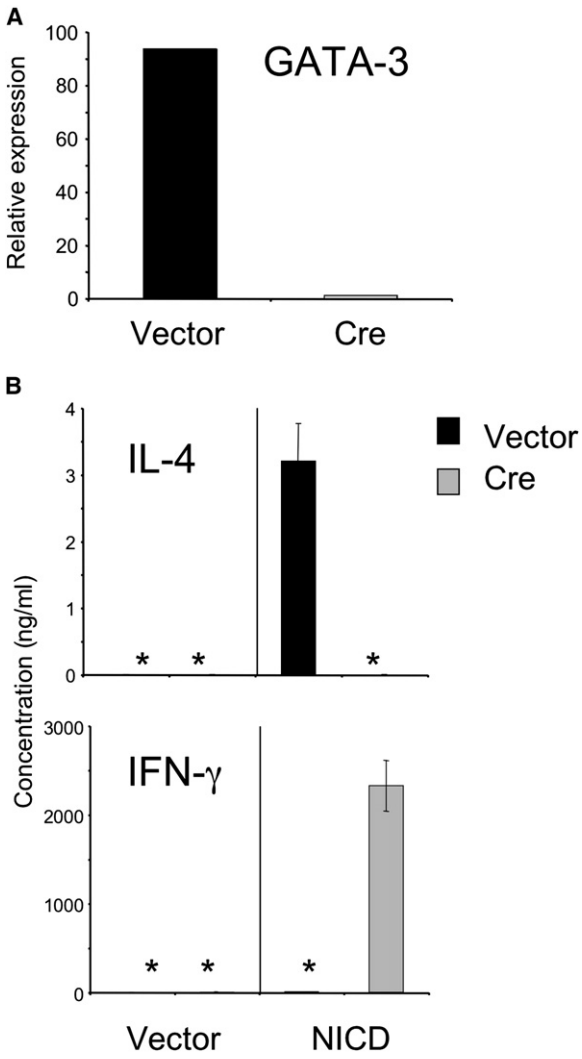

Figure 7. GATA-3 Is Necessary for Notch-Induced Th2 Responses

(A) Naive CD4$^+$ T cells from *Gata3*$^{fl/-}$ mice were activated with splenic APC and antibodies to CD3 and CD28 and infected with control GFP virus or virus encoding Cre linked through an IRES sequence to GFP. After 3 days, GFP$^+$ cells were isolated by FACSorting and RNA was made. Relative abundance of GATA-3 message was determined by real-time PCR with a primer probe set that detects the floxed exon 4 of *Gata3* . Values were normalized against those obtained for HPRT. Bars represent relative GATA-3 mRNA abundance with the value for the Cre sample arbitrarily set to 1. Shown is the mean ± standard deviations from triplicate measurements. Results are representative of two experiments.

(B) Naive CD4$^+$ T cells from *Gata3*$^{fl/-}$ mice were activated as in (A) and infected after 20 hr first with control IRES-GFP (black bars) or Cre-IRES-GFP (gray bars). 6 hr later, cells were transduced with control IRES-Thy1.1 virus (left) or NICD-IRES-Thy1.1 (right). Neutralizing antibodies to IL-4 and IFN-γ (10 μg/ml each) were added to the cultures at the time of the second transduction. 3 days after the last transduction, cells were sorted for GFP and Thy1.1 expression and restimulated with plate-bound anti-CD3 (10 μg/ml). 48 hr supernatants were tested for IL-4 and IFN-γ concentrations by ELISA. Bars represent mean cytokine concentrations ± standard errors of duplicate measurements. These results are representative of three independent experiments.

using both RBP-J-deficient as well as Notch1 and Notch2 double-deficient T cells, we establish that both downstream signaling as well as the Notch receptors themselves are required in Th2 responses. This role of Notch was evident under physiological conditions with extract of *Schistosoma mansoni* eggs, as Th2-inducing adjuvant. Notch was not required in the classical in vitro paradigm for Th2 differentiation (Minter et al., 2005), in which skewing is achieved by addition of a high concentration of IL-4 and blocking antibodies to IFN-γ. We think, however, that these results illustrate a limitation of the cytokine-driven differentiation paradigm. Although this approach has been very useful in delineating many pathways involved in T helper differentiation (and has been used extensively by ourselves for this purpose), it may obscure the physiological role of pathways functioning upstream of these cytokines or under conditions where such strong skewing

conditions are not met. Although exogenous IL-4 may be present in vivo, clearly, its amounts are insufficient to drive Th2 differentiation in the absence of Notch signaling, as evidenced by the profound defect in Th2 induction, for instance, in RBPJ-deficient mice.

We do not think our findings rule out a role for Notch in Th1 differentiation. Our experiments were not designed to rigorously investigate the involvement of Notch in Th1 responses, because SEA does not generally elicit strong Th1 responses (Pearce et al., 2004). We examined the consequence of Notch1 and Notch2 double deficiency in cytokine-driven Th1 differentiation and failed to find a defect. However, it is possible that other Notchs (3 and 4) are sufficient for this response. Our negative data stand in contrast to compelling results showing profound inhibition of Th1 differentiation by gamma secretase inhibitors in vitro (Minter et al., 2005). Also, pronounced Th1 induction is obtained when $CD4^+$ T cells are stimulated with Delta ligands (Amsen et al., 2004; Maekawa et al., 2003; Skokos and Nussenzweig, 2007), and an active Notch allele induced a strong Th1 response when the *Gata3* gene had been deleted. We therefore still consider it likely that Notch has a role in Th1 responses, although it remains unclear under which conditions this pathway normally operates.

How the Notch pathway would regulate such opposite differentiation pathways as Th1 and Th2 is not understood, but our data suggest that the ability of Notch to drive either pathway is dependent on whether or not Notch can activate expression of GATA-3. This might be determined by qualitatively or quantitatively different signaling induced by different ligands and/or by surrounding signals.

The Th1 and Th2 differentiation processes involve both positive feedback and cross inhibitory mechanisms. IFN-γ and IL-4 promote Th1 and Th2 differentiation, respectively, in an auto and paracrine fashion (Murphy and Reiner, 2002). At the same time, these factors induce inhibitory signals toward the opposite differentiation program (Murphy and Reiner, 2002). In this light, it was tempting to speculate that Notch, which controls other differentiation processes through lateral inhibition, promotes default Th2 differentiation by preventing the Th1 differentiation program.

However, a lateral inhibition model seems difficult to reconcile with our finding that Notch actively induces Th1 differentiation in the absence of GATA-3. Furthermore, we have revealed a direct positive connection between Notch and induction of expression of GATA-3, the master regulator of Th2 differentiation. A direct link also exists between Notch and the *Il4* gene: we showed previously that a 3′ enhancer of the *Il4* gene contains conserved Notch-responsive RBP-J sites (Amsen et al., 2004), which was confirmed by others (Tanaka et al., 2006). These direct positive connections between Notch and the induction of expression of key Th2 genes strongly favor an inductive model for Notch-mediated Th2 differentiation, rather than one based on lateral inhibition.

Remarkably little is known about the pathways inducing expression of GATA-3 in T cells. What little has been learned has come from cytokine-driven in vitro differentiation systems, which may override the role of early signals. Regulation of *Gata3* expression is likely to be complex. The genomic region surrounding the *Gata3* gene contains many highly conserved noncoding regions (unpublished observations), and several distal enhancer elements have been described (Burch, 2005).

In $CD4^+$ T cells, expression of the *Gata3* gene is responsive to T cell receptor (TCR) and IL-4 receptor-STAT6 signaling (Murphy and Reiner, 2002). How signaling downstream of these connects to transcriptional activation of the *Gata3* gene has not been established. A good candidate in the TCR-dependent pathway is the transcription factor NF-κB. The role of NF-κB in T helper differentiation is not entirely clear, possibly reflecting distinct functions for different NF-κB family members (Corn et al., 2005), but expression of GATA-3 is reduced in p50-deficient mice (Das et al., 2001). p50 may, perhaps in combination with Bcl3, activate transcription of the *Gata3* gene by binding to a site in the downstream *Gata3* promoter (Corn et al., 2005). It is not known whether, and if so how, p50 is activated specifically under conditions predisposing toward Th2 development. Interestingly, p50 activity is elevated by Notch signaling, which promotes its nuclear retention (Shin et al., 2006). This provides a possible additional mechanism for Notch to promote Th2 responses, independent from the mechanism identified in the present report. Finally, stable expression of GATA-3 may be achieved by a positive-feedback mechanism, in which GATA-3 promotes its own expression (Ouyang et al., 2000). Again, the elements responsible for this have not been identified.

Connections between Notch and GATA factors are not limited to GATA-3; they exist in various cell types and are conserved in phylogeny. For instance, Notch controls expression of the *Drosophila* GATA factor Serpent (Mandal et al., 2004). Expression of the *Gata2* gene in early hematopoietic progenitors is also directly controlled by Notch (de Pooter et al., 2006; Kumano et al., 2001; Robert-Moreno et al., 2005). Importantly, in common lymphoid progenitor cells, Notch signaling induces expression of GATA-3, which is required for commitment to the T cell lineage (Hoflinger et al., 2004; Taghon et al., 2005). It is tempting to speculate that this involves the mechanism identified here by us.

Notch and GATA factors are important regulators of differentiation throughout the metazoan kingdom. In many processes, expression of the latter is connected to instructive differentiation signals received at the cell surface by Notch, a transcription factor and cell-surface receptor at the same time. As we and an accompanying paper (Fang et al., 2007) demonstrate, in T helper cells, this module has been adopted for the induction of Th2-type immunity.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies

Anti-RBP-J (K0043) was from Institute of Immunology Co. (Japan), and anti-CD3 (145-2C11), anti-CD28 (37.1), anti-IL-4 (11B11), anti-IFN-γ

(XMG1.1), NK1.1 (HB101), and anti-Thy1 (Y19) were all from American Type Tissue Culture Collection (Manassas, VA). Recombinant mouse IL-4 and IL-2 were from PharMingen, and recombinant mouse IL-12 was a gift from Wyeth Research. Antibodies for FACS, cytokine, and IgE ELISA were from PharMingen. Other isotype-specific ELISA antibodies were from Southern Biotechnology Associates, Inc. (cat. nr. 5300-04). ELISAs were developed with Horseradish Perozidase Avidin D (Vector Laboratories, Inc., Burlingame, CA) and SureBlue Peroxidase Substrate (KPL, Gaithersburg, MD).

Mice

5- to 8-week-old B6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or NCI (Rockville, MD) and maintained in the Yale University Animal Resources Center. Bred in our colony under SPF conditions: Notch-1 flox (Radtke et al., 1999), Notch2 flox (McCright et al., 2006), RBP-J null (Oka et al., 1995), RBP-J flox (Tanigaki et al., 2002), CD4-Cre transgenic mice (Taconic), STAT6 null, and AND TCR transgenic mice (Jackson Laboratories).The floxed *Gata3*^{F} allele was generated by crossing *Gata3*$^{ex4GFP/+}$ mice (Grote et al., 2006) with the transgenic *FLPe* line (Rodriguez et al., 2000).

Vectors and Constructs

The hCRE-GFP-RV and GFP-RV vectors were generously provided by W. Paul. MSCV Thy1.1 and N1-MSCV-Thy1.1 (containing the entire intracellular tail of human Notch1 starting at amino acid 1748, first amino acids RKRRRQ) were described previously (Amsen et al., 2004). Expression constructs pCMX-RBP-J and pCMX-RBP-J R218H (Kato et al., 1997) were provided by T. Honjo.

In Vitro CD4^{+} T Cell Differentiation

Naive CD44^{low}CD62L^{high}DX5^{-}CD25^{-} CD4^{+} T cells were purified from spleen and peripheral lymph nodes by positive selection with anti-CD4 beads (Miltenyi, cat#130-049-201) followed by FACsorting. Cells were cultured in Bruff's medium (10% FCS, penicillin, streptomycin, and L-glutamine). 10^5 naive CD4^{+} T cells were cultured with 2.5×10^6 irradiated (2000 rad) B10.BR splenocytes obtained by collagenase treatment (Collagenase D, Roche), 10 μg/ml of pigeon cytochrome C (Sigma, cat# C-4011) and 20 μg/ml SEA. For Th1 and Th2 cultures, 2×10^5 naive CD4^{+} cells were activated by 4×10^6 irradiated (2000 rad) T cell- and NK cell-depleted C57Bl/6 splenocytes with soluble anti-CD3 and anti-CD28 (1 μg/ml each), 10 U/ml IL-2 and 3.5 ng/ml IL-12 and 10 μg/ml anti IL-4 (11B11) (Th1 cultures) or 1000 U/ml IL-4 and 10 μg/ml anti IFN-γ (Th2 cultures). After 5 days, viable cells were harvested by fycoll (LSMOL Lymphocyte Separation Medium, Cappel), restimulated at 1×10^5 cells per well (96-well flat-bottom plate, Falcon) with plate bound anti-CD3 (10 μg/ml). Cytokine concentrations (48 hr supernatants) were determined by ELISA. For intracellular cytokine staining, viable effector cells were isolated by fycoll gradient, stimulated with PMA (50 ng/ml), and ionomycin (0.5 μM) and stained with the BD Cytofix/Cytoperm Plus kit (with Golgi Stop) (BD PharMingen).

Retroviral Transductions

Virus was made and transductions were performed as described (Amsen et al., 2004). For double infections, cells were first infected with Cre-expressing virus and 6 hr later with NICD retrovirus. 3 days after transduction, viable cells were isolated by fycoll. GFP-positive and/or Thy-1.1-positive were isolated by FACSorting.

Induction of Anti-SEA Responses

Extracts from *Schistosoma mansoni* eggs were prepared as described (Boros and Warren, 1970). Water-soluble fraction was injected intraperitoneally (50 μg) twice a week for 3 weeks. After an additional 2 week rest period, sera and spleens were collected. To measure T cell responses, CD4^{+} T cells were isolated from spleens and restimulated in vitro with C57Bl/6 splenocytes and 25 μg/ml SEA. Supernatants were collected after 4 days and cytokine concentrations determined by ELISA.

EMSA

Double-Strand Oligos

Double-strand oligos containing the following sequences (with 5′ G overhang) were made: G3P1a, GACCTCTGATGTGCGGTT; mG3P1a, GACCTCTCTTGTGCGGTT; RE, GGGCACTGTGGGAACGGAA. 100 ng of the double-stranded oligos was labeled with Klenow (New England BioLabs) and [γ-^{32}P]dCTP (Perkin Elmer) and purified by column chromatography with Sephadex G-50 (GE-Healthcare-Amersham). 30,000 to 60,000 cpms of labeled oligos (1–5 ng) were incubated with 4 μg nuclear extract from Th2 cells or whole-cell extract from transfected CHO cells. Extracts were prepared as described (Amsen et al., 2004). Reactions were done in the presence of 2 μg of poly dI:dC in binding buffer (100 mM HEPES [pH 7.6], 4 mM EDTA, 2 mM DTT, 20 mM $MgCl_2$, 300 mM KCl, 40% Glycerol, supplemented with proteinase inhibitors). For supershift assays, 1 μg of rat anti-RBP-J (K0043) or isotype control antibody (11B11) was added for 3 hr prior to addition of probe. Samples were resolved on 5% polyacrylamide gels and visualized with HyBlot CL autoradiography film (Denville Scientific Inc.).

RT-PCR and Quantitative PCR

RNA was made with Trizol (Invitrogen, Life Technologies) and further purified with RNeasyTM columns (QIAGEN). RNA was transcribed into cDNA with Oligo(dT)$_{12-18}$ Primers and SuperScript II RNase H-RT Kit as described in the manuals (Invitrogen). *Gata3* exon1a-exon2 splice variant primers: TGTGGGAGCGTCAGCAACAG and AGGGAGAGAGGAATCCGAG; *Gata3* exon1b-exon2 splice variant primers: GAGACTGAGAGAGCGAGACATAG and GGAATCCGAGTGTGACCAC.

Primers and probes for detection of total GATA-3 and HPRT were described (Amsen et al., 2004). Quantitative PCR was performed for 40 cycles with 7500 Real Time PCR System (Applied Biosystems). Relative concentrations were determined on basis of standard curves of cDNA and normalized for HPRT contents with software provided by the manufacturer. HPRT and GATA-3 probes, as well as Power SYBR Green PCR Master Mix, were purchased from Applied Biosystems. Melt curves were run to ensure amplification of a single product.

Chromatin Immunoprecipitation

Chromatin was prepared and precipitated with the ChIP kit from Upstate Cell Signaling (cat# 17-295) essentially as per manufacturer's recommendations with minor modifications: preclearing was performed for 1 hr, and after precipitation, two washes with each of the wash buffers were carried out. Chromatin of 2×10^6 Th2 cells was precipitated per sample with polyclonal goat anti-RBP-J from Santa Cruz Biotechnology (sc-8213) or control goat serum (sc-2028) and salmon sperm-coated protein G-coupled agarose beads (Upstate Cell signaling, cat# 16-201). Precipitation of the GATA-3 promoters was measured by quantitative PCR with the following primers: upstream GATA-3 promoter, 5′-AATGACACTGCCCTGTGGAATG; upstream GATA-3 promoter, 3′-CCGTGCCCATAGAACCTCTTATTG; downstream GATA-3 promoter, 5′-ATTCCCTCCTGCCTGTCCC; downstream GATA-3 promoter, 3′-CAACCCAAACCCGCTCCAG.

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