Mechanisms of Notch signaling specificity in lymphocytes and their leukemic counterparts
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Citation for published version (APA):

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Chapter 5

Notch controls the magnitude of CD4+ T cell responses by promoting cellular longevity

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Abstract

Generation of effective immune responses requires expansion of rare antigen specific CD4+ T cells. The magnitude of the responding population is ultimately determined by proliferation and survival. Both processes are tightly controlled to limit responses to innocuous antigens. Sustained expansion occurs only when innate immune sensors are activated by microbial stimuli or by adjuvants, which has important implications for vaccination. The molecular identity of the signals controlling sustained T cell responses is not fully clear. Here we describe a prominent role for the Notch pathway in this process. Co-activation of Notch allows accumulation of far greater numbers of activated CD4+ T cells than stimulation via T cell receptor and classical co-stimulation alone. Notch does not overtly affect cell cycle entry or progression of CD4+ T cells. Instead, Notch protects activated CD4+ T cells against apoptosis after an initial phase of clonal expansion. Notch induces a broad anti-apoptotic gene expression program, which protects against intrinsic as well as extrinsic apoptosis pathways. Both Notch1 and Notch2 receptors and the canonical effector RBPJ are involved in this process. Correspondingly, CD4+ T cell responses to immunization with protein antigen are strongly reduced in mice lacking these components of the Notch pathway. Our findings therefore show that Notch controls the magnitude of CD4+ T cell responses by promoting cellular longevity.

Keywords
Notch, Delta, CD4 T cell, adjuvant, cell death
Introduction
The precursor frequency of naive CD4+ T cells specific for individual peptide antigens is low. Therefore, effective defense against microbial invaders requires expansion of these cells. This process is tightly regulated to prevent harmful responses to non-infectious self-antigens. Efficient induction of proliferation depends on ligation of the T cell receptor as well as co-stimulatory signals, such as those generated upon interaction of CD28 molecules on T cells with CD80/CD86 molecules on antigen presenting cells (APC). In addition, a major checkpoint determining the efficacy of an immune response lies in the ability to keep activated T cells alive after an initial phase of clonal expansion. This has been shown clearly by monitoring T cell responses to isolated protein antigens. Lacking signatures of microbial danger, such antigens elicit notoriously weak responses, which constitutes a major hurdle for vaccination strategies. The feeble nature of these responses is caused to a large degree by abortion of initially normal expansion due to death of the responding T cells. Responses to protein antigens can be boosted by the use of adjuvants, which induce signals that allow the activated T cells to survive. The identity of these signals is not fully known, although soluble factors such as IL15 can be involved. Identification of the mechanisms controlling viability of activated CD4+ T cells is key for optimizing our ability to manipulate the immune response to our desire, such as in vaccination.

Death of activated CD4+ T cells can be induced via an extrinsic pathway, involving Fas/CD95 mediated activation of caspase 8, which in turn activates effector caspases. In addition, an intrinsic apoptosis pathway exists, in which release of cytochrome C from mitochondria into the cytosol leads to activation of effector caspases. Cytochrome C release depends on Bax and Bak proteins and is inhibited by anti-apoptotic proteins, such as Bcl-2, Bcl-XL and Mcl-1. These anti-apoptotic factors are themselves antagonized by pro-apoptotic BH3 molecules such as Bim, Puma and Noxa and the relative levels of all these proteins determine the fate of the cell. The intrinsic apoptosis pathway controls contraction of the antigen responsive CD4+ T cell repertoire after clearance of the infection when survival cytokines are limiting. Death of CD4+ T cells activated in the absence of adjuvant also depends to a large degree on this intrinsic pathway with critical roles for Bim and to a lesser extent Puma. The extrinsic pathway may contribute to cell death under these conditions when antigen levels are high.

The Notch cell surface receptor controls proliferation and survival of a wide variety of cell types. Signaling by Notch involves cleavage within its transmembrane region by a γ-secretase complex. This allows the intracellular domain of Notch (NICD) to translocate to the nucleus, where it creates a transcriptional activator together with the DNA binding factor RBPJ (also known as CSL) and the MAML co-activator. Five canonical ligands for Notch exist, called Jagged1, Jagged2, Delta1 (DLL1), Delta3 (DLL3) and Delta4 (DLL4), which (except DLL3) activate the same biochemical Notch signaling pathway.
Expression of Notch ligands is induced on APC by stimulation with various microbial products, as well as by interactions with CD4⁺ T cells, supporting a role for this pathway in the control of T cell responses. Indeed, Notch was found to regulate differentiation of CD4⁺ T helper cells. Conflicting results have been reported about the role of Notch in CD4⁺ T cell expansion. Whereas some studies concluded that Notch stimulates proliferation of CD4⁺ T cells, other studies found it to be inhibitory. Notch signaling may have distinct consequences in CD4⁺ T cells under different conditions, but some of the reported discrepancies might also be caused by the experimental approaches used. For instance, some studies targeted the γ-secretase, which does not selectively affect cleavage of Notch only, but also of other γ-secretase substrates. Also, antibodies were used to activate Notch, which may not faithfully mimic the function of natural ligands. Finally, high expression of the active intracellular domain of Notch or the use of high concentrations of recombinant ligands may lead to induction of pathways not normally regulated by Notch.

Here we have revisited the role of Notch on expansion of CD4⁺ T cells. To avoid potential issues of specificity, we complemented gain of function approaches with the use of genetic deficiencies in the Notch pathway. We found that activation of Notch strongly boosts CD4⁺ T cell responses, predominantly by inducing a broad anti-apoptotic program after initial clonal expansion. Correspondingly, CD4⁺ T cell responses to protein antigens in vivo are much reduced after immunization of mice with deficiencies in the Notch pathway. Thus, our results show that Notch positively controls the size of the activated CD4⁺ T cell response by extending cellular longevity.

**Results**

**Notch ligands promote CD4⁺ T cell responses.**

Notch signaling may have different consequences depending on the developmental stage of the T cells. We therefore used purified naïve CD62L⁺CD44⁻CD4⁺ T cells (depleted of NK, NKT, regulatory and activated T cells) to test the consequence of Notch ligation on CD4⁺ T cell expansion. Much greater numbers of viable activated CD4⁺ T cells could be recovered from cultures using DLL4 expressing APC than from cultures using control APC (Figure 1A). This was true despite the presence of activating antibodies to CD28 in all cultures, suggesting that Notch ligands elicit signals that complement classical co-stimulatory signals.

Ectopic expression of ligands for Notch may lead to lateral activation of Notch in the APC, stimulating these cells to produce factors that promote expansion of the CD4⁺ T cells indirectly. To exclude this, we used a reductionist system, in which naïve CD4⁺ T cells were activated in the absence of APC. Recombinant DLL4-Fc strongly promoted recoveries of viable CD4⁺ T cells
Notch controls the magnitude of T helper cell responses by promoting cellular longevity compared to control-Ig cultures (Figure 1B and 1C right). Recombinant DLL1-Fc also augmented the yields from CD4+ T cell cultures, albeit much less potently than DLL4-Fc (Figure 1C). Thus, DLL ligands enhance CD4+ T cell responses through direct effects on CD4+ T cells.

Figure 1. Notch ligands increase recovery of activated CD4+ T cells. (A) Naive CD4+ T cells were stimulated with A20 cells expressing DLL4 (black triangles) or empty vector (open circles) in the presence of 1μg/ml anti-CD28 and different concentrations of anti-CD3. Shown is the number of viable cells recovered at day 4. (B) Naive CD4+ T cells were stimulated with 10μg/ml plate bound anti-CD3, 1μg/ml soluble anti-CD28 and 5μg/ml plate bound control-Ig or DLL4-Fc and the number of viable cells was determined at day 5. (C) Naive CD4+ T cells were stimulated and analyzed as in B with different concentrations of control-Ig, DLL1-Fc (left) or DLL4-Fc (right). Black triangles/bars represent DLL1/4-Fc and open circles/bars control-Ig samples in B and C. Results are representative of 3 (A), more than 10 (B) and two experiments (C).

Notch protects activated CD4+ T cells against cell death.

Stimulation with DLL might enhance cell cycle entry, the rate of proliferation or survival. To distinguish between these possibilities, we labeled naive CD4+ T cells with carboxyfluorescein diacetate succinimidyl ester (CFSE) and followed their proliferation. DLL4-Fc did not change the proportion of cells that failed to divide after two days of culture, suggesting that Notch activation does not affect the threshold for cell cycle entry (Figure 2A and S1). Furthermore, similar populations had divided once or twice at this time point, regardless of the presence of DLL4-Fc (Figure 2A and S1) and cell cycle distribution patterns of cells activated for 2 or 3 days in the presence of DLL4-Fc or control-Ig were indistinguishable (Figure 2B). Production of IL2 was also not affected by DLL4-Fc (Figure S2). Thus, DLL4-Fc did not affect general T cell activation or proliferation. Indeed, expansion of the CD4+ T cells was similar during the first 3 days of culture (Figure 2C).
In sharp contrast, at later time points after activation, DLL4-Fc promoted survival of cells that had already divided (Figure 2A and 2C). After 5 days, the majority of CD4+ T cells in control cultures had died, whereas most cells stimulated with DLL4-Fc were still alive (Figure 2A). Addition of QVD, a broad-spectrum caspase inhibitor, almost completely rescued survival of CD4+ T cells activated with control-Ig to the levels obtained by DLL4-Fc (Figure 2D). These results suggest that DLL4-Fc protects activated CD4+ T cells from apoptosis. Deficiency for Fas/CD95, the receptor involved in the

Figure 2. DLL4-Fc promotes survival of activated CD4+ T cells. Naive CD4+ T cells were stimulated as in Figure 1B. (A) Viability (7AAD) and proliferation (CFSE) were analyzed on different days. (B-E) Black bars represent DLL4-Fc and open bars control-Ig samples. (B) Cell cycle distribution analyzed at day 2 (top) and 3 (bottom). Shown is the mean of three independent experiments + SEM. (C) Recoveries of total (left) and viable cells (right) on different days after activation. (D) Cells were stimulated as in Figure 1B in the presence or absence of 20μM QVD and analyzed at day 5. Percentages of viable cells are shown. Mean of three independent experiments + SEM is shown. (E) Naive CD4+ T cells from Fas- and control mice were activated and analyzed at day 5 as in Figure 1B. A-D are representative of 3 and E of 2 experiments.
extrinsic apoptosis pathway, rescued a proportion of cells activated in the presence of control-Ig. Such deficiency did not, however, further increase the viable cell yields from DLL4-Fc cultures (Figure 2E). This suggests that DLL4-Fc protected cells that would have succumbed to Fas/CD95 mediated killing in the absence of Notch activation. Importantly, recoveries from cultures of Fas/CD95 deficient CD4+ T cells could still be enhanced two-fold by stimulation with DLL4-Fc (Figure 2E), demonstrating that this ligand for Notch also induces protection against cell death mediated by mechanisms not involving Fas/CD95. Thus, these results suggest that Notch induces protection against both extrinsic and intrinsic apoptosis pathways.

Elements of the Notch pathway involved.

To determine which Notch receptors control survival of activated CD4+ T cells, we activated naïve CD4+ T cells deficient for various Notch genes in the presence or absence of DLL4-Fc. Deficiency for Notch1 or Notch2 resulted in decreased, but still measurable survival responses to DLL4-Fc (Figure 3A and 3B). Responsiveness was lost entirely when CD4+ T cells lacked expression of both Notch1 and Notch2 (Figure 3C), showing that these two receptors function redundantly to regulate longevity of CD4+ T cells. This result suggests that, unlike Notch2 on thymocytes, Notch 2 on activated CD4+ T cells can interact with DLL4. Although expression of Notch3 and Notch4 is also induced in CD4+ T cells after activation, combined deletion of Notch3 and Notch4 did not significantly affect survival in response to DLL4-Fc (Figure 3D). Finally, DLL4-Fc mediated survival was entirely dependent on RBPJ (Figure 3E). Thus, both the Notch1 and the Notch2 receptors relay survival signals through the canonical Notch signaling pathway.

Figure 3. DLL4-Fc induced survival depends on Notch1, Notch2 and RBPJ. Naive CD4+ T cells were stimulated and analyzed at day 5 as in Figure 1B. Black bars represent DLL4-Fc samples and open bars control-Ig samples. (A) Cumulative results using CD4+ T cells from 3 individually tested Notch1fl/flCD4-Cre- (N1ko) and 5 Notch1fl/flCD4-Cre+ (WT) mice. (B) Cumulative results using CD4+ T cells from 4 individually tested Notch2fl/flCD4-Cre+ (N2ko) and 5 Notch2fl/flCD4-Cre+ (WT) mice. (C) CD4+ T cells from Notch1fl/flNotch2fl/flCD4-Cre- (N1/2ko) mice. (D) CD4+ T cells from Notch3-/-Notch4-/- (WT) or Notch3-/-Notch4-/- (N3/4ko) mice. (E) CD4+ T cells from RBPJfl/flCD4-Cre+ (WT) or RBPJfl/flCD4-Cre+ (RBPJko) mice. A and B show the mean + SEM (p-values *** <0.0001 (wt) and 0.0005 (N1ko), ** 0.004 (N1ko), * 0.0118 (N2ko) unpaired, two tailed T test. Results shown in C – E are individual mice representative of 3 experiments. (F) Surface expression of ICOS as in (A).
Notch induces a survival program.

Although activation of Notch induces protection against the extrinsic apoptosis pathway, neither expression of Fas (CD95) nor of its ligand (CD178) was reduced by stimulation with DLL4-Fc (Figure 4A). DLL4-Fc did modestly increase cell surface expression of CD25, the high affinity IL-2 receptor γ-chain (Figure 4A). On the other hand, there was no induction of the IL-2/IL-15 receptor β-chain (CD122), the common cytokine receptor γ-chain (CD132) or the IL-7 receptor α-chain (CD127) (Figure 4A). The elevated expression of CD25 in DLL4-Fc stimulated cells could allow stronger IL-2 receptor signaling, which might activate survival pathways. However, although IL-2 promotes survival under some conditions, it also increases the sensitivity of activated CD4+ T cells to the extrinsic apoptosis pathway. In fact, addition of exogenous IL-2 to control cultures reduced viable CD4+ T cell numbers (Figure S3). Therefore, stronger IL-2 receptor signaling is unlikely to explain the superior survival induced by DLL4-Fc, suggesting the existence of other protective mechanisms.

Because RBPJ is required to induce survival of CD4+ T cells (Figure 3E), a transcriptional mechanism is likely involved. We therefore prepared RNA from cells activated with control-Ig or DLL4-Fc to examine the consequences of Notch activation on expression of protective genes. RNA was isolated at a point just before cells started dying in the control-Ig cultures (3 days after activation, see Figure 2C, right). This way, we minimized possible indirect effects caused by different viability between the samples, while maximizing the chance that "protective" RNAs would be expressed. Using a multiplex assay, we measured mRNA expression of members of the Bcl-2 family (which inhibit the intrinsic apoptosis pathway), c-FLIP (an inhibitor of the extrinsic pathway, encoded by Cflar) and the inhibitor of apoptosis (IAP) family (XIAP in particular blocks apoptosis via both intrinsic and extrinsic pathways) (4) (Figure S4). Among these genes, only expression of Bcl2 was significantly induced and this translated into elevated Bcl2 protein (Figure S4, S5 and 4B and 4C). Retroviral expression of Bcl-2 rescued CD4+ T cells activated with control-Ig (Figure 4D). However, the degree of protection was much less than obtained by expression of NICD1 (Figure 4D), especially at later time points, suggesting the activation of additional protective pathways by Notch. One candidate for this function is the IκB family protein Bcl-3, which protects activated CD4+ T cells against cell death. Indeed, DLL4-Fc induced expression of Bcl-3, and retroviral expression of Bcl-3 rescued cells activated with control-Ig from death (Figure 4B and 4D). Again, however, this effect was less pronounced than the protection provided by expression of NICD1.

Since neither Bcl-2 nor Bcl-3 expression alone achieved the strong anti-apoptotic effects of Notch activation, we conducted genome wide transcriptional profiling for the consequences of stimulation with DLL4-Fc on activated CD4+ T cells. For analysis, we made use of the ROMER (ROtation testing using MEan Ranks) method for gene set enrichment analysis, using gene sets representing biological pathways (C2) or gene
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Figure 4. DLL4-Fc induces a broad survival program. Naive CD4+ T cells (Thy1.2+) were stimulated as in Figure 1B for 3 days. (A) Surface expression of markers. Filled grey histograms represent staining controls, dark grey lines specific staining on cells stimulated with control-Ig and black lines specific staining on cells stimulated with DLL4-Fc. (B) Relative abundance of transcripts for Bcl-2 (left) and Bcl-3 (right) was measured by quantitative-RT PCR and normalized against β-actin. Black bars represent DLL4-Fc samples and open bars control-Ig samples. Individual data points from 6 independent experiments are shown. Paired, two-tailed T test ** 0.0018 (Bcl2) and 0.003 (Bcl3). (C) Western blot for Bcl2 (bottom) or β-actin using lysates from cells generated as in (A). (D) After 36 hours of activation cells were transduced with retroviral expression vectors encoding NICD1 (left), Bcl-2 (middle), Bcl-3 (right) (all black bars) or control (open bars) linked to Thy1.1 via an IRES sequence. The percentages of viable Thy1.1+ cells were determined after different periods of culture. Days indicate the time from the start of the culture. (E) Relative expression of selected genes as determined by Illumina mouse gene chip. All genes were significantly induced by DLL4-Fc (p-value<0.01) (F) Surface expression of ICOS as in (A).
ontology categories representing biological processes, molecular functions and cellular compartments (C5) from MSigDB (http://www.broadinstitute.org/gsea/msigdb/). DLL4-Fc stimulation led to enrichment of multiple gene sets related to apoptosis (Table S6 and Supplementary data set 1, 2, 3, 4). Furthermore, stimulation with DLL4-Fc resulted in enrichment for gene sets associated with metabolic, biosynthetic and stress response pathways (Table S6 and Supplementary data set 1, 2, 3, 4), which may all impact cellular survival.

DLL4-Fc induced expression of a number of genes, which were previously described to protect T cells from apoptosis (Figure 4E). For example, deficiency for the Ets-1 transcription factor\textsuperscript{25} or the β-Arrestin adaptor protein (encoded by Arrb1)\textsuperscript{26} resulted in reduced survival of mature CD4\textsuperscript{+} T cells. Transgenic expression of IEX-1 (encoded by ler3) protected CD4\textsuperscript{+} T cells against superantigen mediated depletion\textsuperscript{27}, and both IEX-1 and Faim3 inhibited susceptibility of T cells to CD95/Fas mediated apoptosis induction\textsuperscript{27,28}. Although no pro-survival functions have yet been described for the Pim1 kinase and the TRAF5 adapter in CD4\textsuperscript{+} T cells, these factors were found to control survival of activated CD8\textsuperscript{+} T cells\textsuperscript{29,30}. Likewise, the GILZ transcription factor (encoded by Tcs22d3) protected T cell lines from apoptosis induced by cytokine withdrawal or T cell receptor stimulation\textsuperscript{31,32}, and Ddit4\textsuperscript{33} inhibited dexamethazone induced death of thymocytes. Finally, co-stimulation with DLL4-Fc induced an increase in both mRNA and cell surface levels of ICOS (Figure 4E and 4F), a CD28 family receptor implicated in control of CD4\textsuperscript{+} effector cell survival\textsuperscript{34}. In summary, Notch activates a broad gene expression program to protect activated CD4\textsuperscript{+} T cells from cell death. Due to the diversity of protective mechanisms induced, ectopic expression of individual factors may never achieve the level of protection elicited by Notch.

Figure 5. Notch signaling is required for optimal CD4\textsuperscript{+} T cell responses \textit{in vitro}. Naïve OTII transgenic CD4\textsuperscript{+} T cells were stimulated with Ovalbumin protein presented by splenic CD11c\textsuperscript{+} DCs for 5 days. Viability was analyzed as in Figure 1A. Black bars represent wild type (RBPJ\textsuperscript{fl/fl}CD4-Cre\textsuperscript{−}) and open bars RBPJ knock out (RBPJ\textsuperscript{fl/fl}CD4-Cre\textsuperscript{−}) CD4\textsuperscript{+} T cells from littermate mice. Results are representative of 3 experiments.
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Notch determines the magnitude of CD4+ T cell responses in vivo.

To test whether Notch protects activated CD4+ T cells from cell death in the context of antigen presentation by genuine APC, we introduced T cell receptor (TCR) transgenes into mice with T cell specific deletion of either RBPJ or both Notch1 and 2. RBPJ deficient CD4+ T cells, (expressing the OTII T cell receptor, specific for Ovalbumin peptide in I-A\(^{b}\)), expanded to the same degree as wild type CD4+ T cells in response to presentation of protein antigen by dendritic cells (Figure 5). However, whereas most wild type CD4+ T cells were viable still after 5 days of culture, the majority of the RBPJ deficient CD4+ T cells had died by this time (Figure 5). Similar findings were obtained with CD4+ T cells lacking expression of both Notch1 and Notch2 (using the AND TCR specific for pigeon Cytochrome C peptide in I-E\(^{k}\)) (Figure S7). Therefore, also when activated by professional APC, CD4+ T cells depend on Notch for their survival after initial expansion. We reasoned that this requirement might result in reduced responses of CD4+ T cell populations isolated from Notch deficient mice 8 days after immunization, as fewer antigen specific cells would persist to be reactivated. To test this, we immunized mice with T cell specific deletions in both Notch1 and Notch2 genes with Keyhole Limpet Hemocyanin (KLH) adsorbed to alum, a major adjuvant used for vaccination of humans\(^{35}\). In vitro proliferation of restimulated Notch1/Notch2 double deficient CD4+ T cells was significantly weaker than responses elicited in wild type mice (Figure 6A). Although alum is a clinically relevant adjuvant, it is not a natural adjuvant associated with microbial pathogens. To test whether Notch controls the magnitude of CD4+ T cell responses in the context of a

Figure 6. Notch signaling is required for optimal CD4+ T cell responses in vivo.
Mice were immunized subcutaneously with KLH in alum (A), KLH in SEA (B) or SEA alone (C-D). After 8 days, CD4+ T cells from draining lymph nodes were restimulated in vitro with splenic APC loaded with different concentrations of KLH or SEA as indicated. Proliferation was measured on the basis of \(^{3}H\)-thymidine incorporation during the last 12 hours of 72 hour culture and displayed as cpm. (A-B) Notch1\(^{fl/fl}\)/Notch2\(^{fl/fl}\)/CD4-Cre\(^{-}\) (WT - closed symbols) or Notch1\(^{fl/fl}\)/Notch2\(^{fl/fl}\)/CD4-Cre\(^{-}\) (N1/2 KO - open symbols) (C) Notch1\(^{fl/fl}\)/CD4-Cre\(^{-}\) (WT - closed circles), Notch1\(^{fl/fl}\)/CD4-Cre\(^{-}\) (N1 KO - open circles), Notch2\(^{fl/fl}\)/CD4-Cre\(^{-}\) (WT - closed squares) or Notch2\(^{fl/fl}\)/CD4-Cre\(^{-}\) (N2 KO - open circles) (D) RBPJ\(^{fl/fl}\)/Cre\(^{-}\) (WT - closed circles) and RBPJ\(^{fl/fl}\)/CD4-Cre\(^{-}\) (RBP KO - open circles). Each symbol represents a single mouse. Results are representative of at least 3 experiments.
natural adjuvant, we immunized mice with KLH mixed with extract of the eggs of the helminth parasite *Schistosoma mansonii* (SEA). This extract elicits responses both to antigens present in the extract (Figure 6B, right) as well as to the KLH model antigen (Figure 6B, left). Importantly, responses to both types of antigens were markedly reduced in mice lacking expression of Notch1 and Notch2 in T cells (Figure 6B). On the other hand, no defect was observed upon immunization of mice lacking only Notch1 or Notch2 (Figure 6C), showing that these receptors are redundant for this function. Finally, CD4+ T cell responses were strongly reduced in mice lacking expression of RBPJ (Figure 6D). Thus, the magnitude of the CD4+ T cell response after immunization with protein antigens and different adjuvants depends on Notch1, Notch2 and canonical Notch signaling.

**Discussion**

The ability of the immune system to combat large numbers of microbes, while limiting responses to innocuous antigens, requires tight regulation of the magnitude of the antigen responsive CD4+ T cell population. Our findings show that Notch controls expansion of this population, predominantly by protecting already expanded clones from apoptosis.

The anti-apoptotic activity of Notch in primary CD4+ T cells depends on RBPJ (Figure 3E), suggesting that a transcriptional mechanism is involved. Whether non-transcriptional mechanisms are also involved, based for instance on the previously reported physical interaction of the NICD with factors such as XIAP, Bax, Nur77, p56Lck and PI3K, remains to be tested. Notch induces a transcriptional program which includes genes with protective activities against both extrinsic and intrinsic apoptosis pathways. Thus, the extrinsic pathway is inhibited by Faim3, IEX-1 and TRAF5. For Faim3, this may involve inhibition of caspase 8 recruitment and diversion of Fas/CD95 signaling towards survival pathways. On the other hand, Bcl2, Bcl3 and β-arrestin inhibit the intrinsic apoptosis pathway by preventing Bax/Bak multimerization, release of Bim and inducing expression of the Bcl2 gene, respectively. How other induced factors fit into this scheme is less clear. The leucine zipper transcription factor GILZ may inhibit the intrinsic as well as the extrinsic pathway by repressing the Bim, Fas and FasL genes. The Pim1 kinase inhibits the intrinsic pathway by inactivating the pro-apoptotic BH3 domain protein Bad and stimulates cellular metabolism, which may help activated cells cope with the metabolic demands associated with rapid proliferation. That Notch stimulates metabolic pathways is supported further by the enrichment for various gene sets associated with carbohydrate, lipid and glucose metabolism, glucose transport, biosynthesis and generation of energy (Table S6 and Supplementary data set 1, 2, 3, 4). Although previous studies found Notch to induce expression of BclXL, neither mRNA nor protein levels for this Bcl2 family member were induced by DLL4-Fc (Figure S4 and S8). Also, we did not find evidence for increased
expression of Bmi1 (Figure S9) or decreased expression of Noxa (Figure S4)\(^{20}\).

Some of the anti-apoptotic mediators induced by Notch may be more prominently involved in protecting CD4\(^+\) T cells in vivo than in the reductionist in vitro culture system used in this study. One example may be ICOS. Expression of mRNA for its ligand was undetectable in activated T cells, making a function for this receptor in our T cell cultures unlikely. However, ICOS can promote CD4\(^+\) T cell survival in vivo\(^{34}\) and may therefore be an important player in the program used by Notch upon immunization.

The requirement for specific signals which license CD4\(^+\) T cell expansion helps restrict the generation of aggressive immune responses to those antigens that merit such potentially harmful activity. Adjuvants or microbial structures elicit the production of signals licensing the survival of activated CD4\(^+\) T cells even to non-infectious protein antigens\(^{1,2}\). Notch protects activated CD4\(^+\) T cells at precisely the stage controlled by adjuvants, making it a candidate receptor for this licensing function. In support of this, microbial stimuli induce expression of several Notch ligands on APC\(^{10,12}\) and Notch, like adjuvants, induces a protective program which includes Bcl-3\(^{3,23,40}\). Interestingly, Notch also promotes expansion of CD8\(^+\) T cells, in part by protecting these cells from cell death, although in these cells Notch also seems to enhance proliferation itself (Figure S10). Thus, Notch seems to have a rather general role in controlling the viability of activated T cells. We are currently investigating the importance of this function of Notch in CD8\(^+\) T cell responses in vivo.

In conclusion, our results demonstrate that Notch activates multiple pathways that protect activated CD4\(^+\) T cells against apoptosis. This function of Notch is essential for the generation of full-blown CD4\(^+\) T cell responses upon immunization. Thus, Notch, a conserved decider of cell fate, has been adopted by the immune system to determine whether the presence of antigen deserves the generation of an aggressive immune response.

**Materials and Methods**

**Reagents.** Antibodies are listed in the supplementary material. Q-VD-OPh (QVD) was purchased from R&D (catalog number OPH001). Human Fc-tagged DLL1 and DLL4 protein was produced in HEK293T cells transfected with expression plasmids for DLL1-Fc or DLL4-Fc\(^{21}\). For isolation procedure see supplementary material. Isotype matched control-Ig (Palivizumab, synagis #54874TF) was a gift from AIMM Therapeutics.

**Vectors and constructs.** Bcl-2 was cloned from C57BL6/NCrl CD4\(^+\) T cell cDNA into the MSCV-Thy1.1 vector using BglII and SalI. Bcl-3-MSCV-Thy1.1 and NICD1-MSCV-Thy1.1 were described before\(^{10,23}\).
Chapter 5

**Cell culture.** All cells were grown in IMDM with 10% FCS, 2mM GlutaMAX (Invitrogen), 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen) and 50μM β-Mercaptoethanol.

**Mice.** Six- to eight-week-old C57BL/6NCrl mice were purchased from Charles River. Notch1 flox, Notch2 flox, Notch3 null, Notch4 null, RBPJ flox, CD4-Cre transgenic mice and Faslpr were all backcrossed to C57Bl/6 for over 10 generations. Experimental and control mice were littermates. Cre* mice heterozygous for floxed alleles or homozygous wild type behaved as Cre+ mice. For origins of mice see Supplementary information. Mice were maintained in the animal facility of the AMC under specific pathogen free conditions. All animal experiments were in compliance with EU and national laws and approved by the local ethical committee.

**CD4 T cell activation.** Naïve CD44-CD62L+CD49b-CD25- (Thy1.2+) CD4+ T cells were purified from spleen and peripheral lymph nodes using anti-CD4 microbeads (Miltenyi, cat# 130-049-201) and FACsorting. 5x10^4 naïve CD4+ T cells were cultured with 1μg/ml anti-CD28 in 96-well flat bottom plates coated with 10μg/ml anti-CD3 and 5μg/ml DLL4-Fc, DLL1-Fc or control-Ig or with 2.5 x 10^4 irradiated A20 cells (120Gy) expressing DLL4 or empty vector, 1μg/ml anti-CD28 and different amounts of anti-CD3. Alternatively, 5 x 10^4 naïve CD4+ T cells were cultured with 5 x 10^4CD11c+ dendritic cells (DC), purified from spleen by MACS using anti-CD11c beads (Miltenyi 130-052-001), and Ova protein (50μg/ml). Viability and cell numbers were analyzed by flow cytometry using AnnexinV-APC (BDbiosciences 550474) and 7-amino-actinomycinD (7AAD, eBioscience 00-6993-50). For labeling with 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE-Sigma 21888) naïve CD4+ T cells were washed with cold PBS and stained for 5 minutes at room temperature with 5μM CFSE at 2x10^6 cells/ml in PBS, followed by washing with PBS (1% BSA).

**Retroviral transductions.** PlatE cells were transfected with retroviral expression plasmids using FugeneHD (Promega). Two days after transfection virus containing medium was supplemented with 8μg/ml polybrene and added to CD4+ T cells activated 36 hours earlier. The cells were centrifuged at 700g for 90 minutes at 37°C. After an additional incubation of 2.5 hours at 37°C the medium was changed.

**Cell cycle analysis.** Activated CD4+ T cells were stained with 10μM Draq5 (Biostatus) in medium. After 30 minutes at 37°C cells were analyzed by flow cytometry. Cell cycle distribution was analyzed with the cell cycle tool (FlowJo) using the Watson (pragmatic) model.

**Microarray analysis and quantitative RT-PCR.** RNA was extracted using Trizol followed by RNeasy columns (Qiagen) from CD4+ T cells 3 days after activation. RNA was labeled and hybridized by the W.M. Keck facility (Yale Center for Genome Analysis) on Illumina Mouse BeadChip (Illumina, Inc.) mouse whole-genome expression arrays (MouseRef-8 v2.0). Two pairs of
samples are replicates from one experiment, the third pair comes from an independent experiment. For statistical analysis see Supplementary methods. Data have been deposited in NCBI Gene Expression Omnibus (GEO) in a MIAME compliant format and are accessible through GEO Series accession number GSE35547. cDNA was made with Oligo(dT) and random hexamers using the First Strand cDNA synthesis kit (Fermentas). Quantitative PCR using SYBRgreen (Bio-Rad) was performed using the C1000 Thermal Cycler (Bio-Rad). Relative concentrations were determined based on standard curves and normalization for β-actin contents using the Bio-Rad CFX Manager software. Melt curves ensured amplification of a single product. For PCR primers see supplementary material.

**Immunization.** Mice were immunized subcutaneously with 50μg KLH in alum, KLH with SEA (300μg) or SEA alone (for preparation SEA see Supplementary Methods). After 8 days, CD4⁺ T cells were isolated from draining lymph nodes and stimulated *in vitro* with splenic APC and different concentrations of KLH or SEA. Proliferation was measured by 3H-thymidine incorporation during the last 12 hours of a 72 hour culture period and displayed as counts per minute (cpm).

**Acknowledgements**

We thank Dr. E. Esplugues for help with gene chips, Dr. F. Radtke for DLL-Fc plasmids, AIMM Therapeutics for control-Ig, J. van Laar for help with MLPA, Dr. K. van Gisbergen for lpr mice, Dr. T. Mitchell for Bcl3-MSCV-Thy1.1, Dr. M. Wolkers for PlatE, Dr. S. Rutz for A20-DLL4, D. Picavet for help with genotyping and Dr. J. Magarian Blander for critical reading of the manuscript. This work was supported by a Boehringer Ingelheim PhD fellowship to CH, NIH grant HD034883 to TG. RAF is an investigator of the Howard Hughes Medical Institute. DA was supported by an AMC fellowship and a fellowship from the Landsteiner Foundation for Blood Research.

**References**


Supplementary Information

Figure S1. DLL4-Fc does not affect the proportion of cells in different division peaks. Naive CD4+ T-cells were labeled with CFSE and stimulated as in Figure 1B with control-Ig (open bars) or DLL4-Fc (closed bars). The number (left) and percentage (right) of cells in each CFSE peak was determined on day 2 after activation.

Figure S2. DLL4-Fc does not stimulate production of IL-2. Naive CD4+ T-cells from C57Bl/6 mice were stimulated as in Figure 1B. Supernatants were collected at 12, 16 and 21h after initiation of the culture. IL-2 concentrations were determined by ELISA using anti-IL-2 (JES6-1A12), recombinant mouse IL-2 and anti-IL-2-biotin (JES6-5H4).

Figure S3. Addition of IL-2 diminishes rather than promotes survival of activated CD4+ T-cells. 5x10^4 naïve CD4+ T-cells were activated as in Figure 1B. Recombinant mouse IL-2 was added from the start of the culture in concentrations as indicated in the Figure. After 5 days, survival was determined as in Figure 1B.
Figure S4. Bcl-2 is the only anti-apoptotic factor found by MLPA to be up-regulated by DLL4 stimulation. Naive CD4+ T-cells from C57Bl/6 mice were stimulated as in Figure 1B. Three days after initiation of the culture, RNA was isolated. RNA was extracted using Trizol (Invitrogen) and further purified with RNeasy columns (Qiagen). Multiplex Ligation-Dependent Probe Amplification (MLPA) was done as described earlier (11). Three independent experiments were done. Shown is the relative expression per gene of the DLL4-Fc sample divided by the control-Ig sample. Results are cumulative data from 3 independent experiments. Paired t-test, n=3. Please note that the change in expression of Bcl-G was not reproducible (see Figure. S5).

Figure S5. Bcl-G expression is not significantly down-regulated by DLL4-Fc. Normalized expression data for Bcl-G from individual experiments as measured by the MLPA shown in Figure. S4.
Figure. S6. CD4 T-cells activated by DC require Notch to survive after division. Naïve CD4+ T-cells from Notch1fl/fl Notch2fl/fl CD4-Cre (WT) or Notch1fl/fl Notch2fl/fl CD4-Cre (N1/2ko) AND TCR transgenic mice on a C57Bl/6 background were labeled with CFSE and cultured with B10BR (I-Ek+) splenic CD11c+ cells in the presence of 10μg/ml cytochrome c. Five days after initiation of the culture cells were analyzed by flow cytometry for viability and proliferation using 7AAD and CFSE.

Figure S7. Western blot for Bcl-XL. Naïve CD4+ T-cells were activated as in Figure. 1B. After 3 days, cells were lysed and protein lysates were analyzed by western blot for Bcl-XL and β-actin.

Figure S8. Bmi1 is not regulated by DLL4-Fc. Normalized expression data from samples obtained in 3 different experiments, measured by gene chip.

Figure S9. DLL4-Fc promotes survival of activated CD8 T-cells. Naïve CD8 T-cells were stimulated and analyzed as in Figure. 1B. Paired t-test, two-tailed, n=7 shown is the mean +SEM, the p-values are * (0.014) and ** (0.0079).

Table S1. ROMER analysis of genes regulated by DLL4-Fc. Naïve CD4+ T-cells from C57Bl/6 mice were stimulated as in Figure. 1B. Three days after initiation of the culture RNA was isolated and gene expression was assessed by illumina mouse gene chip. ROMER was used for gene set enrichment analysis. ROMER was applied using gene sets representing biological pathways (C2: BioCarta, KEGG, Reactome) or gene ontology categories representing biological processes, molecular functions and cellular compartments (C5) from MSigDB (http://www.broadinstitute.org/gsea/msigdb/). Shown are significantly enriched gene sets affected by DLL4-Fc stimulation selected for association with apoptotic, metabolic, biosynthetic and stress functions. Lists of all significantly enriched gene sets can be found in Supplementary data sets 1 and 2.
### C2

**UP**

- KEGG_CG_CG_CS: BOSPHOLIPID BIOSYNTHESIS GLOBO SERIES 7 1.00E-04
- REACTOME_CG_CG_CS: GLYCOCEN BREAKDOWN GLYCOGENOLYSIS 11 1.00E-04
- REACTOME_CG_CG_CS: GLUCOSE METABOLISM 38 1.00E-04
- KEGG_CG_CG_CS: BOSPHOLIPID BIOSYNTH LACTO AND NEOGLOBE SER. 14 1.00E-04
- KEGG_PYRUVATE_MERABLISM 24 1.00E-04
- KEGG_GLUTATHNE METABOLISM 31 1.00E-04
- KEGG_GLYCOCEN GLYCOGENOLYSIS 34 1.00E-04
- REACTOME_CG_CG_CS: GLYCOGENOLYSIS 13 1.00E-04
- HYPERCARTA_BI_PATHWAY 12 1.00E-04
- KEGG_MG_MG_CS: SIGNALING PATHWAY 38 1.00E-04
- KEGG_MG_MG_CS: STARCH AND SUCROSE METABOLISM 14 0.0066
- REACTOME_CG_CG_CS: BEROXISOMAL LIPID METABOLISM 14 0.0115
- KEGG_GALACTOSE METABOLISM 17 0.0159
- REACTOME_STEROID METABOLISM 31 0.0352
- REACTOME_MG_MG_CS: SIGNALING 21 0.05

**DOWN**

- REACTOME_APOPTOSIS 102 1.00E-04
- HYPERCARTA_STRESS PATHWAY 24 0.0101
- REACTOME_CG_CG_CS: DEATH RECEPTOR SIGNALING 10 0.0152
- REACTOME_APOPTOSIS 7 0.0277

**MIXED**

- KEGG_MG_MG_CS: STARCH AND SUCROSE METABOLISM 14 1.00E-04
- KEGG_CG_CG_CS: BOSPHOLIPID BIOSYNTHESIS GLOBO SERIES 7 1.00E-04
- KEGG_CG_CG_CS: BOSPHOLIPID BIOSYNTH GLOBLI SERIES 11 1.00E-04
- KEGG_TERPENOID BACKBONE BIOSYNTHESIS 10 1.00E-04
- KEGG_METABOLISM_OF_XENOBIOTICS_BY_CYTOCHROME_250 20 1.00E-04
- HYPERCARTA_BI_PATHWAY 18 1.00E-04
- REACTOME_APOPTOSIS 102 1.00E-04
- REACTOME_CG_CG_CS: FACILITATIVE NA INDEPENDENT GLUCOSE TRANSPORT 6 1.00E-04
- REACTOME_CG_CG_CS: GLYCOCEN BREAKDOWN GLYCOGENOLYSIS 11 1.00E-04
- REACTOME_CG_CG_CS: INTRINSIC PATHWAY FOR APOPTOSIS 24 1.00E-04
- REACTOME_CG_CG_CS: METABOLISM OF CARBOHYDRATES 77 1.00E-04
- REACTOME_CG_CG_CS: PYRUVATE METABOLISM 13 1.00E-04
- REACTOME_CG_CG_CS: TRIACLYGLYERIDE BIOSYNTHESIS 12 1.00E-04
- REACTOME_CG_CG_CS: SYNT_ AND INTERCONV_ OF NUCL_ DI AND TRIPHOSPH. 14 1.00E-04
- KEGG_APOPTOSIS 66 0.0185
- REACTOME_CG_CG_CS: PYRUVATE METABOLISM AND TCA CYCLE 29 0.0445

### C5

**UP**

- MITOCHONDRDIAL OUTER MEMBRANE 16 1.00E-04
- GLYCOLIPID METABOLIC_PROCESS 9 1.00E-04
- NEGATIVE_REGULATION_ APOPTOSIS 101 1.00E-04
- ENERGY_RESERVE METABOLIC_PROCESS 9 1.00E-04
- STEROID METABOLIC_PROCESS 35 1.00E-04
- MEMBRANE LIPID BIOSYNTHETIC_PROCESS 34 1.00E-04
- CELLULAR CARBOHYDRATE METABOLIC_PROCESS 75 1.00E-04
- GLYCOPHOSPHOLIPID METABOLIC_PROCESS 6 1.00E-04
- ANTI_APOPTOSIS 78 1.00E-04
- CARBOHYDRA METABOLIC_PROCESS 106 1.00E-04
- REGULATION_ APOPTOSIS 228 1.00E-04
- NEGATIVE_REGULATION_ PROGRAMMED CELL DEATH 102 1.00E-04
- REGULATION_ PROGRAMMED CELL DEATH 229 1.00E-04
- RESPONSE TO HYPOXIA 18 1.00E-04
- PROGRAMMED CELL DEATH 279 2.00E-04
- APOPTOSIS 278 0.0016

**DOWN**

- APOPTOTIC MITOCHONDIAL CHANGES 9 1.00E-04

**MIXED**

- GLYCOLIPID METABOLIC_PROCESS 9 1.00E-04
- GLYCOPHOSPHOLIPID METABOLIC_PROCESS 6 1.00E-04
- CARBOHYDRA METABOLIC_PROCESS 106 1.00E-04
- RESPONSE TO HYPOXIA 18 1.00E-04
- OXYGEN AND REACTIVE OXYGEN SPECIES METABOLIC_PROCESS 8 1.00E-04
- SUPEROXIDE METABOLIC_PROCESS 5 1.00E-04
- GLYCOPHOSPHOLIPID BIOSYNTHETIC_PROCESS 22 1.00E-04
- PROTEIN METABOLIC_PROCESS 781 1.00E-04
- GLUCOSE CATALABIC PROCESS 7 1.00E-04
- GLUCOSE METABOLIC_PROCESS 17 1.00E-04
- APOPTOTIC_PROGRAM 38 1.00E-04
- NEGATIVE_REGULATION_ METABOLIC_PROCESS 167 1.00E-04
- NEGATIVE_REGULATION_ CELLULAR METABOLIC_PROCESS 166 1.00E-04
- RESPONSE TO STRESS 308 1.00E-04
- CELL PROLIFERATION_GO_0008283 279 1.00E-04
- REGULATION_ METABOLIC_PROCESS 594 1.00E-04
- REGULATION_ CELLULAR METABOLIC_PROCESS 495 1.00E-04
- GLUCAN METABOLIC_PROCESS 6 0.0098
- GLYCOPROTEIN BIOSYNTHETIC_PROCESS 42 0.0142