using the program O²⁹. We used a single CA molecule as the docking unit; the linker between the N-terminal domain and C-terminal domain (residues 146–149) was allowed full flexibility within steric constraints.

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correction

Signalling through CD30 protects against autoimmune diabetes mediated by CD8 T cells

Christian Kurts, Francis R. Carbone, Matthew F. Krummel, Karl M. Koch, Jacques F. A. P. Miller & William R. Heath

Nature **398**, 341–344 (1999).

It has come to our attention that the conclusions drawn in this paper need to be revised. We have found that the effects attributed to CD30 were the result of background gene(s) of 129 origin. Subsequent backcrossing of the CD30-deficient OT-I line by G. Davey in our laboratory segregated the capacity of low numbers of OT-I cells to cause disease from their capacity to express CD30. Our conclusion that signalling through CD30 protects against autoimmune diabetes must be reconsidered as an effect of a background gene(s) that we are currently mapping. □

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Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

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erratum

How self-tolerance and the immunosuppressive drug FK506 prevent B-cell mitogenesis

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The fourth sentence in the Methods section should have read “For stimulation experiments, HEL (Sigma) was used at 500 ng ml⁻¹, goat anti-mu (Jackson Labs) at 10 µg ml⁻¹, FK506 (gift from G. Crabtree laboratory) at 10 ng ml⁻¹, PD98059 (NEB) at 20 µM unless otherwise stated, ionomycin (gift from G. Crabtree laboratory) at 1 µM and EGTA at 3 mM.” □

electrode with single-voltage pulses (100–200 μ s, up to 5 V). The stimulating electrode was typically placed over layer 1 about 500 μ m lateral to the cells. After recordings, slices were fixed and stained as described¹. All results are reported as mean $\pm$ s.e.m.

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Signalling through CD30 protects against autoimmune diabetes mediated by CD8 T cells

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Autoantigens found on pancreatic islets can move to draining lymph nodes, where they are able to cause the activation and consequent deletion of autoreactive T cells by a mechanism termed cross-tolerance^{1,2}. This deletion depends on signalling through CD95 (also known as Fas), a member of the superfamily

of tumour-necrosis-factor receptors³. Here we describe a new mechanism that protects against autoimmunity: this mechanism involves another member of this superfamily, CD30, whose function was largely unknown. CD30-deficient islet-specific CD8-positive T cells are roughly 6,000-fold more autoaggressive than wild-type cells, with the transfer of as few as 160 CD30-deficient T cells leading to the complete destruction of pancreatic islets and the rapid onset of diabetes. We show that, in the absence of CD30 signalling, cells activated but not yet deleted by the CD95-dependent cross-tolerance mechanism gain the ability to proliferate extensively upon secondary encounter with antigen on parenchymal tissues, such as the pancreatic islets. Thus, CD30 signalling limits the proliferative potential of autoreactive CD8 effector T cells and protects the body against autoimmunity.

CD30 is a member of the tumour-necrosis-factor receptor (TNFR) superfamily, and is expressed by activated, but not by resting, B or T cells^{4–8}. Although its function is largely unknown, *in vitro* studies have shown that it has effects on both cell death and cell activation^{7–11}. T cells of the T-helper-2 phenotype may express CD30 (ref. 6), although this has been questioned¹². Mice deficient in CD30 showed a mild impairment in thymic negative selection¹³, but a function for CD30 in the *in vivo* response of mature T cells has yet to be demonstrated.

Here we investigated the role of CD30 signalling in the maintenance of self-tolerance by using a model system in which ovalbumin (OVA)-specific CD8⁺ T cells from the OT-I transgenic line (OT-I cells) were adoptively transferred into unirradiated transgenic mice that expressed OVA in the pancreatic β -cells and the proximal tubular cells of the kidney (RIP-mOVA mice)¹⁴. In this model, wild-type OT-I cells caused diabetes only when adoptively transferred in relatively large numbers (>250,000 cells) (Table 1), with lower doses being effectively tolerated. In contrast, CD30-deficient OT-I cells (OT-I.CD30^{–/–} cells) were highly diabetogenic, with as few as 160 cells causing disease. The diabetogenic potential of such small doses of CD30-deficient cells indicates that CD30 is important in the prevention of autoimmunity mediated by CD8⁺ T cells.

We have shown previously that OT-I cells are slowly deleted by a CD95-dependent mechanism in the weeks following transfer into RIP-mOVA mice³. CD30 deficiency did not impair this slow deletion process (data not shown), but seemed to exert its effect much earlier than did CD95, as indicated by the rapid onset of diabetes as early as 4 days after adoptive transfer. To gain understanding of what was happening in these few days following adoptive transfer, we used flow cytometry to count wild-type and CD30-deficient OT-I cells in the spleen and lymph nodes of RIP-mOVA mice shortly after adoptive transfer (Fig. 1). There was a marked antigen-specific increase (expansion) in the number of CD30-deficient cells, but not of wild-type OT-I cells, peaking on days 4–5 after adoptive transfer into RIP-mOVA mice. This expansion was accompanied by prominent infiltration of the CD30-deficient cells into the pancreas and by β -cell destruction (data not shown). Thus, CD30-deficient cells

Table 1 CD30-deficient OT-I cells cause diabetes in RIP-mOVA mice

No. of injected cells	No. of diabetic mice/no. in group	
	OT-I	OT-I.CD30 ^{–/–}
5,000,000	18/18	5/5
2,000,000	9/10	9/9
1,000,000	4/8	6/6
250,000	0/26	9/10
100,000	0/7	9/11
20,000		12/13
4,000		6/6
800		9/13
160		4/9

Data from several experiments are pooled. Mice were injected with semi-purified OT-I or OT-I.CD30^{–/–} T cells and then examined for diabetes by analysis of urine glucose as described¹⁵. In some experiments, diabetes was confirmed by analysis of blood glucose, and islet destruction was confirmed by histology as described¹⁶. Diabetes onset occurred between days 4 and 10, depending on the number of cells transferred.

proliferated extensively in the few days following adoptive transfer, indicating that CD30 signalling may normally limit the proliferative potential of autoreactive cells.

When transferred into RIP-mOVA mice, OT-I cells respond to antigen sequentially, in two different phases, each of which occurs in a separate site. In the priming phase of the response, naïve OT-I cells are activated by OVA cross-presented on bone-marrow-derived antigen-presenting cells (APCs) in the draining lymph nodes of the kidney and pancreas; then, during the effector phase, activated OT-I cells enter parenchymal tissues and attack cells synthesizing OVA (for example, pancreatic-islet β -cells)^{1,14,15}. Under normal circumstances, OT-I cells primed in this manner generate a very weak effector phase which is able to cause diabetes only when large numbers of OT-I cells ($>250,000$) are adoptively transferred (ref. 15 and Table 1). Lower numbers of OT-I cells are not pathogenic and are eventually deleted through the CD95-dependent mechanism³, thus maintaining self-tolerance.

To determine where CD30 exerted its effect on the proliferation, we studied the response of wild-type and CD30-deficient OT-I cells in both sites of antigen recognition, that is, the pancreatic islets and the draining lymph nodes. To examine proliferation during the effector phase in the pancreatic islets, we adoptively transferred 2×10^5 wild-type or CD30-deficient OT-I cells into RIP-mOVA mice and then 5 days later injected a 6-h pulse of the thymidine analogue bromodeoxyuridine (BrdU), which is incorporated into the DNA of proliferating cells *in situ*. This short pulse was used to ensure that those BrdU-labelled cells found in the pancreas had proliferated in this site. Immunohistological examination of the pancreas revealed many BrdU-positive (proliferating) cells associated with the islets of mice given CD30-deficient OT-I cells, but no BrdU-positive cells in mice given wild-type OT-I cells (Fig. 2). This showed that CD30-deficient cells, in contrast to wild-type cells, proliferated extensively in the pancreatic islets.

Analysis of the proliferation rates of OT-I cells during the priming phase in the pancreatic draining lymph nodes revealed no difference between wild-type or CD30-deficient cells on days 2 or 3 after adoptive

transfer (data not shown). Thus, CD30 did not affect proliferation during the priming phase in the draining nodes. Taken together, these data indicate that the preferential expansion of CD30-deficient cells occurred upon recognition of antigen in the pancreatic islets.

Although we had shown that CD30-deficient cells proliferated preferentially after recognizing antigen on islet cells, it was important to determine whether priming in the draining nodes was a prerequisite for this recognition to occur; that is, was there a link between priming in the nodes and proliferation in the islets? To address this issue, we asked whether expansion of CD30-deficient OT-I cells required antigen recognition in the draining nodes (during the priming phase), in the parenchymal tissues (during the effector phase), or in both tissues. We generated chimaeric mice using two congenic mouse strains, C57Bl/6 (B6) and bm1, which differ at the major-histocompatibility-complex (MHC) locus *H-2K*. OVA can be presented to OT-I cells by the MHC class-I molecule *H-2K^b* (expressed by B6 mice), but not by *H-2K^{bm1}* (expressed by bm1 mice)¹⁶. After introducing bm1 bone marrow into RIP-mOVA mice of the B6 type (bm1 $\rightarrow$ B6), the bone-marrow-derived cells of the recipients were incapable of presenting OVA to OT-I cells, and thus priming in the draining lymph nodes was blocked, whereas presentation of OVA by parenchymal tissues remained functional. Likewise, introduction of B6 bone marrow into RIP-mOVA mice of the bm1 type (B6 $\rightarrow$ bm1) generated chimaeras in which antigen recognition on parenchymal tissues was impossible, but priming in the draining lymph nodes remained unaffected. Finally, generation of B6 $\rightarrow$ B6 chimaeras allowed both the presentation of antigen by the bone-marrow compartment during the priming phase in the draining nodes, and the presentation of antigen by islet β -cells to effector T cells that had migrated to the pancreas. After adoptive transfer of CD30-deficient cells, extensive expansion was seen only in B6 $\rightarrow$ B6 chimaeras, where presentation could take place in both the draining nodes and the pancreas (Fig. 3).

These data indicate first, that priming in the lymph nodes was necessary to allow expansion of CD30-deficient cells (B6 $\rightarrow$ B6 but

Figure 1 Expansion of CD30-deficient OT-I cells on days 4 and 5 after transfer into RIP-mOVA mice. 2×10^6 OT-I (squares) or OT-I.CD30^{-/-} (circles) cells were injected into RIP-mOVA mice (R; filled symbols) or non-transgenic littermates (LM; open symbols). At different time points, the number of OT-I cells present in the recipient mice was determined by flow cytometry after pooling their spleen and lymph-node cells and staining for expression of CD8 and the transgenic T-cell antigen-receptor chains V α 2 and V β 5 as described¹. Data from several experiments are pooled. Each symbol represents one mouse. It is usual for the recovery of cells to be significantly lower than the number adoptively transferred, as only about 10–20% of cells ‘take’ in unirradiated recipients. The decrease in the number of OT-I.CD30^{-/-} cells seen on days 6–7 in RIP-mOVA mice was associated with a general decrease in all T-cell numbers caused by the stress of acute-diabetes onset (data not shown). Of 5 RIP-mOVA mice given 2×10^6 CD30-deficient OT-I cells and followed daily for diabetes onset by testing glucosuria, all 5 became diabetic on day 5.

Figure 2 CD30 deficiency leads to extensive proliferation of OT-I cells in the islets of RIP-mOVA mice. RIP-mOVA mice were injected with **a**, 2×10^5 OT-I.CD30^{-/-} cells or **b**, 2×10^5 OT-I cells. Five days later, recipients were injected intraperitoneally with 1.5 mg BrdU in saline and then killed after a further 6 h. Frozen sections of pancreatic tissue were stained for BrdU incorporation as described²³. Recipients were not diabetic at the time of analysis.

not $\text{bm1} \rightarrow \text{B6}$ chimaeras showed expansion), but second, that, despite the proliferative response in the draining lymph nodes, there was no marked expansion of CD30-deficient cells unless they also recognized antigen on parenchymal cells ($\text{B6} \rightarrow \text{B6}$ but not $\text{B6} \rightarrow \text{bm1}$ chimaeras showed expansion). Thus, the protective (anti-proliferative) effect of CD30 exerts itself at the level of secondary encounter with antigen on parenchymal tissues. The requirement for priming in the draining nodes is most likely to be related to the fact that naïve T cells recirculate only within the secondary lymphoid tissues¹⁷ and will not migrate into the parenchymal tissues unless they are first activated by priming. The ability of CD30-deficient cells to proliferate in response to 'non-professional' APCs suggests that activated CD30-deficient cells have little requirement for co-stimulation by members of the B7 family.

We have shown that CD30 signalling can protect against autoimmunity by preventing extensive expansion of autoreactive CD8⁺ effector T cells during secondary encounter with antigen in parenchymal tissues. CD30 signalling may also alter other functions, such as cytokine production or expression of homing receptors, but such changes are not needed to explain our data because a simple increase in the frequency of precursor OT-I cells leads to diabetes. Analysis of the cytotoxic capacity of CD30-deficient cells *in vitro* revealed that they were no more cytotoxic than wild-type cells (data not shown). Similarly, the *in vitro* proliferative response to OVA-bearing cells showed no enhancement when cells were deficient in CD30 (data not shown). This latter result indicates that the specific characteristics of our *in vivo* model are essential to show the effect of CD30 deficiency.

Our results identify a new mechanism for control of autoimmunity that is distinct from the CD95-dependent deletion of autoreactive CD8⁺ T cells³. Thus, two levels exist for the control of such cells. The first level represents a mechanism of deletion that is dependent on CD95 and is akin to activation-induced cell death. This mechanism is rather slow, however, and results in the genera-

tion of effectors that, before their deletion, can be highly destructive. To limit the destructive potential of these effectors, the immune system uses a second level of control involving CD30, which prevents extensive proliferation of effector cells that encounter antigen on parenchymal tissues such as the islets. Interestingly CD30 signalling leads to degradation of TRAF2, a TNFR-associated factor¹⁸. Without TRAF2, signalling through TNFR-1 is biased towards the apoptotic pathway rather than the TRAF2-dependent NF- κ B-linked activation pathway. Perhaps, in the absence of CD30-mediated degradation of TRAF-2, TNF or other related molecules preferentially signal cell proliferation rather than apoptosis. It is unlikely that CD30 signalling acts indirectly through CD95 to prevent proliferation, as CD95-deficient OT-I cells are only marginally more diabetogenic than wild-type cells (data not shown), whereas very few CD30-deficient cells are required to cause diabetes (Table 1). Another alternative⁹ is that CD30 signalling may prevent proliferation by downregulating the expression of molecules such as CD28. However, as the stimulators of proliferation in our model (the islet cells) are non-professional APCs, which do not express the CD28-binding molecules B7.1 or B7.2, it is unlikely that regulation of CD28 expression is critical. However, CD30-mediated regulation of other molecules involved in cell-cell interactions could provide an explanation.

These results raise the question of why the immune system has evolved a powerful, CD30-dependent, mechanism to stop the proliferation of CD8⁺ T cells in parenchymal tissues. The cross-tolerance pathway is partner to a cross-priming pathway¹⁹ that is capable of inducing immunity to tumours, DNA vaccines and minor antigens²⁰. Perhaps CD30 signalling is suppressed under conditions of inflammation or infection, similar to the decoupling of the CD95 pathway from cross-priming that is seen in the presence of lipopolysaccharide²¹, so that activated CD8⁺ T cells can undergo an extra amplification step in the parenchymal tissue when they encounter infected cells. In contrast, for self-antigens, the CD30 signal would remain functional and severely limit the expansion of autoreactive T cells. It will be interesting to evaluate the potential of blocking CD30 signalling for the augmentation of cytotoxic-T-lymphocyte function during antitumour immunotherapy. □

Methods

Mice. C57B1/6, bm1 , RIP-mOVA¹⁴, CD30-deficient¹³ OT-I and OT-I²² mice were maintained at the Walter and Eliza Hall Institute of Medical Research.

Preparation of lymphocytes for adoptive transfer. OT-I and OT-I.CD30^{-/-} cells were prepared from the lymph nodes of transgenic mice as described¹⁴. Briefly, erythrocytes, B cells and CD4⁺ T cells were depleted by treatment with the heat-stable antigen-specific monoclonal antibody J11d and the CD4-specific monoclonal antibody RL172, followed by complement. Semi-purified cells were analysed by flow cytometry to determine the proportion of OT-I cells, and then injected intravenously into recipient mice.

Bone-marrow chimaeras. To generate bone-marrow chimaeras, 7–8-week-old mice were lethally irradiated (900 cGy) and then reconstituted with 5×10^6 T-cell-depleted bone-marrow cells. The next day, mice were further depleted of T cells by intraperitoneal injection of 100 μl of the Thy1-specific ascites, T24 ascites.

Flow-cytometric analysis. To determine the total number of OT-I cells in recipient mice, we pooled lymph-node (inguinal, brachial, axillary, mesenteric and superficial cervical) and spleen cells of recipient mice, depleted them of erythrocytes and stained them for CD8 and the OT-I T-cell-receptor chains $\text{V}\alpha 2$ and $\text{V}\beta 5$, using the monoclonal antibodies YTS169.4, B20.1 and MR9.4, respectively, as described¹. The number of OT-I cells was then calculated as $\text{percentage CD8}^+ \text{V}\alpha 2^+ \text{V}\beta 5^+ - 1.4\% \times \text{percentage CD8}^+ \times \text{total cell number}$, where 1.4 was the percentage of $\text{CD8}^+ \text{V}\alpha 2^+ \text{V}\beta 5^+$ T cells in a normal mouse, as described¹.

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Figure 3 The expansion of autoreactive CD8⁺ T cells requires their interaction with both bone-marrow-derived APCs and parenchymal tissue cells. 2×10^6 OT-I.CD30^{-/-} were injected into chimaeric RIP-mOVA mice (R) or non-transgenic littermate control mice (LM); these chimaeric mice could present OVA only on their professional APCs ($\text{B6} \rightarrow \text{bm1}$), only on their parenchymal tissue ($\text{bm1} \rightarrow \text{B6}$), or in both compartments ($\text{B6} \rightarrow \text{B6}$). After five days, the number of OT-I cells present in the recipient mice was determined by flow cytometry as described¹. Histological analysis of pancreas sections of these chimaeric mice stained with Gomari's aldehyde fuchsin revealed massive infiltration and nearly complete β -cell destruction in the $\text{B6} \rightarrow \text{B6}$ chimaeras, but not in the $\text{B6} \rightarrow \text{bm1}$ or $\text{bm1} \rightarrow \text{B6}$ chimaeras (data not shown). Thus, expansion of CD30-deficient cells correlates with autoimmunity. Similar experiments, performed with wild-type OT-I cells, did not show cell expansion in any of the chimaeras (data not shown), confirming that expansion was specific to CD30-deficient cells.

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Effects of altered gene order or orientation of the locus control region on human β -globin gene expression in mice

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The five human β -type-globin genes, ϵ , $G\gamma$, $A\gamma$, δ and β , are close together and are regulated by a locus control region (LCR) located at the 5' end of the locus^{1,2}. Here we investigate the functional consequences of this organization with respect to temporal regulation of the individual genes, by using recombination techniques to invert the order of either the genes or the LCR *in vivo*. Our analysis of transgenic mice bearing either normal or mutant transgenes leads to two new observations. First, the position of the

ϵ -globin gene next to the LCR is mandatory for its expression during the yolk-sac stage of erythropoiesis. Second, LCR activity is orientation dependent, and so the LCR does not act as a simple enhancer to stimulate transcription of the globin genes. Thus, in the absence of any change in transgene integration position, transgene copy number, *trans*-acting factors or other resident genetic information, simple inversion of the human genes or the LCR fundamentally alters the transcription of β -type globin genes.

The human β -globin genes are organized in a 5'- ϵ - $G\gamma$ - $A\gamma$ - δ - β -3' array and their expression is transcriptionally controlled, resulting in different haemoglobin tetramers being produced during the embryonic (ϵ -globin), fetal (γ -globin) and adult (β -globin) stages¹. High-level expression of all of the β -like genes is regulated by the LCR, a group of five DNaseI-hypersensitive sites (Fig. 1a) found far 5' of the ϵ -globin genes of humans, galagos, rabbits, goats and mice. In these animals, an embryonic gene is always located closest to the LCR, whereas adult genes are always further away². Previous studies addressing the contribution of gene order to temporal regulation in the locus obtained the following results. First, when two equivalent genes were linked to the LCR, the proximal gene was more abundantly transcribed^{3–5}. Second, when the γ - and β -globin genes were linked to the LCR, they were regulated in appropriate stage-specific manner regardless of gene order, although their relative levels were dependent on their proximity to the LCR⁴. It thus became generally accepted that *trans*-acting factors are the primary determinants of temporal specificity of β -type-globin gene expression, whereas proximity to the LCR modulates the relative expression of the genes by altering expression ratios through promoter competition^{6,7}. However, another possibility is that gene order plays no significant part in developmental activation, and that the principal activity of the LCR is to open chromatin, thereby allowing regulatory sequences throughout the locus equal access to transcription factors⁸. Analyses of deletion mutants of individual hypersensitive sites in the LCR (either transgenes or the endogenous locus) in mice have indicated that hypersensitive sites contribute synergistically to LCR activity and, therefore, that the LCR may function as a single unit containing both strong chromatin-opening and transcriptional-stimulatory activities^{9,10}. Thus we were interested to know whether the LCR exhibits directionality.

To minimize the usual complications of analysing transgenic mice (with respect to gene copy number or effects from the transgene integration site), we used Cre-mediated *loxP* recombination^{11,12} within yeast artificial chromosome (YAC) transgenes¹³ *in vivo*. A *loxP* site was introduced, by homologous recombination in yeast, in inverted orientation between the LCR and the ϵ -globin gene promoter and also 3' of the β -globin gene enhancer, in YAC A201F4.3 (these sites were 48 kilobases (kb) apart; Fig. 1a). To modify the LCR, we placed inverted *loxP* sites 5' of hypersensitive site 5 and 3' of hypersensitive site 1 (16 kb apart; Fig. 1b). YAC DNAs were purified and injected into fertilized eggs to generate transgenic mice. Following founder identification by the polymerase chain reaction (PCR), we determined the copy number and structure of the transgenes by conventional and pulsed-field Southern blots. In the end-fragment assay¹⁴, we detected single fragments when using probes for both left and right vector arms in all the transgenic lines (lines 42 and 31 for gene inversions, and 277 and 264 for LCR inversions; Fig. 1c), indicating that all of the transgenes were both intact and single copy.

To invert the genes or LCR *in vivo*, we collected fertilized eggs produced after mating of female mice to transgenic studs, and then injected these eggs with a Cre expression plasmid. The progeny were genotyped by PCR, and Cre-mediated site-directed recombination between *loxP* sites was monitored on Southern blots (see later). Those animals (Cre F₀) in which *in vivo* recombination was detected were bred again to generate F₁ mice. To confirm the structure of the

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Cytotoxicity assay^{11,23}

STARFISH inhibitor was dissolved in double-distilled, deionized water. Stock solutions of purified SLT-I and SLT-II were prepared at concentrations of 400 ng ml⁻¹ and 2 µg ml⁻¹, respectively, in unsupplemented MEM. Serial dilutions, in unsupplemented MEM, of inhibitor solution were prepared using a 96-well microtitre plate. Next, 5 µl of stock SLT-I or SLT-II solution was added to each well (to 80 µl final volume) of the appropriate rows in the dilution plate. The solution in each of the dilution plate wells was thoroughly mixed and the microtitre plate was incubated for 1 h at 37 °C, after which 20 µl from each well was transferred to the corresponding well of a 96-well microtitre plate containing confluent Vero cell monolayers and 200 µl of MEM supplemented with fetal bovine serum. The Vero cell microtitre plate was incubated for an additional 48 h in a 37 °C incubator in an atmosphere of 5% CO₂/95% air. The Vero cell monolayers were then fixed with methanol and cytotoxicity was measured as described¹¹.

Crystallography

A solution of the complex was made by adding 15 µl of a solution of STARFISH (0.35 mM in water) slowly to 15 µl of SLT-I B subunit (10 mg ml⁻¹, 0.1 M NaCl and 10 mM Tris pH 8.0) with agitation. We mixed this solution with an equal volume of reservoir solution (28% saturated ammonium sulfate, 2% 2-methyl-2,4-pentanediol, 0.1 M NaCl and 0.1 M Hepes pH 7.0) for hanging drops. Crystals grew at room temperature as clusters of needles. Diffraction data, collected on a MAR345 detector mounted on a Rigaku rotating anode generator, extended to 2.23 Å resolution (Table 1). A molecular replacement solution was obtained in AMoRe²⁴ using the B subunit of the crystal structure of the SLT-I B-subunit pentamer complexed with Gb₃ (ref. 6) as a model. The test set was selected in thin shells to limit the bias that may be introduced by the fivefold non-crystallographic symmetry (NCS) present in the asymmetric unit. Refinement was carried out in CNS²⁵ using maximum-likelihood targets²⁶, and strict NCS followed by restrained NCS. Manual intervention in between cycles of refinement using the XTALVIEW package²⁷ and averaged cross-validated SigmaA²⁸ maps allowed the building of part of the STARFISH molecule. The STARFISH molecule is fully occupied in the crystal, but, because of symmetry, parts of the linker are only half occupied in the crystallographic asymmetric unit.

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How self-tolerance and the immunosuppressive drug FK506 prevent B-cell mitogenesis

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Therapy for transplant rejection, autoimmune disease and allergy must target mature lymphocytes that have escaped censoring during their development. FK506 and cyclosporin are immunosuppressants which block three antigen-receptor signalling pathways (NFAT, NFκB and JNK), through inhibition of calcineurin¹, and inhibit mature lymphocyte proliferation to antigen^{2–4}. Neither drug induces long-lived tolerance *in vivo*, however, necessitating chronic use with adverse side effects. Physiological mechanisms of peripheral tolerance to self-antigens provide an opportunity to emulate these processes pharmacologically. Here we use gene-expression arrays to provide a molecular explanation for the loss of mitogenic response in peripheral B-cell anergy, one aspect of immunological tolerance⁵. Self-antigen induces a set of genes that includes negative regulators of signalling and transcription but not genes that promote proliferation. FK506 interferes with calcium-dependent components of the tolerance response and blocks an unexpectedly small fraction of the activation response. Many genes that were not previously connected to self-tolerance are revealed, and our findings provide a molecular fingerprint for the development of improved immunosuppressants that prevent lymphocyte activation without blocking peripheral tolerance.

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Figure 1 Tolerance response to self-antigen, $P < 0.00034$. **a**, Upregulated genes. Each line represents samples from each genotype prepared in parallel on the same day. The left end of each line represents hybridization intensity in naive cells; the right end represents tolerant cells. Five sets of data are from negatively depleted B-cell preparations; two are

from FACS-purified cells. For one experiment, two lines connect a single naive cell sample with two tolerant cell samples. Known concentrations of spiked bacterial transcripts allow an approximate calibration of 5 intensity units per copy per cell ($\pm$ twofold¹⁰) assuming 300,000 transcripts per cell. **b**, Downregulated genes, represented as in **a**.

Figure 2 Activation response to foreign antigen, $P < 0.00018$. **a**, Upregulated genes, 1 h activation, 7 experiments, each line represents 1 experiment. The left end of the line represents mock-stimulated cells; the right end represents Ig^{HEL} cells stimulated with HEL (3 experiments) or non-transgenic cells stimulated with anti- μ (4 experiments). Responses of Ig^{HEL} and non-transgenic B cells were essentially identical (see also Supplementary Information). CD72 is shown but only increased 1.5-fold. BL34 (RGS1) is

represented twice on the arrays; both sets of data are shown. **b**, Downregulated genes, 1 h activation, represented as in **a**. **c**, Upregulated genes, 1 and 6 h activation, 2 experiments, HEL stimulation of Ig^{HEL} transgenic B cells. The left end of the line represents 1 h mock stimulation, the middle of the line 1 h activation and the right end of the line 6 h activation. Genes are in order of exaggerated, sustained and transient increases. **d**, Downregulated genes, 1 and 6 h activation, represented as in **c**.

Homogeneous populations of naive B lymphocytes specific for a well-defined antigen, hen egg lysozyme (HEL), were obtained from immunoglobulin transgenic mice (Ig^{HEL} mice). Tolerant B cells carrying the same Ig^{HEL} receptor and matched for developmental stage were obtained from double transgenic mice that also express soluble HEL as a neo-self-antigen (sHEL:Ig^{HEL} mice). Unlike naive cells, tolerant cells fail to proliferate or make antibody to HEL *in vitro* or *in vivo*^{5,6}. Instead, repeated stimulation by self-HEL causes them to make responses that actively reinforce tolerance, such as altered migration, and Fas-dependent and Fas-independent apoptosis⁵. These responses collectively describe a state of anergy and are maintained by partial signalling downstream of the antigen

receptor^{5,7,8}. Tolerant B cells in the spleen of sHEL:Ig^{HEL} mice have been exposed to self-antigen from their development in the bone marrow throughout their $\sim$ 1-week life span⁵. They appear homogeneous with respect to the aspects of anergy described above, and as measured in single cells by continuous calcium oscillations⁷ and uniformly low expression of the activation markers B7-2 (ref. 9) and CD69.

We first screened for gene-expression changes in tolerant B cells using Affymetrix oligonucleotide expression arrays to monitor 6,500 genes and expressed sequence tags (ESTs) (reviewed in ref. 10). For exemplar accession numbers, raw data and statistical methods, see Supplementary Information. We compared expression

between five tolerant B-cell preparations and four naive B-cell preparations; paired samples were purified by negative selection with magnetic beads. A further two preparations of each were purified by positive selection on a cell sorter. Using an algorithm that requires consistency between both purification methods, expression of only 20 genes was significantly increased and that of 8 genes was significantly decreased in tolerant cells (Fig. 1, $P < 0.00034$). Increased messenger RNA for the light chain V κ 5 segment is consistent with the increased frequency of variant B cells that have rearranged endogenous immunoglobulin genes in sHE-L:lg^{HEL} mice. Increased expression of IgD and Egr-1 in tolerant B cells was confirmed by western blotting, and increased expression of CD72 has been confirmed by flow cytometry (J. Parnes, personal

communication). Aside from Egr-1, none of these genes have previously been connected to self-tolerance.

In parallel, we analysed the same set of 6,500 genes for expression changes in naive Ig^{HEL} B cells during a well-defined system of synchronous activation by HEL *in vitro* (Fig. 2). In 7 independent replicate experiments, mRNAs for only 37 genes were significantly increased and 22 were decreased by 1-hour activation, when compared with control cells cultured in parallel without antigenic stimulus ($P < 0.00018$). Many of these 59 transcripts encode transcriptional regulators. Although a small number have been previously identified as early response genes in B cells^{11–17}, which validates our data, most were not previously known to participate in B-cell activation responses.

Figure 3 Effect of FK506, EGTA and PD98059 on activation response. **a**, FK506 sensitivity, upregulated genes; 5 experiments and genes are shown in increasing order of median FK506 sensitivity. The left end of the line represents mock stimulation, the middle of the line represents 1 h activation, and the right end of the line represents 1 h activation in the presence of FK506. Three experiments are Ig^{HEL} cells stimulated with HEL with or without FK506; two experiments are non-transgenic cells stimulated with anti-mu with or without FK506. **b**, FK506 sensitivity of downregulated genes, represented as in **a**. **c**, EGTA compared with FK506. For 1 h upregulated genes (Fig. 2a), relative induction in the presence of EGTA was calculated as average (antigen/EGTA – mock)/(antigen – mock)

of 2 experiments of Ig^{HEL} cells stimulated with HEL with or without EGTA. Relative induction in the presence of FK506 was calculated as median of (antigen/FK506 – mock)/(antigen – mock) over five experiments. An outlier, MyD116, is labelled. **d**, Effect of PD98059. Upper two panels, regulation of 17 1-hour response genes in the presence of various concentrations of PD98059 (PD). Lower panel, three transcripts upregulated by both foreign and self-antigen are sensitive to PD98059. First four columns, non-transgenic B cells stimulated with anti-mu for each gene; last three columns, Ig^{HEL} transgenic B cells stimulated with HEL.

Gene expression was much more extensively altered after 6 hours of activation. Although mRNAs for many of the 1-hour-induced genes had decreased by this time (for example, *Egr-1*, *Pac-1*, *c-fos*, *Fos B*; Fig. 2e), others in the early response set showed sustained or exaggerated responses at 6 hours (for example, *A1* and *MIP-1a/b*; Fig. 2c; *LKLF* and *GILZ*, Fig. 2d). About 300 additional genes had increased mRNA and 200 had decreased mRNA at this time, consistent with movement to G1 phase of cell cycle, including upregulation of *CDK4* and *cyclin D2* (ref. 18).

Comparison of the activation response with tolerance showed that most of the transcripts regulated by self-antigen are also regulated after 1 or 6 hours of activation. For a detailed comparison, including analysis of the effects of tissue culture, see Supplementary Information. Shared transcript changes include *NAB2* and *neurogranin*, which were comparably upregulated in both types of response, and *Egr-1*, *Egr-2*, *Gfi-1*, *cyclin D2* and *Cctq*, which were more highly induced during activation. A subset of the tolerance response genes was not regulated after activation, including *Aeg-2*/*Crisp-3*, a secreted molecule encoded in the MHC and regulated by *Oct-2* (ref. 19). Conversely, the activation response was almost completely blocked in tolerance: only 16 out of more than 500 transcript changes caused by antigen in 6 hours of activation were also regulated by self-antigen in tolerant cells ($P < 0.05$, fold change

>1.8 , at least 1 out of 2 experiments with sorted cells).

In the B-cell activation cultures, we stimulated a third group of cells in the presence of a clinically relevant concentration of FK506 to determine how well immunosuppression mirrors the molecular effects of B-cell anergy. Out of the 59 transcript changes detected 1 hour after B-lymphocyte activation, only one-third were efficiently suppressed by this dose of FK506 (Fig. 3). Some early response genes (for example, *gadd153*) were superinduced. As FK506 affects transcript profiles in yeast independently of calcineurin²⁰, we tested whether chelation of extracellular calcium had a similar effect on suppression of B-cell activation. Activation of naive B cells in the presence of EGTA reproduced the effects of FK506 (Fig. 3c), confirming that FK506 affected transcript levels through a calcium/calcineurin-dependent pathway. We did not note any significant FK506-induced changes in transcripts other than those altered in the antigen activation response.

Transcript changes in B-cell activation that are suppressed by FK506 and EGTA can be assigned downstream of calcium/calcineurin, and further subdivided on the basis of their expression in tolerant cells (Fig. 4a). The low calcium oscillations in tolerant cells are sufficient to induce nuclear translocation of NFAT but not to activate NFkB nor JNK^{7,21}, although all three pathways are dependent on calcium/calcineurin. Thus, FK506-sensitive upregulation of genes not altered in tolerant cells implies signalling through NFkB or JNK (for example, *A1*, *MIP1*, *LSIRF*, *c-myc*), whereas FK506-sensitive genes that are also upregulated in tolerance would be expected to be downstream of NFAT (for example, *Egr-2* and *CD72*). Upregulation of *A1* has recently been described²² as downstream of *c-rel*, which is consistent with our data.

The ERK pathway is also activated in B-cell anergy and activation. To determine downstream transcriptional effects of ERK, we titrated the MEK inhibitor PD98059 (ref. 23) in a B-cell activation experiment. Upregulation of *Egr-1* was totally inhibited by 20 μ M PD98059 and was 50% inhibited by 5–10 μ M, consistent with the potency of PD98059 against recombinant MEK²³ (Fig. 3d). Regulation of other early response genes was less sensitive than *Egr-1*. Induction of three transcripts (*Egr-1*, *NAB2* and *Gfi-1*) that are upregulated by both self-antigen and foreign antigen was sensitive to PD98059 (Fig. 3d) but insensitive to FK506 (Fig. 3a). Continuous activation of the ERK pathway by self-antigen thus appears to have distinct transcriptional consequences (Fig. 4a).

Our findings provide the first molecular explanation for how self-tolerance prevents lymphocyte mitogenesis (Fig. 4b). Given the continuous signalling through the ERK and NFAT pathways in response to self-antigen⁷, it is remarkable how few mitogenic response genes are triggered in anergic B cells. The loss of mitotic response to antigen is explained by failure to upregulate *LSIRF*, a B-cell myeloma protooncogene²⁴ that is an essential transcription factor for B-cell mitogenic responses²⁵, and failure to upregulate *A1*, an anti-apoptotic protein that is sufficient and apparently necessary to block apoptotic responses to antigen in B cells²². The block in induction of the B-cell lymphoma protooncogene, *c-myc*, is also likely to contribute because increased expression of *c-myc* is sufficient to promote B-cell blastogenesis in transgenic mice²⁶. Likewise, tolerant B cells lacked any downregulation of the inhibitory transcription factors *LKLF* and *BKLF*. A decrease in levels of *LKLF* may be an obligatory, although apparently not a sufficient, step in B-cell activation as T cells that are deficient in *LKLF* have a spontaneously activated cell-surface phenotype²⁷. By comparison, it is surprising how little of the early mitogenic response is suppressed by FK506 given its ability to block foreign-antigen-stimulated NFAT, NFkB and JNK⁷. Inhibition of *A1* induction by FK506 may alone be sufficient to explain the anti-mitogenic effects of FK506, as activation in the presence of FK506 is associated with increased B-cell death⁴. It will be interesting in future studies to examine how the activation gene pattern shifts to the tolerance pattern when naive B cells are induced to become anergic over several days of continuous

Figure 4 Expression profiles connect genes to pathways and immune responses. **a**, Summary table of biochemical pathways in tolerant and naive cells exposed to foreign antigen in the presence or absence of FK506 and PD98059. **b**, Summary of key differences in response to antigen during tolerance, activation and immunosuppression. Font size reflects mRNA or protein expression level relative to mock-stimulated cells (immunosuppression and activation panels) or naive cells (tolerance panel). N' granin, neurogranin; YPⁿ, tyrosine phosphorylation.

exposure to antigen^{5,8}.

Many of the genes associated with the tolerance response might be predicted to have negative regulatory functions for maintaining the tolerant state (Fig. 4b). Egr-1 and Egr-2 are transcription factors implicated in B-cell mitogenesis^{11,12}, but their mitogenic activity is likely to be repressed in tolerant cells by relatively high expression of NAB2, an Egr family inhibitor²⁸. Neurogranin and pcp-4 inhibit calmodulin²⁹ and may regulate the downstream effects of calcium oscillations in tolerant cells^{7,21}. IgD represents the primary receptor isotype on tolerant B cells, through which repeated binding of self-antigen triggers low calcium oscillations. IgD signalling may be decreased relative to naive cells⁶ by increased levels of CD72, which recruits the inhibitory tyrosine phosphatase SHP-1 (ref. 30) (Fig. 4b).

Inhibition of Egr-2 and CD72 by FK506 shows that this drug interferes with components of the active self-tolerance response, which may limit its efficacy in establishing or restoring tolerance in autoimmunity and transplantation. To develop more effective drugs, the unique transcript signature in anergic cells could be used in high-throughput assays as a surrogate marker for tolerance. A desired small molecule would suppress members of the subset of activation-only early response genes defined here, while leaving unaffected the subset of early response genes that also participate in tolerance, so that tolerance would be actively (re-)established. □

Methods

B-cell purification and stimulation

For each cell preparation, splenic B cells were purified and pooled from several mice at room temperature in 1% bovine calf serum in RPMI. The spleen cells were stained with CD4, CD8 and Mac-1 FITC-conjugated antibodies (Caltag) and depleted of T cells and macrophages with sheep anti-FITC magnetic beads (Perseptive Biosystems). The remaining cells were 85–95% B220 positive and were either lysed immediately (naive and tolerant cell preps) or stimulated in RPMI with 1% serum at 37 °C at $(2-3) \times 10^6$ cells ml⁻¹. For stimulation experiments, HEL (Sigma) was used at 500 ng ml⁻¹, PD98059 (NEB) at 20 μM unless stated otherwise, ionomycin (a gift from G. Crabtree) at 1 μM and EGTA at 3 mM. The concentration of FK506 was chosen to be within the range maintained in the blood of kidney and liver transplant patients receiving FK506 (also called Tacrolimus and Prograf; information on dosing from <http://www.fujisawa.com/info/medinfo/mnpginst.htm>). Cells were pre-incubated for 45 min with PD98059, 15 min with FK506 and 2 min with EGTA before addition of HEL or anti-μ. Mock stimulations were carried out by addition of carrier alone for stimuli or inhibitors. At the end of the incubation, the cells were pelleted by centrifugation, resuspended in a minimal volume of medium (~50 μl) by pipetting and lysed in 0.5–1 ml Trizol (Gibco BRL). Naive and tolerant B cells were also purified by cell sorting for B220 positive, CD21^{medium} cells. Marginal zone cells (CD21 high) were excluded from the gate. Between 10^7 and 5×10^7 magnetic-bead-selected B cells, or $(1.5-3) \times 10^6$ cell-sorter-purified B cells were used for RNA preparation.

RNA preparation, array hybridization and data analysis

RNA labelling and array hybridization were done as described¹⁰. For detailed methods and statistical analysis, see Supplementary Information.

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Localizative of apical epithelial determinants by the basolateral PDZ protein Scribble

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The generation of membrane domains with distinct protein constituents is a hallmark of cell polarization. In epithelia, segregation of membrane proteins into apical and basolateral compartments is critical for cell morphology, tissue physiology and cell signalling. *Drosophila* proteins that confer apical membrane identity have been found^{1,2}, but the mechanisms that restrict these determinants to the apical cell surface are unknown. Here we show that a laterally localized protein is required for the