INFLAMMATORY BOWEL DISEASE

CD4+ T cell mediated intestinal immunity: chronic inflammation versus immune regulation

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Background: Several studies have suggested that chronic inflammatory bowel disease may be a consequence of antigen specific recognition by appropriate T cells which expand and induce immunopathology.
Aims: We wished to investigate whether autoreactive CD4+ T cells can initiate the disease on recognition of enterocyte specific antigens directly and if induction of mucosal tolerance occurs.
Methods: Transgenic mice (VILLIN-HA) were generated that showed specific expression of haemagglutinin from influenza virus A exclusively in enterocytes of the intestinal epithelium. To investigate the impact of enterocyte specific haemagglutinin expression in an autoimmune environment, we mated VILLIN-HA mice with T cell receptor (TCR)-HA mice expressing an α/β-TCR, which recognises an MHC class II restricted epitope of haemagglutinin, and analysed the HA specific T cells for induction of autoimmunity or tolerance.
Results: In VILLIN-HA×TCR-HA mice, incomplete central deletion of HA specific lymphocytes occurred. Peripheral HA specific lymphocytes showed an activated phenotype and increased infiltration into the intestinal mucosa, but not into other organs of double transgenic mice. Enterocyte specific lamina propria lymphocytes showed a dose dependent proliferative response on antigen stimulation whereas the proliferative capacity of intraepithelial lymphocytes was reduced. Mucosal lymphocytes from VILLIN-HA×TCR-HA mice secreted lower amounts of interferon γ and interleukin (IL)-2 but higher levels of tumour necrosis factor α, monocyte chemoattractant protein 1, and IL-6. Mucosal immune reactions were accompanied by broad changes in the gene expression profile with expression of proinflammatory genes, but strikingly also a remarkable set of genes discussed in the context of peripheral induction of regulatory T cells, including IL-10, Nrp-1, and Foxp3.
Conclusions: Enterocyte specific antigen expression is sufficient to trigger a specific CD4+ T cell response leading to mucosal infiltration. In our model, progression to overt clinical disease was counteracted most likely by induction of regulatory T cells.

The intestinal immune system has to discriminate between harmless antigens derived from nutrients or bacterial flora on the one hand and harmful antigens derived from pathogens on the other hand. Therefore, induction and maintenance of mucosal tolerance is of indispensable importance to avoid inappropriate immune responses in the gut.[1] Central tolerance induction takes place in the thymus where clonal deletion of potentially autoreactive T cells occurs.[2 3] However, in a few cases, autoreactive T cells escape thymic deletion but these T cells are usually rendered anergic due to the absence of costimulatory signals on their target tissue in the periphery.[4] However, because of the huge variety of antigens and the large number of lymphoid cells in the intestine, minor dysfunctions of mucosal immune homeostasis may induce an intestinal immune response resulting in inflammation and chronic disease.[5] Additional tolerance mechanisms must exist to tightly control these inappropriate immune responses. It has been shown that maintenance of tolerance in the gut can be mediated by naturally occurring $CD4^+CD25^+$ or $CD4^+CD45RB^{low}$ regulatory T cells which suppress uncontrolled immune responses, most likely towards luminal antigens.[6] This is achieved by secretion of regulatory cytokines such as interleukin (IL)-10 and transforming growth factor (TGF)-β.[7] Regulatory T cells suppress intestinal pathology mediated by T cells but until now it remains unclear how they elicit their effector function in vivo.[8]

Both loss of integrity in the epithelial barrier or breakdown of peripheral tolerance may initiate inflammatory processes in the intestinal mucosa.[7 9] Furthermore, transfer of $CD4^+CD45RB^{high}$ T cells into T cell receptor (TCR)-β and δ chain defective mice that are T cell deficient leads to atrophic changes and goblet cell transformation in the small intestinal epithelium.[10] Additional efforts to examine the tight balance between tolerance and inflammation in the intestine depend on the availability of better defined mouse models as complex interactions between gut associated lymphoid tissue (GALT), endogenous microbial flora, and potential pathogens on the one hand, and undefined specificities and low frequencies of endogenous T cells on the other hand hamper further progress.

In this study, our aim was to test the hypothesis that antigen specific $CD4^+$ T cell recognition of a single epithelial self antigen is sufficient to trigger an inflammatory cascade, resulting in histological manifestation in the intestine, and investigate whether regulatory mechanisms may suppress

Abbreviations: GALT, gut associated lymphoid tissue; HA, haemagglutinin; IBD, inflammatory bowel disease; IEC, intestinal epithelial cells; IEL, intraepithelial lymphocytes; IFN-γ, interferon γ; IL, interleukin; LPL, lamina propria lymphocytes; LT, lymphotoxin; MCP-1, monocyte chemoattractant protein 1; MLN, mesenteric lymph node; OVA, ovalbumin; PBS, phosphate buffered saline; PCR, polymerase chain reaction; RT, reverse transcription; TCR, T cell receptor; TGF, transforming growth factor; TNF, tumour necrosis factor

inflammation and maintain homeostasis. Therefore, we generated VILLIN-HA transgenic mice expressing the A/PR8/34 haemagglutinin (HA) from influenza A under the control of the enterocyte specific VILLIN promoter in the intestine.[11–13] To establish an autoimmune environment, these mice were crossed with mice expressing a transgenic TCR specific for the MHC class II restricted peptide HA110-120. Moreover, immunological and molecular characterisation of autoreactive $CD4^+$ T cells isolated from the periphery, lamina propria, and intestinal epithelium of double transgenic mice was performed by cellular assays and microarray analysis.

MATERIALS AND METHODS

Mice

TCR-HA transgenic (6.5) mice expressing an α/β-TCR recognising the MHC class II ($H\text{-}2E^d$:$HA_{110–120}$) restricted epitope of the HA protein have been described previously.[14] VILLIN-HA transgenic animals were generated using a construct containing the VILLIN promoter to direct expression of the influenza virus A/PR8/34 haemagglutinin to epithelial cells along the entire crypt-villus axis,[12] a 9 kb regulatory domain (construct kindly provided by Sylvie Robine, Institut Curie, Paris, France), and the complete HA sequence. VILLIN-HA transgenic mice were crossed under specific pathogen free conditions into the BALB/c background for at least six generations. Integration of the transgene was detected by polymerase chain reaction (PCR) analysis of genomic DNA using a villin promoter specific 5′ primer and a HA specific 3′ primer. Mice aged 8–24 weeks were used for histological analysis and 12–16 week old mice were used for cellular and molecular characterisation of 6.5^+CD4^+ T cells.

Antibodies and flow cytometry

The monoclonal antibody 6.5 (α-TCR-HA) was purified from hybridoma supernatant. All other antibodies are from BD Bioscience (San Jose, California, USA). Two and three colour flow cytometry was performed on a FACSCalibur and analysed by CellQuestPro software (BD Bioscience). For gene expression profiling, 6.5^+CD4^+ T cells were sorted with the MoFlow cells sorter (Cytomation, Fort Collins, Colorado, USA).

Expression analysis by RT-PCR

To analyse HA mRNA expression in different tissues of VILLIN-HA transgenic mice, total RNA was isolated using the TriFast FL reagent (PeqLab Biotechnology, Erlangen, Germany). Alternatively, RNA was isolated with the RNeasy Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. cDNA was synthesised using oligo-dT primers and MLV reverse transcription (RT) polymerase (Invitrogen, Karlsruhe, Germany). PCR was performed using HA internal 5′ and 3′primers.

Western blot analysis

For studying HA protein expression in intestinal epithelial cells, whole cell lysates were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis, followed by blotting on a nylon membrane and incubation with the HA specific antibody CMI1.2. A lysate of HA producing recombinant *Escherichia coli* was included as an internal control.

Histology

Organ sections were stained with haematoxylin and eosin (4 µm sections). Immunohistochemistry for T lymphocytes was performed by α-CD3 antibody clone CD3-12 (Serotec Ltd, Kidlington, UK) and the avidin-biotin complex method with diaminobenzidine as chromogen. Immunohistochemistry sections were counterstained with haematoxylin.

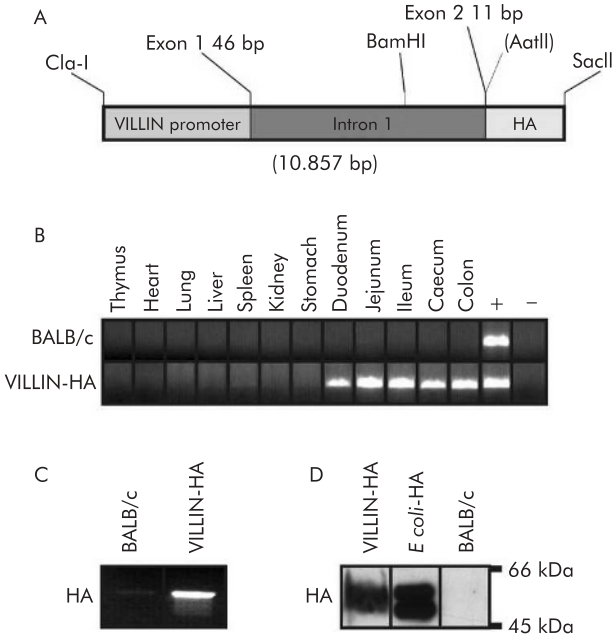

Figure 1 Intestine specific haemagglutinin (HA) expression in VILLIN-HA transgenic mice. (A) Targeting construct. The 10.857bp Cla-I /SacII fragment comprising the VILLIN promoter followed by the HA protein (from influenza virus A/PR/8/34) was used for generation of (C57BL/6JxDBA/2) F1-transgenic mice. Mice were crossed on a BALB/c background. (B) HA mRNA expression analysis by reverse transcription-polymerase chain reaction in different organs of transgenic and control mice. The upper panel shows HA mRNA expression in BALB/c mice (negative control) and the lower panel in VILLIN-HA transgenic mice. DNA templates from transgenic and non-transgenic animals were used as positive and negative controls. (C) Semiquantitative HA mRNA expression analysis of intestinal epithelial cells from VILLIN-HA transgenic and BALB/c mice. (D) Western blot analysis of HA expression in the gut. As a positive control a lysate of HA producing recombinant *Escherichia coli* was used.

Preparation of lymphocyte populations

Intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) were isolated as described previously.[15] For isolation of LPL, the small intestine was cut into small pieces followed by sequential stirring in medium to remove mucus and the epithelial layer. LPL were released by digestion at 37℃ with collagenase. Lymphocytes were collected by density centrifugation. For isolation of IEL, the gut was opened longitudinally and the mucosa was scraped off and then dissociated by stirring in medium and dithiothreitol (1 mM) at 37℃. After centrifugation, the pellet was vortexed for three minutes in HANKS containing 10% fetal calf serum. The cell suspension was rapidly passed through a buffered glass wool column. Eluted cells were collected by centrifugation.

Isolation of intestinal epithelial cells (IEC)

IEC were isolated as described previously.[16] Briefly, the small intestine was isolated, rinsed with phosphate buffered saline (PBS) and opened longitudinally. Mucus was removed by treatment with 1 mM dithiothreitol for 15 minutes. After washing with PBS, the mucosa was placed in calcium and magnesium free Hanks' balanced salt solution containing 1.5 mM EDTA and tumbled for 10 minutes at 37℃. The supernatant was collected, the remaining mucosa was vortexed in PBS, and this supernatant was also collected and pooled cells were washed with PBS.

Proliferation assay

For antigenic stimulation of 6.5^+CD4^+ T cells, 5×10^5 cells from spleen and the mesenteric lymph node (MLN) were

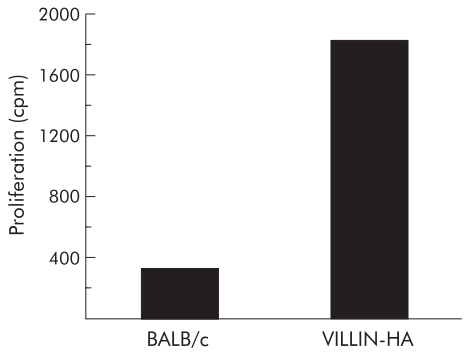

Figure 2 Antigen presenting capacity of intestinal epithelial cells from VILLIN-HA transgenic mice. Intestinal epithelial cells were isolated from VILLIN-HA transgenic and BALB/c mice and incubated for at least 72 hours with haemagglutinin (HA) specific CD4+ T cells. Proliferation was measured by ^{3}[H] thymidine incorporation.

cultured in the presence or absence of 10μg/ml HA peptide 110–120.[17] ^{3}[H] thymidine incorporation over the last 15 hours of a 48 hour culture was measured by scintillation counting. In case intestinal lymphocytes were used as responders, 10^5 LPL or IEL were cultured with different amounts of the HA peptide and 5×10^5 feeder cells. After 48 hours, proliferation of the cells was estimated by culturing the cells in the presence of 1 μCi per well ^{3}[H] thymidine for an additional 16 hours.

For IEC stimulation experiments, 2×10^5 IEC from VILLIN-HA and BALB/c mice were cultured with 4×10^4 CD4+ T cells enriched from TCR-HA splenocytes and cultured for 72 hours. Proliferation was measured by ^{3}[H] thymidine incorporation for at least 16 hours.

Cytometric bead array

Quantification of cytokines in culture supernatants was performed using the cytometric bead array kit (BD Bioscience). Data acquisition was performed by flow cytometry using a FACSCalibur. Acquired data were analysed using BD Bioscience Cytometric Bead Array software.

DNA microarrays and real time RT-PCR

Total RNA from sorted 6.5+CD4+ T cells was isolated using the RNAeasy kit (Qiagen). Quality and integrity of total RNA isolated from 10^5 sorted T cells was assessed by running all samples on an Agilent Technologies 2100 Bioanalyser (Agilent Technologies, Waldbronn, Germany). Expression analysis was performed according to the Affymetrix small sample target protocol. Real time RT PCR for expression of IL-10, Nrp-1, and FoxP3 was performed as described previously.[18]

RESULTS

Generation of VILLIN-HA transgenic mice

In order to investigate the consequences of intestine specific antigen expression on the outcome of mucosal homeostasis, we generated transgenic mice expressing the HA from influenza virus A as model antigen under control of the enterocyte specific VILLIN promoter (fig 1A).[11 12] This promoter directs protein expression to undifferentiated and mature epithelial cells in the small intestine and colon.[12] VILLIN-HA mice were analysed for HA expression in different organs by RT-PCR. HA mRNA was detectable throughout the whole gut but not in other organs (fig 1B). To specify HA expression, intestinal epithelial cells of VILLIN-HA mice and BALB/c control mice were isolated and analysed for HA mRNA. We found a high level of HA mRNA expression in intestinal epithelial cells from VILLIN-HA transgenic mice compared with BALB/c controls (fig 1C). Finally, HA protein expression in the intestinal epithelium was confirmed by western blot analysis (fig 1D).

Stimulatory capacity of intestinal epithelial cells from VILLIN-HA mice

To clarify if intestinal HA expression is efficient to elicit a HA specific T cell response, intestinal epithelial cells of VILLIN-HA and BALB/c control mice were incubated with 6.5+CD4+ T cells in vitro for at least 72 hours and proliferation was measured by ^{3}[H] thymidine incorporation. We clearly demonstrated that intestinal epithelial cells of VILLIN-HA mice but not of BALB/c induced a proliferative response of TCR-HA transgenic T cells (fig 2).

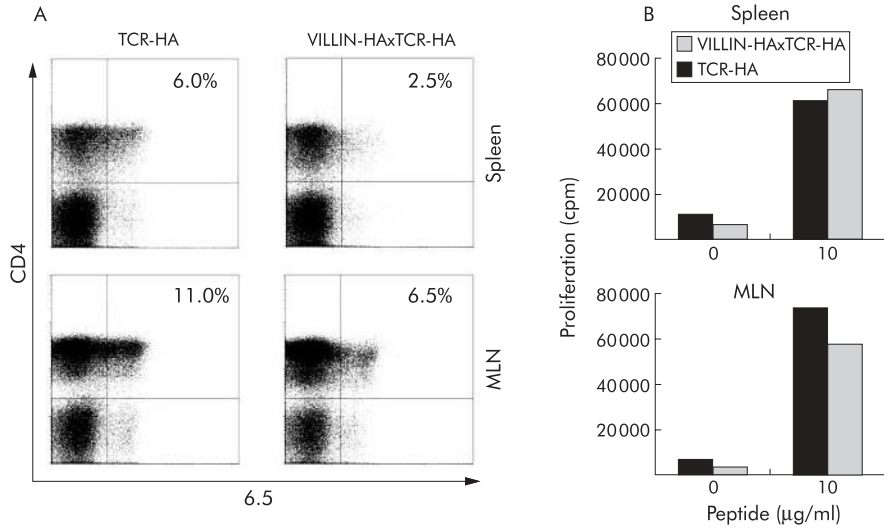

Figure 3 Haemagglutinin (HA) specific CD4+ T cells in the periphery of VILLIN-HA×T cell receptor (TCR)-HA mice and their proliferative capacity. (A) VILLIN-HA×TCR-HA and TCR-HA control mice were sacrificed, and spleen and mesenteric lymph node (MLN) cells were isolated and stained for 6.5 and CD4 expression to measure the percentage of transgenic T cells in the different compartments. (B) Proliferative capacity of HA specific CD4+ T cells from VILLIN-HA×TCR-HA mice in spleen and MLN. Splenic and lymph node cells from VILLIN-HA×TCR-HA and TCR-HA control mice were isolated and identical numbers of antigen specific 6.5+CD4+ T cells from the spleen and MLN were used for in vitro proliferation assays in the presence or absence of 10 μg/ml of HA peptide. Proliferation was measured by ^{3}[H] thymidine incorporation.

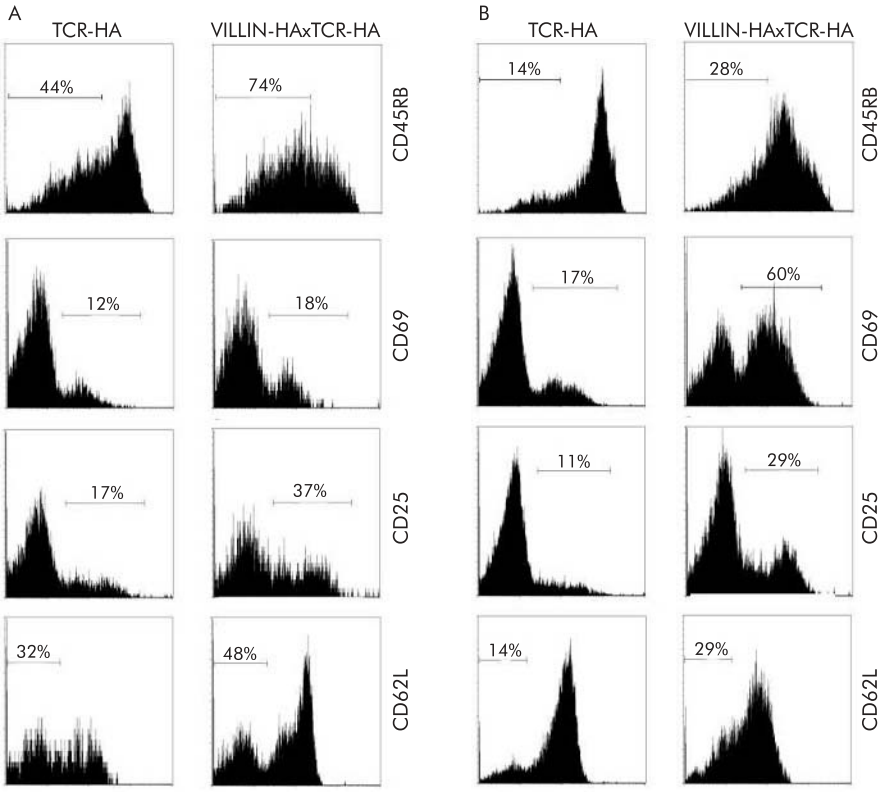

Figure 4 Activation pattern of 6.5$^+$ CD4$^+$ T cells from double transgenic VILLIN-HA×T cell receptor (TCR)-HA mice compared with TCR-HA mice. 6.5$^+$CD4$^+$ T cells were isolated from the spleen and mesenteric lymph node (MLN) of VILLIN-HA×TCR-HA and TCR-HA mice, respectively. Lymphocytes were stained for 6.5 and CD4 expression on splenocytes (A) and MLN (B) as well as for CD25, CD45RB, CD62L, and CD69 antibodies. Cells were gated for 6.5 and CD4 expression and analysed regarding expression of the different activation markers by FACS.

Enterocyte specific CD4$^+$ T cells from VILLIN-HA×TCR-HA mice mature in the thymus and have an activated phenotype

The prerequisite for the development of autoimmunity is inefficient thymic deletion of autoagressive T cells. Therefore, the key question to answer was whether 6.5$^+$CD4$^+$ T cells mature in the thymus of VILLIN-HA×TCR-HA double transgenic mice and can be found in peripheral lymphoid organs. Thus T cells from spleen and MLN of VILLIN-HA×TCR-HA and TCR-HA control mice were analysed for expression of the transgenic TCR (fig 3A). Indeed, incomplete clonal deletion of HA specific CD4$^+$ T cells results in maturation of potentially autoreactive T cells in the periphery of VILLIN-HA×TCR-HA mice. Analysis of peripheral 6.5$^+$CD4$^+$ T cells from VILLIN-HA×TCR-HA mice for expression of activation and memory markers revealed an activated phenotype of these cells (fig 4).

We next addressed whether mature 6.5$^+$CD4$^+$ T cells in the periphery are functional with respect to their proliferative capacity on antigen encounter. This is of particular interest as we have previously shown that expression of HA under control of the Ig-κ promoter by haematopoietic cells resulting in constant antigen expression both in thymus and in the periphery leads to tolerance rather than inflammation.[19]

To this end, T cells from the spleen and MLN of VILLIN-HA×TCR-HA and TCR-HA control mice were stimulated in vitro with the specific HA peptide. FACS analysis and normalisation of cell numbers ensured that the same percentage of 6.5$^+$CD4$^+$ T cells from double and single transgenic mice were used for the experiments. No differences in their capacity to proliferate on stimulation with their cognate peptide could be observed between T cells isolated from VILLIN-HA×TCR-HA and TCR-HA mice (fig 3B).

Histology of the small intestine

Morphological evaluation of intestinal tissue sections from VILLIN-HA×TCR-HA mice aged 8–24 weeks revealed increased numbers of IEL and LPL in the intestine (fig 5A). Comparing the absolute numbers of LPL and IEL in the ileum of VILLIN-HA×TCR-HA and TCR-HA mice revealed increased infiltration of lymphocytes into the lamina propria of double transgenic mice (threefold increase) whereas the IEL compartment showed only a slight infiltration of lymphocytes in VILLIN-HA×TCR-HA mice (fig 5B). However, tissue damage to the epithelial cell layer was not observed after BrdU staining (fig 6), suggesting a mild form of mucosal inflammation or induction of T cell tolerance in the periphery.

Functional characterisation of intestinal autoreactive CD4$^+$ T cells

To evaluate the responsiveness of 6.5$^+$CD4$^+$ T cells isolated from the small intestine of double transgenic mice to antigenic stimulation, IEL and LPL from VILLIN-HA×TCR-HA as well as TCR-HA mice were stimulated in vitro with HA peptide. Proliferation was measured by ^{3}[H] thymidine incorporation and culture supernatants were analysed for several cytokines by cytometric bead array. Whereas IEL and LPL from TCR-HA mice as well as LPL from VILLIN-HA×TCR-HA transgenic mice proliferated in a dose dependent manner, the proliferative capacity was drastically reduced and even abrogated in IEL from VILLIN-HA×TCR-HA mice (fig 7). Compared with control mice, antigen stimulated 6.5$^+$CD4$^+$ LPL from diseased mice secreted significantly lower amounts of IFN-γ and IL-2 on in vitro stimulation (fig 8A), both of which are cytokines normally involved in induction of gut inflammation.[20] Additionally, IEL from VILLIN-HA×TCR-HA mice secreted lower levels of IFN-γ compared with control mice. In contrast, in LPL and IEL from double transgenic mice, basal level secretion of TNF-α, MCP-1, and IL-6, which are also discussed as important mediators in the context of inflammatory bowel disease (IBD), was considerably increased.

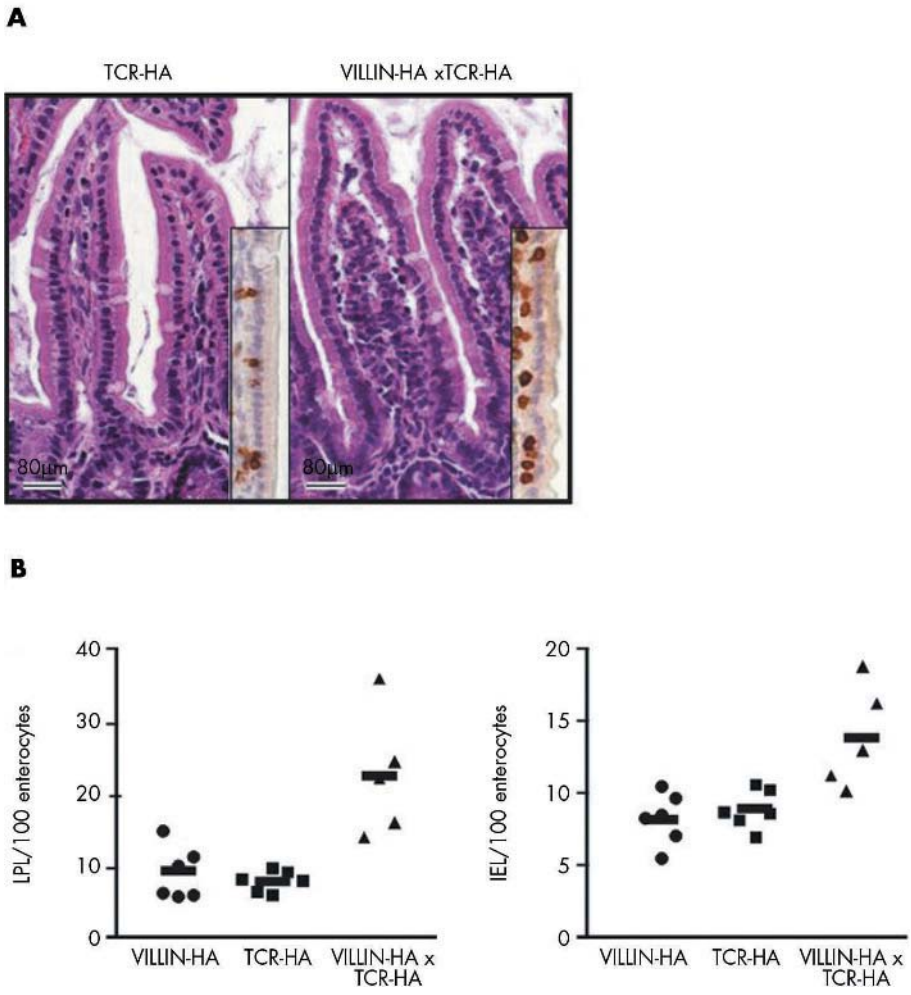

Figure 5 VILLIN-HA×T cell receptor (TCR)-HA double transgenic mice were characterised by infiltration of lymphocytes into the lamina propria and intestinal epithelium. (A) Intestinal villi were distended by increased numbers of lymphocytes (left panel) compared with intestinal villi of TCR-HA transgenic mice (right panel). Similarly, the number of intraepithelial lymphocytes (IEL) was increased (insets). Insets show α-CD3 immunohistochemistry on paraffin embedded tissues. The avidin-biotin complex method with diamino-benzidine was used as substrate (brown colour) with haematoxylin counterstain (blue nuclei). Ileum: haematoxylin and eosin (H&E) stain; scale bar 80µm. (B) Increased number of lamina propria lymphocytes (LPL) and IEL. Lymphocytes were counted in H&E stained sections of 12–16 week old mice and are reported per 100 enterocytes. Individual animals are indicated for double transgenic VILLIN-HA×TCR-HA mice, TCR-HA mice, and for VILLIN-HA control mice.

Global gene expression profiling

As enterocyte specific antigen expression obviously has a strong impact on the function of autoreactive $CD4^+$ T cells, we extensively characterised these T cells by global gene expression profiling. 6.5^+CD4^+ T cells from the epithelium and lamina propria of the small intestine of four individual VILLIN-HA×TCR-HA and four TCR-HA mice were isolated by FACS sorting and RNA was subjected to differential gene expression analysis using Affymetrix MG-U74Av2 oligonucleotide arrays. The advantage of this technology is that every gene analysed is represented by 16 independent probe pairs which together establish the basis for statistical evaluations of the respective signals. Therefore, only those genes that are reproducibly regulated are included in the analysis. Based on this approach, we obtained a comprehensive overview of the functional gene classes involved in maintenance of mucosal immune homeostasis, including surface antigens, regulators of transcription and translation, secreted or signalling molecules, and genes involved in cell cycle, apoptosis, and survival. Selected genes are highlighted and summarised in table 1 and fig 9.

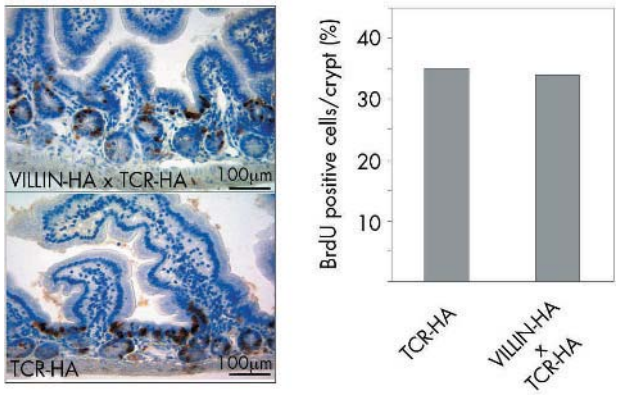

Figure 6 No tissue damage occurred in the intestinal epithelial layer. T cell receptor (TCR)-HA and VILLIN-HA×TCR-HA mice were injected with 1 mg of BrdU via the intraperitonal route. After 24 hours the small intestine was harvested and processed for paraffin sections. Immunohistochemical staining of BrdU was performed using the BrdU In-situ Detection Kit (BD Bioscience). Proliferating cells in crypts that incorporated BrdU can be identified by the dark brown colour in their nuclei (left panel). Counted BrdU positive cell/crypt in TCR-HA and VILLIN-HA×TCR-HA transgenic mice are depicted in the right panel.

As expected, many of the genes found to be regulated have been described previously in the context of intestinal inflammation. Some proinflammatory genes were specifically regulated in both LPL and IEL. In agreement with published data, integrin $\alpha_E\beta_7$ expression was upregulated in 6.5^+CD4^+ LPL and IEL from inflamed tissue of VILLIN-HA×TCR-HA transgenic mice compared with cells from control mice. It has been shown that changes in $\alpha_E\beta_7$ expression in Crohn's disease and ulcerative colitis patients versus controls are of pathological relevance and that this may be one of the earliest events in the pathogenesis of this disease.[21] A wide variety of

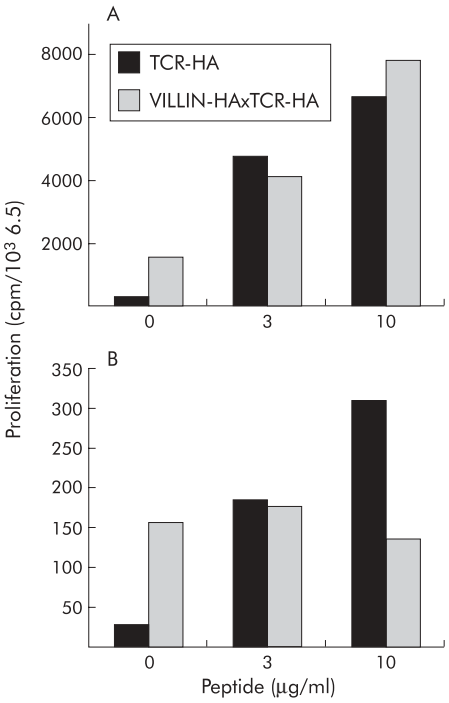

Figure 7 Reduced proliferative capacity of haemagglutinin (HA) specific intestinal epithelial cells (IEL) from VILLIN-HA×T cell receptor (TCR)-HA mice. Lamina propria lymphocytes (LPL) (A) and IEL (B) were isolated from VILLIN-HA×TCR-HA and TCR-HA control mice and stimulated in vitro with the corresponding haemagglutinin (HA) peptide. Thymidine incorporation was measured in cpm per 1000 $6.5^{+}CD4^{+}$ T cells.

members of the TNF receptor superfamily were differentially expressed on LPL and IEL from double transgenic mice. Especially, Tnfrsf7 and Tnfrsf9 were upregulated on LPL and IEL from inflamed tissue. These molecules are known to be expressed in elevated numbers of peripheral lymphocytes in IBD.[22 23] LPL and IEL do not resemble a homogenous T cell population; each population has its own phenotype with specialised function. According to these characteristics, the gene expression profile of these cells may differ. Indeed, many genes were found to be exclusively regulated in LPL of VILLIN-HA×TCR-HA transgenic mice. Although $\alpha_E\beta_7$ was upregulated in both LPL and IEL, expression of various other integrins was significantly increased only in LPL from inflamed tissue. Integrins are involved in lymphocyte homing to the intestinal mucosa and it has been demonstrated that their expression is often enhanced on intestinal inflammation.[24–26] Besides genes exclusively regulated in LPL, many proinflammatory genes exist whose expression level is only changed in the IEL of double transgenic mice. One example is expression of the CD7 surface molecule. In line with published data which show that the frequency of $CD7^{+}$ T cells is significantly increased in IBD, expression of CD7 in IEL of VILLIN-HA×TCR-HA mice was upregulated. In addition, the expression level of STAT3 was increased in IEL. STAT3 has been shown to be directly linked to secretion of IL-6 in IBD.[27] This is in accordance with the finding that LPL and IEL from VILLIN-HA×TCR-HA secrete higher amounts of IL-6 compared with control cells. Intestinal inflammation is often initiated by failure of mucosal lymphocytes to undergo preprogrammed cell death.[28] In agreement with this, expression of the antiapoptotic *bcl2* gene family was significantly upregulated in IEL of the inflamed tissue. Many other genes were found to be significantly up- or downregulated in the inflamed intestine compared with healthy donors, such as CD83, PTGER4, or CCR7 and numerous others which have been discussed in the context of IBD.[29–32]

In addition to genes that are associated with intestinal inflammation, a large number of genes previously described as playing a role in immune regulation and induction of regulatory T cells in the intestine have also been identified. The major finding was that expression of IL-10 and IFN-γ, both of which are mediators playing important roles in the

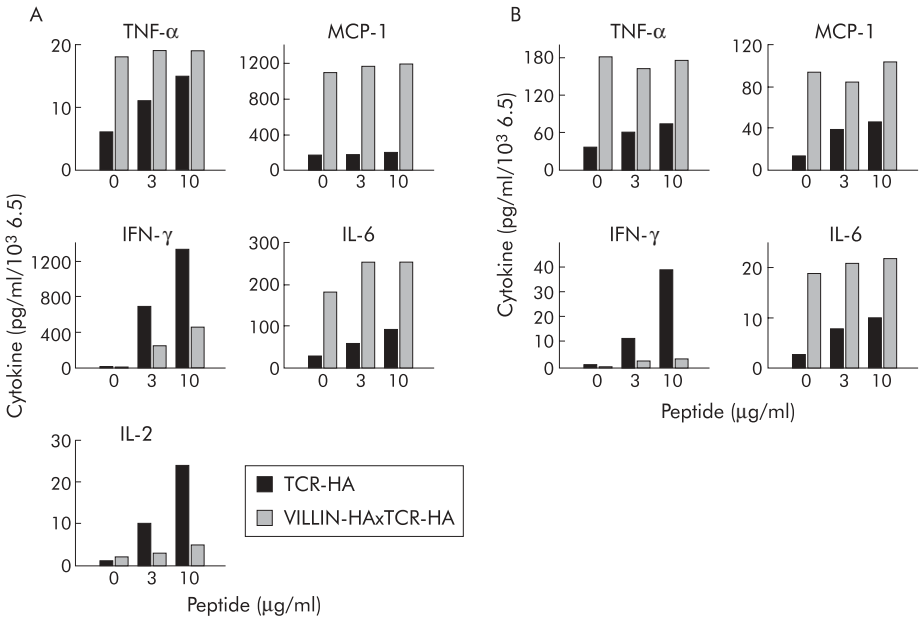

Figure 8 Haemagglutinin (HA) specific $CD4^{+}$ T cells from the infiltrated mucosa differed in cytokine profile. Lamina propria lymphocytes (LPL) (A) and intestinal epithelial cells (IEL) (B) from T cell receptor (TCR)-HA and VILLIN-HA×TCR-HA mice were stimulated in vitro with the HA110-120 peptide. Culture supernatants were analysed for several cytokines using the cytokine bead array from BD Bioscience. Cytokine quantities are depicted as pg/ml per 1000 $6.5^{+}CD4^{+}$ intestinal T cells. TNF-α, tumour necrosis factor α; MCP-1, monocyte chemoattractant protein 1; IFN-γ, interferon γ; IL, interleukin.

Table 1 Selected genes differentially expressed in LPL and IEL from VILLIN-HA×TCR-HA and TCR-HA mice

Name	Regulation	Signal intensity				Cluster in fig 9
		LPL stg	LPL dtg	IEL stg	IEL dtg	
LPL and IEL proinflammatory						
$\alpha_E\beta_7$	↑	135	399	1206	2075	A
S100a6	↑	158	1627	71	478	A
Snx9	↑	159*	357	82*	483	A
Tnfrsf7	↑	673	1377	436	1114	A
CD83	↑	878	1225	237	992	A
Tnfrsf9	↑	174	423	33*	197	A
LPL proinflammatory						
Itgb7	↑	1273	2356	4276	3097	C
Itga4	↑	96	274	42	76	C
PTGER4	↓	650	221	1628	1090	D
EGR2	↓	1941	655	143	122*	F
IEL proinflammatory						
CD7	↑	76*	58*	216	1945	E
BCL2L13	↑	258	242	197	441	E
STAT3	↑	2033	1807	184	1107	E
LPL and IEL anti-inflammatory						
CST7	↑	70	468	725	1197	A
Areg	↑	274	488	71	530	A
Nrp-1	↑	25*	87	69*	269	A
IL-10	↑	288	745	144	713	A
IFN-γ	↓	179	20	730	143	B
IL-7r	↓	2430	567	2318	620	B
LPL anti-inflammatory						
Tnfrsf18/GITR	↑	2384	7324	669	772	C
LT-β	↓	2113	358	1805	1695	D
CCR5	↓	4212	1928	877	981	D
IL-17	↓	867	298	256	377	D
ICOS	↓	1696	562	169	126	D
IEL anti-inflammatory						
ANXA1	↑	28*	24*	69*	233	E
CCL3	↓	357	485	1256	649	F
CCR7	↓	329	309	1215	455	F
IL-6rα	↓	177	159	653	209	F
ICAM1	↓	126	77	190	65*	F
LPL and/or IEL pro-/anti-inflammatory						
PD1	↑	349	1446	87*	247	A
KLRG1	↑	194	380	77	78	C
CCL5	↓	18622	15975	3550	1424	D
Lag3	↑	69*	36*	45*	388	E
Tnfrsf4/OX40	↑	1386	5804	398	1494	A

Stg, T cell receptor (TCR)-HA; dtg, VILLIN-HA×TCR-HA; LPL, lamina propria lymphocytes; IEL, intraepithelial lymphocytes.
*Absent, defined by the Affymetrix software algorithm.
Genes in italics represent established markers of regulatory T cells.
Results are from pooled individual mice (n>3).

regulation of progression of IBD, were significantly regulated in the IEL and LPL of diseased mice. IL-10 was highly upregulated in LPL and IEL from double transgenic mice compared with control mice. IL-10 has a major role in the regulatory network of cytokines controlling mucosal tolerance. Using a murine knockout model it has been shown that IL-10 prevents the development of intestinal inflammation.[33] Furthermore, application of IL-10 to diseased mice abrogated clinical signs or suppressed inflammation in the intestine.[34] In addition, IFN-γ, which plays a key role in induction of IBD, was downregulated in mucosal lymphocytes of VILLIN-HA×TCR-HA transgenic mice. Recently, we described Nrp-1 as an activation independent marker exclusively expressed on regulatory T cells.[18] LPL and IEL from VILLIN-HA×TCR-HA double transgenic mice showed elevated expression of Nrp-1. Furthermore, expression of other proinflammatory cytokines such as LT-β and IL-17 in LPL from double transgenic mice was also downregulated. It has been shown that Tnfrsf18 is predominantly expressed on $CD4^+CD25^+$ regulatory T cells.[35] This member of the TCR receptor superfamily was significantly upregulated in LPL of double transgenic mice. Also, IEL showed differential gene expression resembling induction of regulatory mechanisms to maintain homeostasis. Genes involved in IBD induction such as CCR7, IL-6rα, or ICAM were downregulated in IEL. The entire data set of this microarray experiment is accessible as MIAME format online (www.gbf.de/array).

Expression of IL-10, Nrp-1, and Foxp3 in intestinal lymphocytes

The data presented here strongly suggest that intestinal epithelial cell expression of HA antigen induces activation of HA specific T cells counteracted by induction of tolerance. To further confirm this hypothesis, IL-10 and Nrp-1 mRNA expression in 6.5^+CD4^+ LPL was confirmed by real time RT-PCR. Our analysis revealed that Nrp-1 as well as IL-10 expression was significantly upregulated in LPL of VILLIN-HA×TCR-HA double transgenic mice following the expression pattern of naturally occurring $CD4^+CD25^+$ regulatory T cells (fig 10). Recently, the transcription factor Foxp3 has been identified as being essential for the development and function of regulatory T cells.[36] Similar to Nrp-1, Foxp3 expression was also found to be upregulated in LPL from double transgenic mice suggesting that chronic antigen

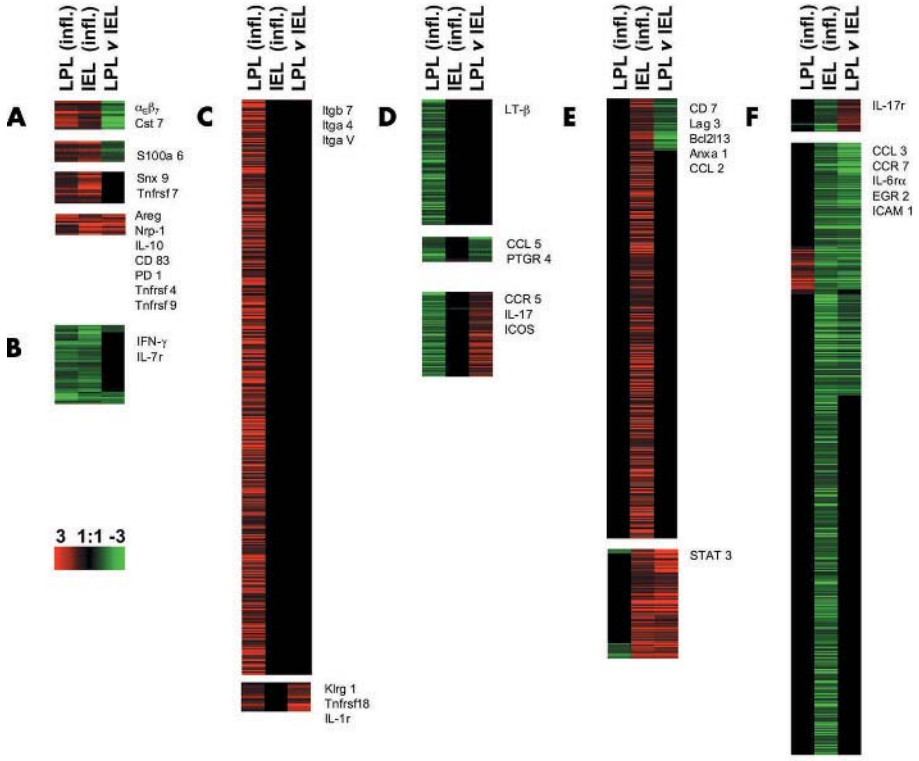

Figure 9 Global gene expression profiling of haemagglutinin (HA) specific CD4+ T cells. Cluster analysis of genes differentially expressed in 6.5+CD4+ T cells isolated from lamina propria (LPL) and epithelium (IEL) of infiltrated VILLIN-HA×T cell receptor (TCR)-HA as well as healthy TCR-HA mice. Red indicates induction of gene expression, green indicates repression. The brighter the colour the stronger the factor of gene regulation (+3, bright red; −3, bright green). Black indicates no changes. Inclusion into this heat map required at least a 1.5-fold difference in inducible gene expression. LPL (infil.) represents genes differentially expressed in 6.5+CD4+ T cells from the infiltrated lamina propria of VILLIN-HA×TCR-HA mice compared with the LPL of healthy TCR-HA donors. IEL (infil.) represents gene differentially expressed in 6.5+CD4+ T cells from the epithelium of VILLIN-HA×TCR-HA mice compared with TCR-HA. LPL *v* IEL characterises basal level expression of genes by LPL compared with IEL of healthy TCR-HA mice. Cluster (A): Genes upregulated in the LPL and IEL of VILLIN-HA×TCR-HA mice on mucosal infiltration. Cluster (B): Genes downregulated in LPL and IEL during infiltration. Cluster (C): Genes exclusively upregulated by LPL from double transgenic mice. Cluster (D): Genes downregulated in self reactive LPL CD4+ T cells in the infiltrated gut. Cluster (E): Genes exclusively upregulated by IEL from infiltrated tissue. Cluster (F): Genes downregulated by IEL from VILLIN-HA×TCR-HA mice. Results are from pooled individual mice (n>3).

stimulation in VILLIN-HA×TCR-HA is controlled by regulatory T cells (fig 10).

DISCUSSION

Despite the fact that T cells with an autoaggressive character are involved in the development of intestinal inflammation, it has been difficult to identify self proteins that may play a role in the aetiology or chronicity of IBD and to asses the impact of antigen specificity. An important aim of this study was to test the hypothesis that antigen specific CD4+ T cell recognition of a single epithelial self antigen is sufficient to trigger an inflammatory cascade in the intestine and to investigate whether regulatory mechanisms may suppress inflammation and maintain homeostasis. Therefore, a transgenic mouse expressing HA in enterocytes of the intestinal epithelium was generated. Concomitant expression of HA and a MHC class II restricted T cell receptor specific for HA in VILLIN-HA×TCR-HA mice is sufficient to induce a specific

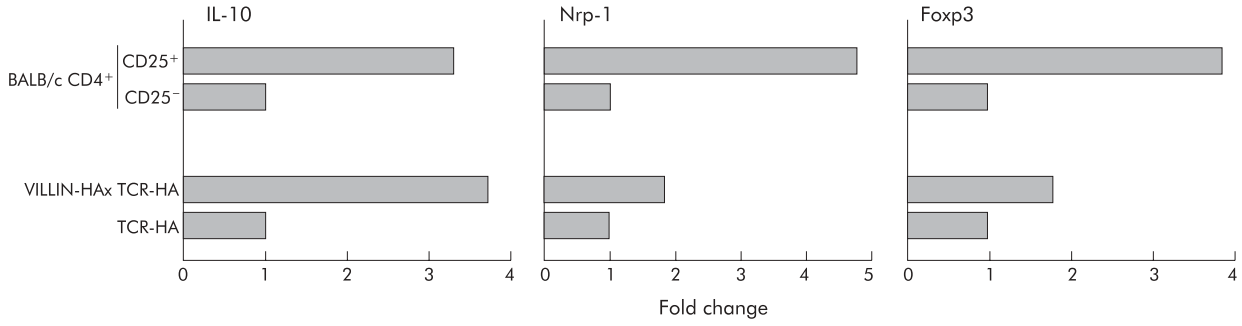

Figure 10 Interleukin (IL)-10, Nrp-1, and Foxp3 expression pattern of 6.5+CD4+ lamina propria lymphocytes (LPL). 6.5+CD4+ LPL were sorted from T cell receptor (TCR)-HA and VILLIN-HA×TCR-HA transgenic mice. Total RNA was prepared, reverse transcribed, and mRNA expression levels determined in real time reverse transcription-polymerase chain reaction (RT-PCR) assays. Mean relative regulation is indicated. Results are from pooled individual mice (n>3) and obtained in duplicate real time RT-PCR assays. RPS9 mRNA expression served as a housekeeping gene control.

CD4 T cell response leading to infiltration of lymphocytes into the mucosa.

Autoimmune diseases are believed to be under complex genetic regulation but all require some form of escape from self tolerance. In VILLIN-HA×TCR-HA double transgenic mice, 6.5^+CD4^+ transgenic T cells could be detected in the peripheral lymphatic organs (fig 2A). This finding was not unexpected as it has been described previously that expression of the HA antigen in pancreas[37 38] or in haematopoetic cells[39] and concomitant expression of the MHC class II restricted TCR specific for HA does not lead to complete deletion of 6.5^+ T cells. A possible explanation for the escape from central tolerance might involve coexpression of two different TCR by the same cell. Due to allelic inclusion of TCR-α genes, self reactive T cells may leave the thymus leading to autoimmunity.[38] To exclude the fact that T cells were rendered in an activated, but anergic state, we compared the proliferative capacity of 6.5^+CD4^+ T cells recovered from spleen and MLN (fig 2B). As no differences in ^{3}[H] thymidine incorporation were observed, we postulated that 6.5^+CD4^+ T cells should be functional in vivo.

In the recently published GFAP-HA×CL4-TCR model, mice died within a few days after birth, caused by a hyper acute jejuno-ileo-colitis mediated by transgenic $CD8^+$ T cells that recognise enteric glia.[40] Based on pathological observations in VILLIN-HA×TCR-HA transgenic mice demonstrating some features of inflammation (that is, expression of proinflammatory mediators) accompanied by marked infiltration of CD3 lymphocytes in the LPL and IEL compartment, we concluded that our model mimics the chronic state of so-called physiological inflammation in which the gut is poised for, but actively restrained from, full immunological responses. During the course of infections in the normal host, full activation of GALT occurs but is rapidly superseded by downregulation of the immune response. Apparently, the immunological balance in our model stands on the edge. On the one hand, activation and infiltration of lymphocytes in the intestine occurs, but on the other hand, regulatory mechanisms seem to counteract uncontrolled progression of intestinal inflammation. In IBD this process is not regulated normally.

When mucosal lymphocytes are stimulated via the TCR they normally respond only poorly and activation seems to be dependent on CD2/CD28 stimulation to result in proliferation and cytokine secretion.[41 42] Interestingly, IEL and LPL from TCR-HA mice as well as LPL from VILLIN-HA×TCR-HA transgenic mice proliferate in an antigen dose dependent manner. In contrast, the antigen specific capacity of IEL from double transgenic mice to proliferate was abrogated with a high background proliferation even without antigenic stimulation (fig 7). In general, the cytokine profile in IBD shows some characteristic differences depending on the type of disease. Crohn's disease is associated with a Th1 cytokine pattern, characterised by IFN-γ, TNF-α, and IL-12 secretion.[20 43] In ulcerative colitis, the cytokine profile is less restricted and appears to be a modified Th2 response.[20] In the VILLIN-HA×TCR-HA transgenic mouse model, antigen stimulated 6.5^+CD4^+ LPL and IEL secreted lower amounts of IFN-γ and IL-2 on in vitro stimulation compared with control mice (fig 8). These data suggested suppression of proinflammatory mediators in the intestine of double transgenic mice. However, basal level secretion of TNF-α, MCP-1, and IL-6, which are all important mediators in induction of IBD, was considerably increased in LPL and IEL from double transgenic mice (fig 8). These unusual cytokine patterns may denote a steady state between regulatory and pathological mechanisms being active in the intestine. To consider this hypothesis in more detail, global gene expression analysis of HA specific LPL and IEL from the intestine of VILLIN-HA×TCR-HA or from TCR-HA control mice was performed.

Infiltration of the mucosa in VILLIN-HA×TCR-HA transgenic mice was accompanied by broad changes in the gene expression pattern of autoreactive LPL and IEL. The profiling revealed differential expression of proinflammatory genes, as well as a remarkable set of genes discussed in the context of immune regulation. Different types of regulatory T cells, Th3 cells, $CD4^+CD25^+$ or $CD4^+CD45RB^{low}$ T cells, or $CD8^+$ suppressor T cells[44–47] have been described as being responsible for controlling intestinal inflammation. These regulatory cells induce immunosuppression in surrounding T cells, most likely by secretion of regulatory cytokines such as IL-10 or TGF-β, and inhibit inappropriate immune responses towards harmless mucosal antigens.[48 49] Quantitative real time RT-PCR clearly demonstrated upregulation of IL-10 in LPL from VILLIN-HA×TCR-HA mice. Moreover, increased expression of the marker genes for regulatory T cells Nrp-1[18] and Foxp3[36] in LPL from double transgenic mice supports the idea that chronic mucosal antigen exposure may lead to the development of regulatory T cells in vivo. Further studies will clarify the role of regulatory T cells in the downregulation of mucosal inflammation in the VILLIN-HA×TCR-HA transgenic mouse model. Our genome wide transcriptome of mucosal lymphocytes from inflamed and normal tissue provides a focused starting point for the further elucidation of genetic and mechanistic aspects of intestinal inflammation and immune regulation (all data will be freely accessible at www.gbf.de/array).

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REFERENCES

1 **Nagler-Anderson C**. Man the barrier! Strategic defences in the intestinal mucosa. *Nat Rev Immunol* 2001;**1**:59–67.
2 **Kisielow P**, Teh HS, Bluthmann H, *et al*. Positive selection of antigen-specific T cells in thymus by restricting MHC molecules. *Nature* 1988;**335**:730–3.
3 **Von Boehmer H**, Aifantis I, Gounari F, *et al*. Thymic selection revisited: how essential is it? *Immunol Rev* 2003;**191**:62–78.
4 **Melamed D**, Friedman A. Direct evidence for anergy in T lymphocytes tolerized by oral administration of ovalbumin. *Eur J Immunol* 1993;**23**:935–42.
5 **Monteleone I**, Vavassori P, Biancone L, *et al*. Immunoregulation in the gut: success and failures in human disease. *Gut* 2002;**50**(suppl 3):III60–4.
6 **Sakaguchi S**. Regulatory T cells: mediating compromises between host and parasite. *Nat Immunol* 2003;**4**:10–1.
7 **Powrie F**, Correa-Oliveira R, Mauze S, *et al*. Regulatory interactions between CD45RBhigh and CD45RBlow CD4+ T cells are important for the balance

between protective and pathogenic cell-mediated immunity. *J Exp Med* 1994;**179**:589–600.
8 **Maloy KJ**, Powrie F. Regulatory T cells in the control of immune pathology. *Nat Immunol* 2001;**2**:816–22.
9 **Hermiston ML**, Gordon JI. Inflammatory bowel disease and adenomas in mice expressing a dominant negative N-cadherin. *Science* 1995;**270**:1203–7.
10 **Dohi T**, Fujihashi K, Koga T, *et al.* T helper type-2 cells induce ileal villus atrophy, goblet cell metaplasia, and wasting disease in T cell-deficient mice. *Gastroenterology* 2003;**124**:672–82.
11 **Caton AJ**, Stark SE, Shih FF, *et al.* Transgenic mice that express different forms of the influenza virus hemagglutinin as a neo-self-antigen. *J Clin Immunol* 1995;**15**(suppl 6):106–12S.
12 **Pinto D**, Robine S, Jaisser F, *et al.* Regulatory sequences of the mouse villin gene that efficiently drive transgenic expression in immature and differentiated epithelial cells of small and large intestines. *J Biol Chem* 1999;**274**:6476–82.
13 **Wilson IA**, Skehel JJ, Wiley DC. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 A resolution. *Nature* 1981;**289**:366–73.
14 **Kirberg J**, Baron A, Jakob S, *et al.* Thymic selection of CD8+ single positive cells with a class II major histocompatibility complex-restricted receptor. *J Exp Med* 1994;**180**:25–34.
15 **Guy-Grand D**, Griscelli C, Vassalli P. The mouse gut T lymphocyte, a novel type of T cell. Nature, origin, and traffic in mice in normal and graft-versus-host conditions. *J Exp Med* 1978;**148**:1661–77.
16 **Rogler G**, Daig R, Aschenbrenner E, *et al.* Establishment of long-term primary cultures of human small and large intestinal epithelial cells. *Lab Invest* 1998;**78**:889–90.
17 **Hackett CJ**, Dietzschold B, Gerhard W, *et al.* Influenza virus site recognized by a murine helper T cell specific for H1 strains. Localization to a nine amino acid sequence in the hemagglutinin molecule. *J Exp Med* 1983;**158**:294–302.
18 **Bruder D**, Probst-Kepper M, Westendorf AM, *et al.* Frontline: Neuropilin-1: a surface marker of regulatory T cells. *Eur J Immunol* 2004;**34**:623–30.
19 **Buer J**, Lanoue A, Franzke A, *et al.* Interleukin 10 secretion and impaired effector function of major histocompatibility complex class II-restricted T cells anergized in vivo. *J Exp Med* 1998;**187**:177–83.
20 **Fiocchi C**. Inflammatory bowel disease: etiology and pathogenesis. *Gastroenterology* 1998;**115**:182–205.
21 **Elewaut D**, De Keyser F, Cuvelier C, *et al.* Distinctive activated cellular subsets in colon from patients with Crohn's disease and ulcerative colitis. *Scand J Gastroenterol* 1998;**33**:743–8.
22 **Croft M**. Costimulation of T cells by OX40, 4-1BB, and CD27. *Cytokine Growth Factor Rev* 2003;**14**:265–73.
23 **Raedler A**, Fraenkel S, Klose G, *et al.* Elevated numbers of peripheral T cells in inflammatory bowel diseases displaying T9 antigen and Fc alpha receptors. *Clin Exp Immunol* 1985;**60**:518–24.
24 **Hornquist CE**, Lu X, Rogers-Fani PM, *et al.* G(alpha)i2-deficient mice with colitis exhibit a local increase in memory CD4+ T cells and proinflammatory Th1-type cytokines. *J Immunol* 1997;**158**:1068–77.
25 **Podolsky DK**, Lobb R, King N, *et al.* Attenuation of colitis in the cotton-top tamarin by anti-alpha 4 integrin monoclonal antibody. *J Clin Invest* 1993;**92**:372–80.
26 **Sun FF**, Lai PS, Yue G, *et al.* Pattern of cytokine and adhesion molecule mRNA in hapten-induced relapsing colon inflammation in the rat. *Inflammation* 2001;**25**:33–45.
27 **Wang L**, Walia B, Evans J, *et al.* IL-6 induces NF-kappaB activation in the intestinal epithelia. *J Immunol* 2003;**171**:3194–201.
28 **De Maria R**, Boirivant M, Cifone MG, *et al.* Functional expression of Fas and Fas ligand on human gut lamina propria T lymphocytes. A potential role for the acidic sphingomyelinase pathway in normal immunoregulation. *J Clin Invest* 1996;**97**:316–22.
29 **Campbell JJ**, Murphy KE, Kunkel EJ, *et al.* CCR7 expression and memory T cell diversity in humans. *J Immunol* 2001;**166**:877–84.
30 **Kabashima K**, Saji T, Murata T, *et al.* The prostaglandin receptor EP4 suppresses colitis, mucosal damage and CD4 cell activation in the gut. *J Clin Invest* 2002;**109**:883–93.
31 **Otten K**, Dragoo J, Wang HC, *et al.* Antigen-induced chemokine activation in mouse buccal epithelium. *Biochem Biophys Res Commun* 2003;**304**:36–40.
32 **Te Velde AA**, van Kooyk Y, Braat H, *et al.* Increased expression of DC-SIGN+IL-12+IL-18+ and CD83+IL-12-IL-18- dendritic cell populations in the colonic mucosa of patients with Crohn's disease. *Eur J Immunol* 2003;**33**:143–51.
33 **Kuhn R**, Lohler J, Rennick D, *et al.* Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 1993;**75**:263–74.
34 **Steidler L**, Hans W, Schotte L, *et al.* Treatment of murine colitis by Lactococcus lactis secreting interleukin-10. *Science* 2000;**289**:1352–5.
35 **Shimizu J**, Yamazaki S, Takahashi T, *et al.* Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol* 2002;**3**:135–42.
36 **O'Garra A**, Vieira P. Twenty-first century Foxp3. *Nat Immunol* 2003;**4**:304–6.
37 **Degermann S**, Reilly C, Scott B, *et al.* On the various manifestations of spontaneous autoimmune diabetes in rodent models. *Eur J Immunol* 1994;**24**:3155–60.
38 **Sarukhan A**, Lanoue A, Franzke A, *et al.* Changes in function of antigen-specific lymphocytes correlating with progression towards diabetes in a transgenic model. *EMBO J* 1998;**17**:71–80.
39 **Lanoue A**, Bona C, Von Boehmer H, *et al.* Conditions that induce tolerance in mature CD4+ T cells. *J Exp Med* 1997;**185**:405–14.
40 **Cornet A**, Savidge TC, Cabarrocas J, *et al.* Enterocolitis induced by autoimmune targeting of enteric glial cells: a possible mechanism in Crohn's disease? *Proc Natl Acad Sci U S A* 2001;**98**:13306–11.
41 **Boirivant M**, Marini M, Di Felice G, *et al.* Lamina propria T cells in Crohn's disease and other gastrointestinal inflammation show defective CD2 pathway-induced apoptosis. *Gastroenterology* 1999;**116**:557–65.
42 **Targan SR**, Deem RL, Liu M, *et al.* Definition of a lamina propria T cell responsive state. Enhanced cytokine responsiveness of T cells stimulated through the CD2 pathway. *J Immunol* 1995;**154**:664–75.
43 **Elson CO**, Holland SP, Dertzbaugh MT, *et al.* Morphologic and functional alterations of mucosal T cells by cholera toxin and its B subunit. *J Immunol* 1995;**154**:1032–40.
44 **Miller A**, Lider O, Roberts AB, *et al.* Suppressor T cells generated by oral tolerization to myelin basic protein suppress both in vitro and in vivo immune responses by the release of transforming growth factor beta after antigen-specific triggering. *Proc Natl Acad Sci U S A* 1992;**89**:421–5.
45 **Mowat AM**, Lamont AG, Strobel S, *et al.* The role of antigen processing and suppressor T cells in immune responses to dietary proteins in mice. *Adv Exp Med Biol* 1987;**216A**:709–20.
46 **Roncarolo MG**, Levings MK. The role of different subsets of T regulatory cells in controlling autoimmunity. *Curr Opin Immunol* 2000;**12**:676–83.
47 **Sakaguchi S**. Regulatory T cells: key controllers of immunologic self-tolerance. *Cell* 2000;**101**:455–8.
48 **Ludviksson BR**, Seegers D, Resnick AS, *et al.* The effect of TGF-beta1 on immune responses of naive versus memory CD4+ Th1/Th2 T cells. *Eur J Immunol* 2000;**30**:2101–11.
49 **Strober W**, Kelsall B, Marth T. Oral tolerance. *J Clin Immunol* 1998;**18**:1–30.