

# MODULATING THE INTESTINAL IMMUNE SYSTEM: THE ROLE OF LYMPHOTOXIN AND GALT ORGANS

T W Spahn, T Kucharzik

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The gut associated immune system fences off potentially harmful intestinal antigens from the systemic circulation and induces systemic tolerance against luminal antigens. Intestinal immune responses against luminal antigens include IgA secretion and induction of regulatory cells. Unlike few other cytokines, lymphotoxin  $\alpha/\beta$  regulates the development of intestinal lymphoid organs. The embryonic development of Peyer's patches, postnatal lamina propria B cell development, and isolated lymphoid follicle development all depend on lymphotoxin  $\beta$  receptor interactions. Lymphotoxin  $\alpha/\beta$  signalling also contributes to the development of mesenteric lymph nodes. In addition, intestinal inflammation is suppressed by inhibition of lymphotoxin  $\beta$  signalling, an observation which has initiated clinical studies using this treatment principal. Intestinal follicular lymphoid organs are sites of antigen presentation. Antigen presenting cells tune the delicate balance between intestinal immune tolerance and inflammation. Therefore, gut associated lymphatic organs and factors regulating their development are critical for the prevention of adverse immune reactions to intestinal antigens. This review provides an overview on the role of lymphotoxin and the gut associated lymphatic organs in the regulation of oral tolerance and intestinal inflammation.

## INTRODUCTION

Intestinal mucosal surfaces are exposed to alimentary and bacterial antigens of the intestinal flora. The physiological immune response towards intestinal antigens is non-harmful to the entire organism and includes induction of systemic immune tolerance and IgA secretion. Inflammatory bowel disease (IBD) is associated with activation of the local intestinal and systemic immune responses. In various animal models of IBD, uncontrolled immune responses following intestinal injury result in mucosal insult. Colitis is associated with loss of tolerance against intestinal antigens, which also contributes to perpetuation of local and systemic inflammatory immune responses. Characterisation of intestinal inflammatory cytokine pathways has provided valuable tools to modulate the activity of IBD.

While inhibition of tumour necrosis factor receptor (TNFR) interactions is effective in controlling IBD, inhibition of interactions between the TNF family molecule lymphotoxin  $\beta$  (LT $\beta$ , bound in LT $\alpha_1\beta_2$ ) with its receptor (LT $\beta$ R) is currently being investigated as a potential IBD treatment. LT $\alpha_1\beta_2$ -LT $\beta$ R interactions control the development and function of the intestinal immune system. During embryonic development, LT $\beta$ R ligation is critical for the formation of gut associated lymphoid tissue (GALT). Postnatal LT $\alpha_1\beta_2$ -LT $\beta$ R ligation controls the development of lamina propria (Lp) B cells. In adult mice, experimental colitis can be suppressed by inhibition of LT $\alpha_1\beta_2$ -LT $\beta$ R signalling.

Presentation of antigens to immune effector cells is concentrated at sites of organised mucosal lymphoid follicles. The hallmark of organised GALT is the presence of lymphoid follicles. Therefore, GALT is the intestinal frontier of the systemic immune response. Figure 1 provides a schematic overview of the lymphoid organs of the intestinal immune system. As GALT organs are sites where antigen is presented to professional antigen presenting cells (APCs), they are critical for the decision between inflammation and tolerance. On the mucosal intestinal surface, only a thin layer of epithelial cells (IECs) separates the gut lumen from the mucosal immune system. Despite their barrier function, IECs provide a variety of transport functions to deliver information about the external milieu. Peyer's patches (PP) are highly specialised "gut-type" lymphoid follicles in the small intestinal wall that contain naïve B cells, follicular dendritic cells (FDCs), and T cell rich areas. PPs are covered by the follicle associated epithelium (FAE) which contains specialised epithelial cells, called M cells. M cells sample luminal antigen directly into the PP, bypassing IECs. Isolated lymphoid follicles (ILF) and cryptopatches are additional intestinal lymphoid aggregates. ILFs are lymphoid aggregates in the antimesenteric wall of the small intestine which have been described in mice, humans,<sup>1</sup> and other species. Colonic ILFs have also been reported in mice.<sup>2</sup> Similar to PPs, ILFs consist of segregated B and T cell areas. They possess

See end of article for authors' affiliations

Correspondence to:  
Dr T Spahn, Department of  
Medicine B, Albert-  
Schweitzer-Str 33, D-48129  
Münster, Germany;  
spahn@uni-muenster.de

► Inhibition of interactions between the TNF family molecule lymphotoxin  $\beta$  with its receptor is currently being investigated as a potential IBD treatment.

germinal centres and an overlying FAE containing M cells.<sup>2</sup> Because of these structures, ILFs are suggested to be inductive sites for mucosal immune responses. Murine ILFs are up to 20 times more frequent than PPs and develop postnatally in a  $LT\alpha_1\beta_2$ - $LT\beta R$  dependent fashion.<sup>3</sup>

Intestinal cryptopatches (CP) are small clusters of interleukin 7 receptor ( $IL-7R$ )<sup>+</sup> lymphocytes in the Lp of the small and large intestine. There is substantial evidence indicating that gut CP develop progenitor T cells for extrathymic descendants which subsequently migrate to the intraepithelial lymphocyte (IEL) compartment.<sup>4-6</sup> Colonic lymphoid follicles are present in the large intestine. IEL and Lp lymphocytes are distinct intestinal immune cell populations. Mesenteric lymph nodes (MLNs) provide a second line of defence as organised lymphoid tissue filtering the mesenteric lymph vessels.

Extraintestinal mucosal surfaces also develop specialised secondary lymphoid organs such as the nasal associated lymphoid tissue (NALT) and bronchus associated lymphoid tissue in the respiratory tract. Unlike PP, which develop during gestation, NALT organs develop after birth (reviewed by Mebius<sup>7</sup>).

Studies of the TNF cytokine family, especially  $LT\alpha/\beta$  and its receptor, have provided a better understanding of factors regulating the development of the lymphoid GALT organs and Lp B lymphocytes. Furthermore,  $LT\alpha_1\beta_2$ - $LT\beta R$  interactions are involved in the control of intestinal inflammation. Investigators have thus been provided tools to differentially address the role of these organs in the regulation of intestinal tolerance and inflammation. This review provides an overview on recent studies covering the development of GALT organs, their role in experimental models of IBD and oral tolerance (OT), and the implications of  $LT\alpha/\beta$  for the control

of GALT organ formation and maintenance of an anti-inflammatory intestinal milieu.

## STRUCTURE AND DEVELOPMENT OF GALT ORGANS

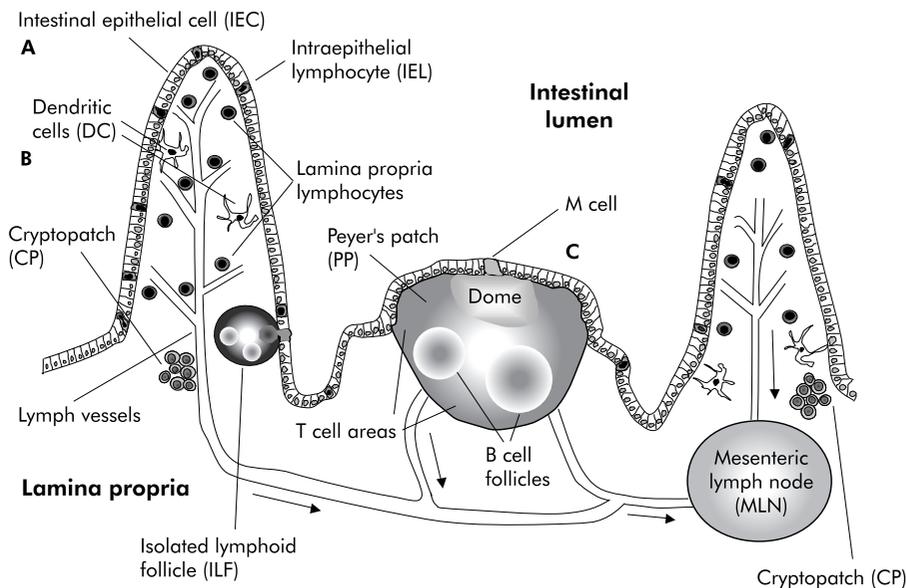
### Development of secondary lymphoid organs in the intestine: lymphotoxin and IL-7 play a predominant role in GALT development

#### Overview and time course

Part of the organised GALT such as PPs seems to be genetically determined as they form at predictable sites during embryogenesis. Additionally, PPs accumulate in mice and human as they age. Colonic patches appear in disease (IBD).<sup>8</sup> However, the triggers for this postnatal accumulation of intestinal secondary lymphoid tissues are not well understood. The development of PPs and MLNs is controlled by various cytokines and chemokines. Table 1 summarises gene deficient mice with defects in GALT development. Lymph nodes (LN) develop from lymph sacs by endothelial cell budding from veins, which are subsequently colonised by connective tissue. Lymph sacs give rise to the lymphatic vasculature by sprouting of lymphatic vessels (reviewed by Mebius<sup>7</sup>). MLNs are the first to develop in embryogenesis (gestational days 10.5–15.5), indicating a pivotal role of this organ for the entire organism. However, PPs develop late in gestation (gestational days 16–21). Intestinal CPs, intestinal Lp B cells, and NALT organs develop postnatally.<sup>7</sup> Figure 1 provides an overview on the different GALT organs.

### Central role of TNF/lymphotoxin for GALT development

TNF family cytokines, especially LT and the  $LT\beta$  receptor ( $LT\beta R$ ), play a critical role in the development of secondary lymphoid organs.  $LT-\alpha$  forms soluble homotrimers ( $LT\alpha_3$ ) and heterodimers ( $LT\alpha_1\beta_2$ ,  $LT\alpha_2\beta_1$ ) with the membrane molecule  $LT\beta$ .  $LT\alpha_3$  similar to TNF binds to the TNF receptors I (55 kDa) and II (75 kDa). LIGHT is another ligand of  $LT\beta R$ . Figure 2 depicts the different LT molecules and their receptors.  $LT\alpha_1\beta_2$  is expressed on haematopoietic cells,



**Figure 1** Schematic overview of the lymphoid elements of the gut associated lymphatic system. Peyer's patches (PP) and mesenteric lymph nodes (MLN) are organised intestinal lymphoid follicles. (A–C) Pathways of intestinal antigen uptake: luminal antigen can be taken up by (A) intestinal epithelial cells, (B) interdigitating lamina propria dendritic cells, and by (C) M cells. The lymphatic drainage of PP and villus lamina propria goes to the MLNs (direction of lymph flow indicated by arrows).

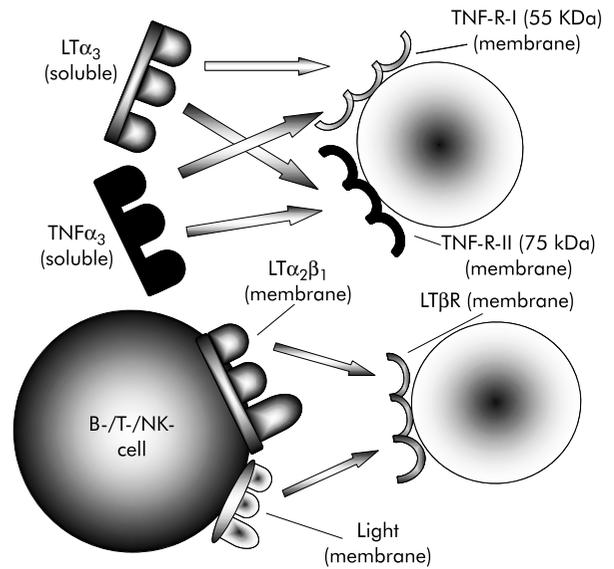
including T cells, B lymphocytes, and natural killer (NK) cells. The various biological effects of  $LT\alpha_1\beta_2$  are mediated by interaction of these circulating cells with  $LT\beta R$ , which is expressed on resident mesenchymal and stromal cells.<sup>9</sup>  $LT\alpha_1\beta_2$ - $LT\beta R$  interactions are completely or partially disrupted in mice with targeted deletion of the respective cytokine and receptors genes ( $LT\alpha$  gene deficient ( $-/-$ ),  $LT\beta$ - $-/-$ ,  $LT\beta R$ - $-/-$  mice), or in mice lacking  $LT\alpha\beta$  expressing cell populations ( $\mu$ MT mice without mature B cells). Competitive inhibition of  $LT\beta R$  binding by soluble  $LT\beta R$ -IgG fusion protein ( $LT\beta R$ IgG) is another effective means of blocking  $LT\beta R$  signalling. Figure 3 depicts the biological effects of  $LT\beta R$  mediated signalling and its inhibition.

**The role of IL-7 in GALT development**

An early event in LN and PP formation is the occurrence of interleukin 7 receptor ( $IL-7R$ )<sup>+</sup>  $CD3^-CD4^+CD45^+$  cells, which are progenitors of APCs and NK cells. Ligation of  $IL-7R$  and of TNF related activation induced cytokine (TRANCE) induces  $LT\alpha_1\beta_2$  expression on these cells.<sup>9-12</sup>  $IL-7R^+$  cells colocalise with vascular cell adhesion molecule (VCAM)-1<sup>+</sup> cell clusters.  $LT\alpha_1\beta_2$ - $LT\beta R$  interaction induces VCAM expression which can be inhibited by soluble  $IL-7R$  or blockade of  $LT\alpha_1\beta_2$ - $LT\beta R$  signalling pathways during embryogenesis.<sup>13</sup> Ablation of  $IL-7$  or of the  $IL-7$  signalling pathway results in mice deficient in PP.<sup>13-16</sup> Transgenic expression of  $IL-7$  in the intestine of  $IL-7$  gene deficient ( $-/-$ ) mice restored PP formation.<sup>17</sup> Gestational treatment of mice with anti- $IL-7R\alpha$  antibody by a single injection on day 15.5 post-conception abrogated PP formation in progeny of treated mice.<sup>18</sup>

**GALT defects secondary to inhibition of  $LT\alpha_1\beta_2$ - $LT\beta R$  and TNF-RI (55 kDa) signalling**

Signalling through the  $LT\beta R$  by  $LT\alpha_1\beta_2$  is critical for PP development (fig 2). Disruption of  $LT\alpha/\beta$  heterodimer formation in  $LT\alpha$ - $-/-$  mice results in loss of PP and of all



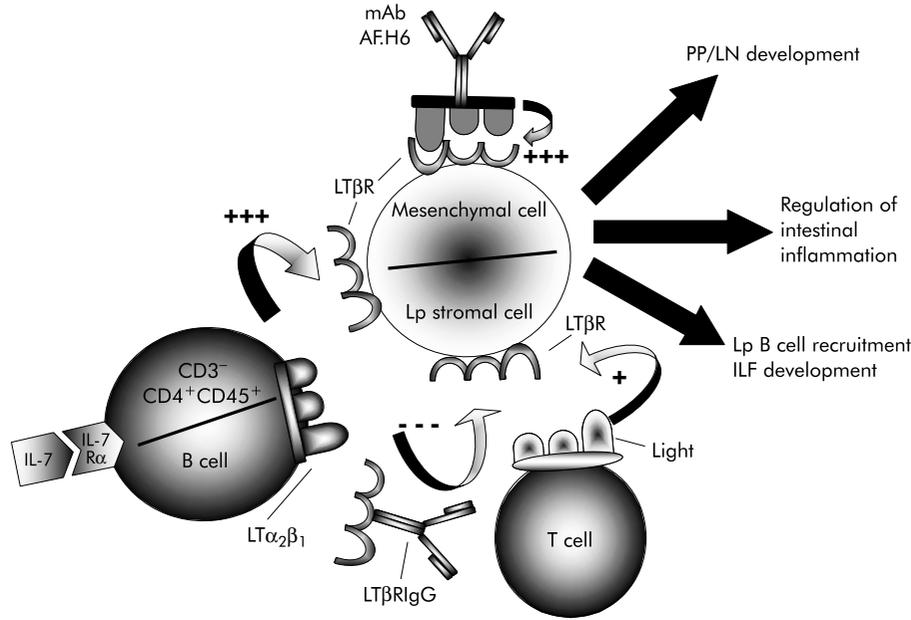
**Figure 2** Schematic overview of  $LT\alpha_1\beta_2$ - $LT\beta R$  ligand receptor interactions. Soluble  $LT\alpha_3$  interacts with the TNF receptors I (55 kDa) and II (75 kDa) similar to soluble  $TNF\alpha_3$ .  $LT\alpha_1\beta_2$  heterodimers and LIGHT are ligands for the membrane bound  $LT\beta R$  which is expressed on mesenchymal cells.  $LT\alpha/\beta$ , -R, lymphotoxin  $\alpha/\beta$ , -receptor; NK, natural killer; TNF, tumour necrosis factor; TNF-R, TNF receptor.

LN.<sup>19-20</sup> Gestational inhibition of  $LT\alpha_1\beta_2$ - $LT\beta R$  interactions inhibits PP formation.<sup>21-23</sup> Combined inhibition of  $LT\beta R$  and TNF-R-I (55) receptor-ligand interactions prevents the formation of PP and most LN.<sup>24</sup> Conversely, stimulation of  $LT\beta R$  in  $LT\alpha$ - $-/-$  mice by agonist anti- $LT\beta R$  antibodies (fig 3) induces formation of MLN.<sup>24</sup>  $LT\beta R$ - $-/-$  mice develop a phenotype similar to that observed in  $LT\alpha$ - $-/-$  mice.<sup>25</sup> Figure 4 provides a schematic overview on the effects of gestational

**Table 1** Mice with defects in organised gut associated lymphoid tissue development induced by gene defects or by gestational or postgestational treatment (modified from Mebius<sup>7</sup>)

Gene deficient/treated mice	PP	MLN	Reference
<i>LTα</i> - $-/-$ , <i>LTβR</i> - $-/-$ , <i>Nik</i> - $-/-$ , <i>aly</i> / <i>aly</i>	-	-	19 20 25 81-83
<i>NFκB2</i> - $-/-$	-	+	84
<i>Relb</i> - $-/-$	-	-	85
<i>LTβ</i> - $-/-$	-	+	21 26 86
<i>Light</i> - $-/-$ x <i>LTβ</i> - $-/-$	-	<MLN than <i>LTβ</i> - $-/-$	34
<i>TNF</i> - $-/-$	Reduced number	+	31 87
<i>TNFR-I (55 kDa)</i> - $-/-$	Reduced number	+	30 31
<i>LTα</i> - $-/+$ x <i>LTβ</i> - $-/-$	-	+	88
<i>Gα<i>α</i> i2</i> - $-/-$	Reduced in number	+	89 90
<i>Ikka</i> - $-/-$	-	Not done	91
<i>Trance</i> - $-/-$ , <i>Trancer</i> - $-/-$ , <i>Traf 6</i> - $-/-$	+	-	92-95
<i>Ikaros</i> - $-/-$	-	-	96 97
<i>Rorγ</i> - $-/-$	-	-	98 99
<i>IL7R</i> - $-/-$ , <i>Jak3</i> - $-/-$ , <i>γc</i> - $-/-$	-	+	12 13 16
<i>Id2</i> - $-/-$	-	-	100
<i>IL-7</i> - $-/-$	-	?	15
<i>Cxcr5</i> - $-/-$ , <i>Cxcl 13</i> - $-/-$	0-2	Reduced number	101 102
Gestational <i>LTβR</i> IgG treatment	-	+	22
Gestational <i>LTβR</i> IgG and <i>TNFR</i> IgG treatment	-	- or few	24

*aly*, lymphoplasia inducing gene; *CC*, *CXC/R/L*, *CC*, CX chemokine/receptor/ligand; *G α*α* i2*, G protein  $\alpha i2$ ; *Id2*, inhibitor of the helix loop helix;  $\gamma c$ , common cytokine receptor  $\gamma$  chain; *Ikaros*, member of the Kruppel family of zinc finger DNA binding proteins; *Ikka*, inhibitor of  $\kappa B\alpha$ ; *IL-7*, interleukin 7; *Jak3*, janus kinase 3; *LTα/β*, -R, lymphotoxin  $\alpha/\beta$ , -receptor; *LTβR*IgG, lymphotoxin  $\beta$  receptor-IgG fusion protein; MLN, mesenteric LN; *NFκB*, nuclear factor  $\kappa B$ ; *NIK*, NFκB inducing kinase; *Relb*, NFκB family transcription factor; *Rorγ*, retinoid related orphan receptor  $\gamma$ ; PP, Peyer's patches; *TNF*, tumour necrosis factor; *TNFR*, tumour necrosis factor receptor; *TRAF*, TNF receptor associated factor; *TRANCE*(R), TNF related activation induced cytokine (receptor).



**Figure 3** Biological effects of lymphotoxin (LT) $\alpha_1\beta_2$ -LT $\beta$ R interaction. LT $\alpha_1\beta_2$  heterodimers expressed on CD3<sup>-</sup>CD4<sup>+</sup>CD45<sup>+</sup> cells (lymph node (LN) and Peyer’s patch (PP) development) or on B cells (lamina propria (Lp) B cell development), T cells, and natural killer cells interact with LT $\beta$ R expressed on mesenchymal cells (LN and PP development) or Lp stroma cells (Lp B cell development) and induce signalling, as indicated (+ sign). Expression of LT $\alpha_1\beta_2$  on CD3<sup>-</sup>CD4<sup>+</sup>CD45<sup>+</sup> cells is upregulated by ligation of the interleukin 7 receptor (IL-7R) with interleukin 7 (IL-7). LT $\beta$ R mediated signalling is induced (as indicated by a + sign) by ligation of LT $\beta$ R with agonistic antibody (mAb) AF.H6 and inhibited by soluble LT $\beta$ R- IgG fusion protein (IgG) (as indicated by a - (minus) sign). The tumour necrosis factor family cytokine LIGHT is a transmembrane molecule expressed on T cells which binds to LT $\beta$ R. LIGHT signalling through LT $\beta$ R is not required for PP formation but contributes to MLN formation in a LT $\alpha_1$ LT $\beta_2$  dependent fashion. Black arrows indicate effects of LT $\beta$ R mediated signalling on gut associated lymphoid tissue development and intestinal inflammation. Ligation of LT $\beta$ R is critical for PP and LN development, formation of small intestine isolated lymphoid follicles (ILF), and recruitment of B cells into the Lp compartment. LT $\beta$ R mediated signalling is involved in the regulation of intestinal inflammation as LT $\beta$ RIgG treatment abrogated transfer colitis.

blockade or stimulation of LT $\beta$ R on the formation of GALT organs.

Interactions between LT $\alpha_1\beta_2$  and LT $\beta$ R are critical for recruitment of B cells into the Lp. There is dramatically reduced IgA secretion in LT $\alpha$ <sup>-/-</sup>,<sup>19, 20</sup> LT $\beta$ <sup>-/-</sup>,<sup>26</sup> and LT $\beta$ R<sup>-/-</sup><sup>25</sup> mice. Reconstitution of irradiated LT $\alpha$ <sup>-/-</sup> mice with wild-type bone marrow (BM) restored IgA secretion in BM chimera while PP/LN defects persisted.<sup>27, 28</sup> Thus PP and MLN are not required for IgA secretion. Post-gestational inhibition of LT $\alpha_1\beta_2$ -LT $\beta$ R interactions inhibits migration of B cells into the Lp.<sup>28</sup> Conversely, reconstitution of irradiated LT $\alpha$ <sup>-/-</sup> mice with wild-type or RAG2<sup>-/-</sup> BM restores the Lp B cell population<sup>28</sup> and IgA secretion.<sup>27</sup> LT $\beta$ R expression on Lp stromal cells is crucial for restoration of B cell recruitment and IgA production. Expression of LT $\alpha_1\beta_2$  on B cells contributes to PP formation as inhibition of B cell maturation in  $\mu$ MT mice impairs the development of PP.<sup>29</sup>

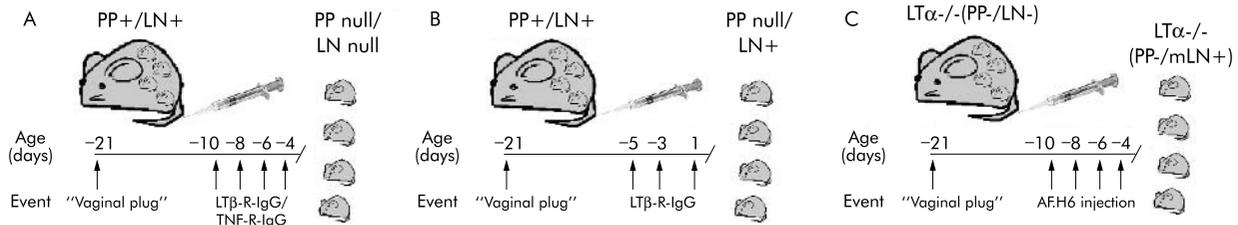
Signalling through the TNF-R-I also contributes to PP development as TNF-R-I (55)<sup>-/-</sup> mice develop small PP.<sup>30, 31</sup>

ILFs are small intestinal lymphoid follicles consisting of T and B cell areas and an overlying FAE containing M cells.<sup>2, 32</sup> The role of these follicles in intestinal immune regulation are as yet unknown. In the human small intestine, there are also isolated lymphoid structures with an epithelium resembling the FAE, MHC class II<sup>+</sup> dendritic cells (DC), memory T cells, and B cells but no immature lymphocytes.<sup>1</sup> Human inflammatory bowel disease is associated with induction of secondary lymphoid follicles in the colon<sup>8</sup> resembling ILFs. In ulcerative colitis (UC) there is lymphoid follicular hyperplasia of so-called basal lymphoid aggregates.<sup>33</sup> These follicles are characterised by abnormal follicular architecture

and unusual immunophenotypes with an increased proportion of apoptosis resistant cells and CD4<sup>-</sup>CD8<sup>-</sup>  $\gamma\delta$  T cell receptor  $\alpha$  (TCR)<sup>+</sup> cells. Lorenz *et al* demonstrated that the formation of ILFs depends on interactions between LT $\alpha_1\beta_2$  and LT $\beta$ R on non-BM derived cells and intact TNF-RI function.<sup>3</sup>

**Other cytokines and chemokines involved in GALT development and maintenance**

In addition to LT $\alpha_1\beta_2$  and IL-7, LIGHT, a second ligand of the LT $\beta$ R, also contributes to GALT organ formation.<sup>34</sup> CXC chemokine ligand 13 (CXCL13, B lymphocyte attractant, BCL) and its ligand, CX chemokine receptor 5 (CXCR5), control the entry of B cells into LNs.<sup>35-39</sup> T cell recruitment into lymphoid tissues is also controlled by CC chemokine receptor 7 (CCR7)-CC chemokine ligand 19 (CCL19) interactions. Another important chemokine is macrophage inflammatory protein 3 $\alpha$  (CCL20) which is expressed by the FAE and can be induced under inflammatory conditions. CCL20 is the only known chemokine ligand for CCR6. CCL20 has chemotactic activity for B and T cell subpopulations and myeloid DCs that bear CCR6.<sup>40</sup> The highly specific interaction of CCL20 and CCR6 and the predominant expression of CCL20 in the FAE suggest that this receptor-ligand pair may be involved in maintenance of organised MALT. Indeed, we recently observed morphological abnormalities in PP from CCR6<sup>-/-</sup> mice and a marked reduction in M cells in these mice (own unpublished observations). Most recently it was shown that regulated IEL development depends on CCR6 expression in lymphoid precursors in intestinal cryptopatches.<sup>41</sup>



**Figure 4** Effect of gestational treatment with soluble cytokine receptor fusion proteins (tumour necrosis factor receptor (TNF-R)-I (55 kDa)), lymphotoxin  $\beta$  receptor-IgG fusion protein (LT $\beta$ R-IgG), and agonist anti-LT $\beta$ R antibody treatment on the formation of gut associated lymphoid tissue tissues. (A) Combined inhibition of TNF-R-I- and LT $\beta$ R signalling by gestational intravenous treatment of TNF-R-I (55 kDa) and LT $\beta$ R-IgG, 10–4 days prior to birth, abrogates formation of Peyer's patches (PP) and most lymph nodes (LNs) (PP null/LN null) in progeny of treated mothers. (B) Selective abrogation of PP formation (PP null/LN+) in wild-type mice is mediated by injection of mice with LT $\beta$ R-IgG 5–3 days prior to birth and within 24 hours after birth. (C) Formation of mesenteric lymph nodes (mLN) but not of PP (PP-/mLN+) is induced in progeny of PP and LN deficient LT $\alpha$ -/- mice by gestational treatment with agonist anti-LT $\beta$ R antibody AF.H6 on days 10–4 prior to birth.

Additional genes and cytokines/chemokines relevant for the organisation of secondary lymphoid tissues are summarised in table 1.

In summary, gestational development of organised GALT organs is predominantly controlled by TNF family cytokines and IL-7 under a tight time frame. Yet poorly defined factors contribute to the increase in PPs after birth. ILF development and Lp B cell recruitment while being regulated by the same factors develop after birth, suggesting a higher priority of MLNs and PPs than ILFs and Lp B cells in the evolutionary schedule. While LT $\alpha$ ,  $\beta_2$ -LT $\beta$ R and IL-7 are major regulators for PP development and important contributors to MLN formation, TRANCE and LIGHT are dispensable for GALT organ formation. Homing of T and B cells to PPs and MLNs is controlled by CCR7-CCL19 and CXCL13-CXCR5 interactions.

### PROFESSIONAL APCs IN ORGANISED GALT ORGANS

Both PP and MLN consist of T and B cell areas which are infiltrated by distinctive sets of DCs. In general, DCs are distinguished as myeloid CD8 $\alpha$ <sup>-</sup>CD11b<sup>+</sup> and lymphoid CD8 $\alpha$ <sup>+</sup>CD11b<sup>-</sup>. PP contain a unique set of CD8 $\alpha$ <sup>-</sup>CD11b<sup>-</sup> DC. CD8 $\alpha$ <sup>-</sup>CD11b<sup>-</sup> DC and CD8 $\alpha$ <sup>-</sup>CD11b<sup>+</sup> cells are present in the dome of PP.<sup>42–44</sup> CD8 $\alpha$ <sup>-</sup>CD11b<sup>+</sup> PP DC predominantly secrete IL-10. Ligand of PP DC with receptor activator of nuclear factor  $\kappa$ B (NF $\kappa$ B) (RANK) induces IL-10 secretion while splenic DC respond with IL-12 secretion.<sup>45</sup> Similarly, PP DC are prone to induce T helper (TH2) responses in antigen specific T cells and receptor activator of NF $\kappa$ B ligand (RANK-L) stimulation enhances OT.<sup>44</sup> MLN DCs most likely migrate from mucosal surfaces to the MLN. Secretion of IL-10 and transforming growth factor  $\beta$  (TGF- $\beta$ ) by MLN DC and stimulation of antigen specific CD4<sup>+</sup> T cells for IL-10 and TGF- $\beta$  have recently been demonstrated in mice following oral antigen.<sup>46</sup> <sup>47</sup>

### THE ROLE OF GALT ORGANS IN INTESTINAL INFLAMMATION: IBD IS FOCUSED AT SITES OF HIGHLY CONCENTRATED LYMPHOID TISSUE

#### Appendix in intestinal inflammation

There is a striking focus of intestinal inflammation in the ileocaecal region, a site with highly concentrated aggregation

- ▶ Gestational development of organised GALT organs is predominantly controlled by TNF family cytokines and IL-7 under a tight time frame.

of secondary lymphoid tissue. In humans, ileocaecal colitis is common in IBD, such as Crohn's disease (CD) or backwash ileitis in UC. However, infectious agents such as *Yersinia enterocolitica* and *Mycobacteria* also cause pseudoappendicitis and mesenteric lymphadenitis.<sup>48</sup> <sup>49</sup> The terminal ileum is also a preferential site of inflammation in several animal models of IBD.<sup>50</sup> <sup>51</sup> Circumstantial evidence indicates that the appendix plays a pivotal role in the development of CD and UC. There is a negative association between appendectomy and the onset and severity of these two diseases.<sup>52</sup> <sup>53</sup> Appendiceal inflammation is observed in half of colectomy specimens from patients with UC. The appendiceal epithelium shows intense upregulation of HLA class II molecules and activation of macrophages<sup>54</sup> in specimens from UC, but not in acute appendicitis. Thus the appendix might serve as a priming site for UC.

T cell receptor  $\alpha$  chain gene deficient (TCR $\alpha$ -/-) mice develop spontaneous colitis which is similar to human UC.<sup>55</sup> Cells in the appendix associated lymphoid follicle (ALF) proliferate and B cells secrete autoantibodies against tropomyosin.<sup>55</sup> In humans, tropomyosin might also act as an autoantigen. In UC, there is also increased secretion of antibodies against tropomyosin.<sup>56</sup> In TCR $\alpha$ -/- mice there was more B cell proliferation and a higher level of autoantibody production in ALF tissue than in PP tissue.<sup>55</sup> Appendectomy at a young age inhibited the development of colitis in TCR $\alpha$ -/- mice and diminished the number of cells in MLN.<sup>55</sup>

There is severe colitis in both IL-2-/- and IL-2R $\alpha$  chain-/- mice, which is similar to human UC.<sup>57</sup> <sup>58</sup> Half of all IL-2-/- mice die within the first nine weeks after birth after developing splenomegaly, lymphadenopathy, and autoimmune anaemia. One hundred per cent of surviving animals develop pancolitis with thickening of the entire colon during the next 6–15 weeks. Similar to colitis in TCR $\alpha$ -/- mice, there are autoantibodies which are directed against colonic antigens.

Lymphoid hyperplasia is a feature of chronic dextran sodium sulphate (DSS) induced colitis.<sup>59</sup> The severity of acute DSS induced colitis was reduced in mice which had undergone appendectomy prior to disease induction.<sup>60</sup> Thus two different animal disease models provide consistent data indicating that the appendix might play a pivotal role in the induction of experimental IBD.

Table 2 summarises studies on the role of GALT organs in experimental intestinal inflammation.

► There is a striking focus of intestinal inflammation in the ileocaecal region, a site with highly concentrated aggregation of secondary lymphoid tissue.

**Role of  $LT\alpha_1\beta_2$ - $LT\beta R$  interactions, lymphoid follicles, PP, and MLN in the development and outcome of murine colitis models: blockade of  $LT\beta R$  signalling can abrogate experimental colitis**

Experimental colitis induced by transfer of TH1-type CD45RB<sup>hi</sup> cells or reconstitution of Tg<sub>e</sub>26 mice with wild-type BM is attenuated by  $LT\beta R$ IgG treatment during disease induction.<sup>61</sup> The effect of  $LT\beta R$ IgG treatment on these two models of TH1 mediated colitis was similar to that of anti-TNF treatment. As a consequence of this study, anti- $LT\beta$  directed treatment of CD is currently being investigated.

A more recent study reported the course of TNBS colitis in mice made deficient of PP and colonic lymphoid follicles secondary to gestational inhibition of  $LT\alpha_1\beta_2$ - $LT\beta R$  interactions.<sup>62</sup> Mice deficient in colonic patches developed focal ulcers with TH1-type responses whereas lesions in normal mice were of a diffuse mucosal type with both TH1- and TH2-type cytokine production.<sup>62</sup> Thus colonic lymphoid follicles and PP provide help to control intestinal inflammation in trinitrobenzene sulphonic acid (TNBS) induced colitis.

Treatment of adult mice with  $LT\beta R$ IgG prior to disease induction and following the TNBS enema was associated with loss of PP and a reduction in DCs in the colon. Interferon  $\gamma$ -/- mice undergoing such treatment were protected from TNBS colitis. Peri-inductional treatment of wild-type mice with  $LT\beta R$ IgG induced hypertrophy of colonic patches following the TNBS enema. Thus TH2-type colitis is dependent on the presence of colonic patches or of  $LT\alpha_1\beta_2$ - $LT\beta R$  interactions.

Thus both TH1-type and TH2-type cytokine mediated types of experimental colitis can be controlled by inhibition of the

$LT\alpha_1\beta_2$ - $LT\beta R$  pathway although the mechanism may vary in different disease models.

Data currently available indicate that inhibition of  $LT\beta R$  signalling suppresses intestinal inflammation by altering the local intestinal and splenic lymphoid microarchitecture. There are fewer DCs in colonic follicles and in the spleen following  $LT\beta R$ IgG treatment.<sup>62-63</sup> Inhibition of  $LT\beta R$  signalling reduces the number and size of follicular DCs, germinal centres, and marginal zones in colonic follicles, all of which are important to mount full scale B cell responses.<sup>62</sup> Figure 5 provides an overview of the known and potential mechanisms modulating intestinal inflammation by inhibition of  $LT\beta R$  signalling.

We have recently studied acute DSS induced colitis in mice without PP and in mice lacking PP and MLN.<sup>64</sup> We found more severe colitis in mice deficient in PP and LN (PP/LN null), in both  $LT\alpha$ -/- mice and in PP/LN null mice secondary to gestational treatment with  $LT\beta R$ IgG and TNFR1gG.<sup>64</sup> There was hyperplasia and induction of colonic lymphoid follicles in  $LT\alpha$ -/- mice and PP/LN null mice, suggesting an LT independent pathway of formation of colonic lymphoid tissue induced during colonic inflammation. Colonic lymphoid patches observed in  $LT\alpha$ -/- mice showed ill defined T and B cell areas while colonic follicles of wild-type PP/LN null mice showed distinct T and B cell zones in the colonic follicles, predominantly consisting of B cells. Clinically, DSS induced colitis was similar in wild-type and mice without PP but with MLN (PP null/LN+ mice), although there was also induction of colonic lymphoid tissue in PP null/LN+ mice. Thus PPs alone do not regulate DSS induced colitis whereas the presence of MLN is critical in this regard. The differences observed using mice with GALT defects are most likely due to the different animal models and strains used.

M cells are specialised epithelial cells of the FAE of PP. M cells have a high capacity for transcytosis of microorganisms

**Table 2** Studies using animal models with mice deficient of organised gut associated lymphoid tissue (GALT) organs and/or lymphotoxin  $\alpha/\beta$  pathway

Animal model	GALT organ defect	Outcome in course of intestinal inflammation	Reference
Spontaneous colitis in TCR $\alpha$ -/- mice	Appendix/ALF	Appendectomy protects against disease if performed at a young age	55
Interleukin-2/- mice, interleukin-2 R $\alpha$ chain/- mice	Lymph node enlargement	Development of colitis similar to UC. Anti-colon antibodies, T and B cell activation	57-58
TNBS colitis in wild-type mice	PP/colonic follicles*	Focal ulcers, TH1 response instead of TH1/TH2 response	62
TNBS colitis in $\gamma$ -IFN/- mice	$LT\beta R$ IgG treatment†	Loss of PP, reduction of DC in the colon, decreased disease severity in TH2-type colitis	62
Oxazolone colitis	Wild-type mice	Inflammation of distal colon, mucosal inflammation at early stage (48 h), transmural inflammation at late stage (7 days)	103-104
CD45RB <sup>hi</sup> T cell transfer colitis	$LT\beta R$ IgG treatment†	Decreased disease severity	61
Tg <sub>e</sub> 26 T cell transfer colitis	$LT\beta R$ IgG treatment†	Decreased disease severity	61
DSS induced colitis	Appendectomy	Decreased disease severity	60
DSS induced colitis	$LT\alpha$ -/- mice w/o‡ PP/MLN	Increased disease severity. Induction of colonic lymphoid tissue	64
DSS induced colitis	Wild-type mice w/o‡ PP/MLN	Increased disease severity. Induction of colonic lymphoid tissue	64
DSS induced colitis	Wild-type mice w/o‡ PP with MLN	No effect on disease severity	64
<i>Citrobacter rodentium</i> induced infectious colitis	TNF-R-1 (55 kDa) gene deficient mice with small PP	Increased intestinal secretion of infectious agent clearance of bacteria and survival similar to wild-type mice	105

ALF, appendix associated lymphoid follicle; DC, dendritic cell; DSS, dextran sodium sulphate;  $LT\alpha/\beta$ , -R, lymphotoxin  $\alpha/\beta$ , -receptor;  $LT\beta R$ IgG, lymphotoxin  $\beta$  receptor-IgG fusion protein; MLN, mesenteric lymph node; PP, Peyer's patch; TH, T helper; TNBS, trinitrobenzene sulphonic acid; TNF-R, tumour necrosis factor receptor; UC, ulcerative colitis.

\*Mice were from a litter of mice which had been treated during gestation with  $LT\beta R$ IgG.

†Mice were treated at adult age.

‡w/o, without; induction of PP/MLN defect by gestational treatment with  $LT\beta R$ IgG/TNFR1-IgG.

► Data currently available indicate that inhibition of LT $\beta$ R signalling suppresses intestinal inflammation by altering the local intestinal and splenic lymphoid microarchitecture.

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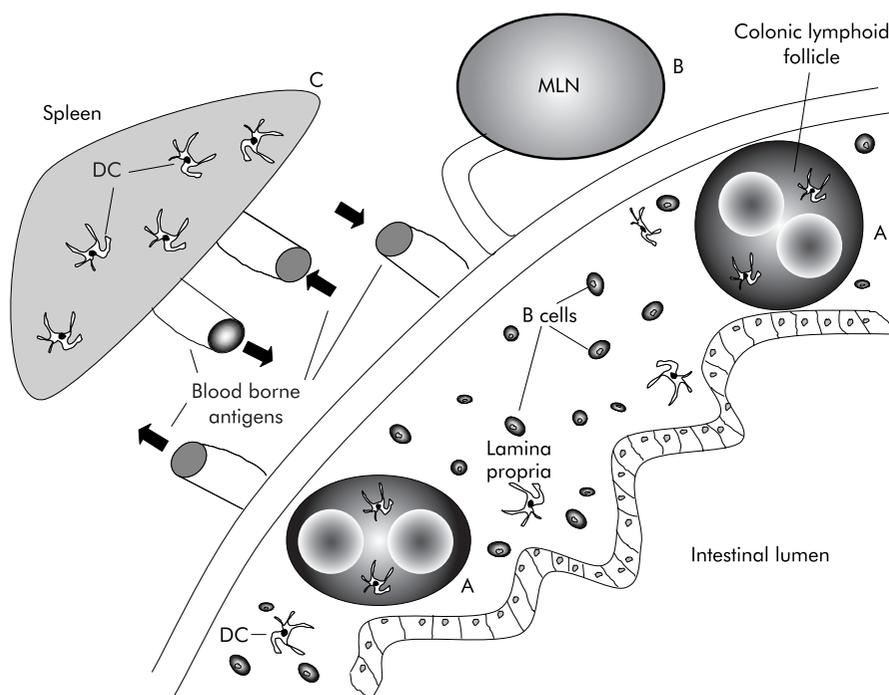
and macromolecules and serve as an antigen sampling system (reviewed by Kucharzik and colleagues<sup>65</sup>). Experimental ileitis in the rat induces the formation of M cells, which subsequently undergo apoptosis.<sup>66</sup>

### THE ROLE OF LYMPHOTOXIN AND GALT ORGANS IN INTESTINAL IMMUNE TOLERANCE: MLN ARE CRITICAL AND PP MIGHT BE REDUNDANT FOR ORAL TOLERANCE

Immune tolerance is defined as induction of "any mechanism by which a potentially injurious immune response is prevented, suppressed, or shifted to a non-injurious class of immune response".<sup>67</sup> OT is a mechanism of tolerance induction, and OT has been demonstrated by the specific suppression of cellular and/or humoral immune responses to an antigen by prior administration of the antigen by the oral route. OT probably serves as a mechanism to prevent the development of adverse immune reactions against intestinal and nutritional antigens. The site/s of intestinal antigen presentation for OT induction is still not clearly defined. Table 3 summarises studies on the role of GALT organs in the induction of OT. IECs are the frontier of antigen contact and present antigen in vitro to CD8<sup>+</sup> T cells.<sup>68-69</sup> Such IEC activated CD8<sup>+</sup> T cells have regulatory properties<sup>70</sup> but their in vivo function for OT remain obscure.  $\gamma\delta$  TCR<sup>+</sup> IEL play a

role in induction of OT as TCR  $\delta$  chain gene deficient mice and mice treated with anti- $\gamma\delta$ -TCR antibodies have a defect in induction of OT.<sup>71-73</sup>  $\mu$ MT mice are deficient in mucosal B cells and can be orally tolerated.<sup>46</sup> LT $\beta$ <sup>-/-</sup>, LT $\alpha$ <sup>+/-</sup>/LT $\beta$ <sup>+/-</sup>, and  $\mu$ MT mice deficient in PP can be orally tolerated.<sup>46-74</sup> Thus the absence of LT $\beta$  itself does not abrogate induction of OT. Surgical removal of PP in rats failed to abrogate OT.<sup>75</sup> Gestational treatment of mice with LT $\beta$ RiGg renders progeny of treated mice deficient in PP. If such PP null mice were generated by intravenous treatment on gestational days 14 and 17, tolerance induction was abrogated.<sup>76</sup> It is of note that such treatment similarly abrogated suppression of delayed type hypersensitivity, proliferative, total IgG responses, and TH1- and TH2-type responses against OVA, suggesting induction of a TH0-type immune response. OT to haptens was not abrogated in this study. However, in PP null mice generated by intravenous LT $\beta$ RiGg treatment on days 16 and 18, OT was intact.<sup>74-77</sup> The different observations indicate that the time course of gestational LT $\beta$ RiGg treatment is critical to regulate different, yet poorly defined, pathways of intestinal immune responses, resulting in maintenance or loss of OT. Lp and/or MLN DCs which are prone to prime for regulatory T cells might be one potential target of early gestational LT $\beta$  inhibition. Postpartal treatment with LT $\beta$ RiGg has been shown to inhibit colonic FDC and DC development.<sup>62</sup> Further work will be necessary to address these unresolved questions.

LT $\alpha$ <sup>-/-</sup> and TNF/LT $\alpha$ <sup>-/-</sup> mice without PP and MLN and Balb/c mice without PP and MLN (PP/LN null mice) secondary to gestational treatment of pregnant mice with LT $\beta$ RiGg and TNFRiGg are resistant to induction of OT.<sup>74-77</sup>



**Figure 5** Known and potential mechanisms of systemic inhibition of lymphotoxin receptor (LT $\beta$ R) signal transduction resulting in control of colitis. (A) Loss of follicular dendritic cells (DC), B cell follicles, and DC content in colonic lymphoid follicles results in down modulation of local intestinal immune response. Lamina propria (Lp) B cells, T cells, and natural killer cells expressing LT $\alpha$ <sub>1</sub> $\beta$ 2 are inhibited in their contact with LT $\beta$ R expressing Lp stromal cells and might thus be dysfunctional. (B) Loss of follicular DC, B cell follicles, and DC content in mesenteric lymph nodes (MLN) is possible following lymphotoxin  $\beta$  receptor-IgG fusion protein (LT $\beta$ RiGg) treatment and might result in immunosuppression. (C) Blood borne intestinal antigens are carried to the spleen. LT $\beta$ RiGg treatment diminishes the content of splenic DC and disturbs splenic architecture resulting in loss of ability to present antigen (bacterial antigens, T cell independent antigen type II).

**Table 3** Studies investigating the role of gut associated lymphoid tissue (GALT) organs in induction of intestinal tolerance and immunity

Animals used	GALT organ defect*	Defect in other lymphoid organs	Effect on oral tolerance/immunisation	Reference
$\gamma\delta$ T cells depleted TCR- $\delta$ -chain-/-	IEL w/o $\gamma\delta$ T cells IEL w/o $\gamma\delta$ T cells	General down modulation of $\gamma\delta$ TCR General absence of $\gamma\delta$ T cells	OT abrogated Low dose OT abrogated, high dose OT intact	71 72 73
$\mu$ MT mice TNF $\alpha$ -/- PP null rats PP null/MLN+ mice‡	Lack of PP, lack of Lp B cells Small PP Surgical removal of PP Lack of PP	General absence of B cells Spleen†	OT intact OT intact OT intact OT to hapten intact OT to low and high dose OVA intact OT to OVA abrogated Oral immunisation with CT/OVA intact	46 74 75 76 74 77 76 106
PP/MLN null mice§ LT $\beta$ -/-	Lack of PP and MLN Lack of PP, MLN present. Lack of Lp B cells	Lack of all LN Absence of peripheral LN cells	OT abrogated OT intact	77 74
LT $\alpha$ +/-LT $\beta$ +/- LT $\alpha$ -/-/TNF $\alpha$ / LT $\alpha$ -/-	Lack of PP, MLN present PP-, MLN-, lack of Lp B cells	Absence of peripheral LN Spleen¶, absence of all LN	OT intact OT abrogated	74 74
LT $\alpha$ -/- w MLN reconstituted**	PP-, MLN +	Spleen¶, absence of all LN except MLN	OT restored	77

CT, cholera toxin; MLN, mesenteric lymph node; Lp, lamina propria; LT $\alpha$ / $\beta$ , -R, lymphotoxin  $\alpha$ / $\beta$ , -receptor; OT, oral tolerance; PP, Peyer's patch; TCR $\alpha$ -/-, T cell receptor  $\alpha$  chain gene deficient mice; TNF, tumour necrosis factor

\*w, with; w/o, without.

†Absence of marginal zones.

‡Induction of PP defect by gestational treatment with LT $\beta$ RlgG.

§Induction of PP/MLN defect by gestational treatment with LT $\beta$ RlgG and TNFRlgG.

¶Absence of primary B cell follicles, marginal zones, germinal centre, follicular dendritic cells.

\*\*Reconstitution of MLN by gestational treatment of LT $\alpha$ -/- mice with agonistic anti-LT $\beta$ R-antibodies.

However, peripheral immune tolerance could be induced by the intraperitoneal route in all PP/LN deficient mice used in these studies, suggesting that the defect in OT is situated at the GALT. Gestational signalling at the LT $\beta$ R by treatment with agonistic anti-LT $\beta$ R antibody AF.H6 induces formation of MLN in LT $\alpha$ -/- mice without inducing PP formation. OT was restored in progeny of AF.H6 treated LT $\alpha$ -/- mice, suggesting that the lack of MLN but not of PP and of the cytokine LT $\alpha$  is critical for OT induction.<sup>77</sup>

Intestinal DCs might play a pivotal role in induction of OT. We assume that intraluminal antigens that pass the intestinal epithelial barrier are taken up by DCs and then migrate through draining lymph vessels to MLN, where they present antigen to T cells. Alpan and colleagues<sup>46</sup> reported priming of naive T cells for TH2 immune responses by MLN derived DC. Oral antigen was administered to in vivo pulsed MLN derived DC from mice with and without PP. In contrast, DC from popliteal LN failed to prime for TH2 T cell responses following subcutaneous footpad antigen administration. Rescigno and colleagues<sup>78</sup> reported that DC penetrate gut epithelial monolayers to sample bacteria and might thus provide an M cell independent pathway for intestinal antigen uptake and presentation. It is possible that the lack of OT observed in mice without PP and MLN<sup>74-77</sup> is also related to a LT $\beta$ R dependent block of recruitment of CD4<sup>+</sup>CD3<sup>-</sup> inducer cells, DC, or other cells to the MLN anlage or the Lp compartment, which are required for intestinal tolerance induction.

More recent studies by Blumberg (personal communication) indicate a third pathway of antigen transport through the intestinal epithelial cell barrier using the intestinal neonatal Fc receptor (FcRn) as a shuttle service. Antigen

bound to IgG can be transported through IECs from the basolateral to the apical side and vice versa. Luminal antigen transported to the basolateral side of IECs primes MLN and hepatic APCs to induce antigen specific T cell responses.

**SUMMARY: A MODEL FOR GALT ORGANS IN INTESTINAL INFLAMMATION AND TOLERANCE**

Recent studies have shed new light on the mechanisms underlying the development of the organised GALT. TNF family cytokine members are among the master regulators of intestinal lymphoid development. Gestational LT $\alpha$ ,LT $\beta$ <sub>2</sub>-LT $\beta$ R interactions are critical for the development of PP whereas blockade of the LT $\alpha$ ,LT $\beta$ <sub>2</sub>-LT $\beta$ R pathway in adolescence is pivotal for Lp B cell development.

GALT organs are the intestinal branch of the organised lymphoid system. PP are sites of IgA production and thus contribute to local and systemic immunity against intestinal antigens. PPs with the FAE and M cells provide a pathway for uptake and processing of particulate antigens. As tolerance to soluble antigens could be induced in various gene deficient mice without PP and with MLN, PP are probably redundant for induction of OT. More likely soluble antigen is predominantly taken up by IECs, interdigitating Lp DCs and the FcRn to shuttle through the intestinal barrier. Antigen primed Lp DCs then migrate through the intestinal lymph vessels to MLNs. Antigen presentation in MLNs causes tolerance under physiological conditions. Tolerance is mediated by the induction of regulatory TH3-type<sup>79</sup> cells and T<sub>reg</sub> cells,<sup>80</sup> probably by secreting TGF- $\beta$  and IL-10. Different mechanisms involved in tolerance induction include anergy and deletion of antigen specific cells. Intestinal inflammation is associated with loss of the immunological balance between pro- and anti-inflammatory

► OT probably serves as a mechanism to prevent the development of adverse immune reactions against intestinal and nutritional antigens.

► Intestinal DCs might play a pivotal role in induction of OT.

- GALT organs are the intestinal branch of the organised lymphoid system.

cytokines. Interestingly, both TH1-type and TH2-type cytokines are potent mediators of intestinal inflammation, as indicated by the TNBS and oxazolone models of colitis. There is evidence for control of both inflammatory conditions by TGF- $\beta$ . The change in the intestinal cytokine milieu in IBD contributes to induction of inflammatory T cell responses by Lp DC cells migrating to the MLN. We assume that MLNs serve as a site where DCs prime T cells for anti-inflammatory T cell responses as the course of acute DSS colitis was more severe in mice without MLNs than in those with MLNs. Human IBD and experimental colitis in mice are associated with immune activation in all GALT organs. Similar to MLNs, the appendix and the ALF seem to be critical for the development of both IBD and experimental colitis in animals as appendectomy attenuates both conditions.

Inhibition of LT $\alpha_1\beta_2$ -LT $\beta$ R signalling in TH1 and TH2 mediated experimental colitis suppresses intestinal inflammation by mechanisms yet poorly defined.

Future studies are necessary to address the mechanisms by which intestinal inflammation is down modulated following post-gestational inhibition of the LT $\alpha_1\beta_2$ -LT $\beta$ R pathway.

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This review is dedicated to Professor Wolfram Domschke on the occasion of his 60th birthday.

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## Authors' affiliations

T W Spahn, T Kucharzik, Department of Medicine B, Münster University Hospital, 48149 Münster, Germany

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