SCIENCE ALERT

Peyer's patch organogenesis—cytokines rule, OK?

Pasparakis M, Alexopoulou L, Grell M, Pfizenmaier K, Bluethmann H, Kollias G. Peyer's patch organogenesis is intact yet formation of B lymphocyte follicles is defective in peripheral lymphoid organs of mice deficient for tumor necrosis factor and 55-kDa receptor. *Proc Natl Acad Sci USA* 1997; **94:** 6319–23.

Abstract

Targeted inactivation of genes in the tumor necrosis factor (TNF)/lymphotoxin (LT) ligand and receptor system has recently revealed essential roles forthese molecules in lymphoid tissue development and organization. Lymphotoxin-αβ (LTαβ)/lymphotoxin-β receptor (LTβ-R) signaling is critical for the organogenesis of lymph nodes and Peyer's patches and for the structural compartmentalization of the splenic white pulp into distinct B and T cell areas and marginal zones. Moreover, an essential role has been demonstrated for TNF/p55 tumor necrosis factor receptor (p55TNF-R) signaling in the formation of splenic B lymphocyte follicles, follicular dendritic cell networks, and germinal centers. In contrast to a previously described essential role for the p55TNF-R in Peyer's patch organogenesis, we show in this report that Peyer's patches are present in both TNF and p55TNF-R knockout mice, demonstrating that these molecules are not essential for the organogenesis of this lymphoid organ. Furthermore, we show that in the absence of TNF/p55TNF-R signaling, lymphocytes segregate normally into T and B cell areas and a normal content and localization of dendritic cells is observed in both lymph nodes and Peyer's patches. However, although B cells are found to home normally within Peyer's patches and in the outer cortex area of lymph nodes, organized follicular structures and follicular dendritic cell networks fail to form. These results show that in contrast to LTαβ signaling, TNF signaling through the p55TNF-R is not essential for lymphoid organogenesis but rather for interactions that determine the cellular and structural organization of B cell follicles in all secondary lymphoid tissues.

Comment

It seems that wherever one turns in mammalian biology, from conception through normal development to death, cytokines of immunological interest have become inserted as important regulators of tissue activity. Or have they? The truth is that at various stages the evolving immune system has borrowed widely to turn existing regulatory and effector molecules to new advantages.

The tumour necrosis factor (TNF) and TNF receptor (TNFR) families

The TNF and TNFR families of molecules[1–3] consist of the ligands TNF, lymphotoxins alpha and beta (LT-α, LT-β), nerve growth factor (NGF), Fas ligand (FasL), CD27 ligand, CD30 ligand, CD40 ligand, OX40 (CD134) ligand, and 4–1BB ligand and their receptors (which, in most cases, are ligand specific). Of the ligands, TNF, LT-α and NGF are secreted products, which in each ease associate to form biologically active homotrimers (LT-α_3 in the case of LT-α). Both TNF and LT-α_3 are approximately equally favoured ligands for two receptors, TNFR I (55 kD) and TNFR II (75 kD). LT-β is a membrane protein that is not secreted and has not been observed as a homotrimer. Expression of LT-β on the cell surface seems to require co-expression of LT-α and their association in the heterotrimers LT-$\alpha_2\beta_1$ (minority) or LT-$\alpha_1\beta_2$ (majority). LT-$\alpha_2\beta_1$ may signal via either of the TNF receptors but LT-$\alpha_1\beta_2$ signals via a specific receptor (LTβ-R; also known as TNFR related protein, $TNFR_{rp}$).

The TNF ligand family are, in general, inducers of either cell proliferation or cell death and the receptors for some are expressed by a wide range of tissues. It would not, therefore, have been surprising if loss of function in ligand/receptor pairs in this family by natural mutations or through gene targeting had caused significant defects in general embryonic morphogenesis. In fact, this has not been the case, even when a double knockout of the genes encoding TNF-α and LT-α has prevented all signalling by these cytokines or by LT-β (table 1). Nevertheless, loss of function in genes encoding the ligand/receptor pairs FasL/Fas; TNF-α, LT-α/TNFR I and LT-α,β/LTβ-R is accompanied by morphological abnormalities that are limited to the lymphoid tissues. In the cases of TNF-α and LT, it is uncertain whether the morphological changes are primarily defects in morphogenesis or whether they are secondary to functional deficiencies in the behaviour of various differentiated cells of the lymphoid system (as seems to be the case in natural mutations of either Fas or FasL).

Organogenesis of Peyer's patches in mice lacking TNF-α or the TNFR I

A recent publication by Pasparakis and colleagues examines the gross anatomical, histological and cellular abnormalities that follow targeted disruption of the genes encoding TNF-α and one of its receptors, TNFR I (p55TNF-R). From the foregoing discussion (table 1) it can be seen that TNF-$\alpha^{-/-}$ and TNFR $I^{-/-}$ mice need not exhibit equivalent phenotype because the TNFR I also serves LT-α. Both homozygous knockout mutants (on mixed 129Sv X C57BL/6 background) had normal thymic morphology and function, grossly normal lymph nodes (LN) but had Peyer's patches (PP) that were reduced both in size and number. Peyer's patches were flat, they appear from the illustrations to lack subdivision into distinct follicles, while distortion of normal morphology (lack of clear follicle associated epithelium and organised patch associated villi) was more evident in the TNFR $I^{-/-}$ mice. Nevertheless, the PP contained both B and T cells and there was some segregation of T cells into small presumptive interfollicular areas. Dendritic cells were present within these concentrations of T cells and also within the rudimentary dome areas, an interesting observation in light

TABLE 1 *Predicted activities of TNF/LT ligands in gene knockout mice*

	Residual effector function					
Gene targeted	*TNF-α*[a]	*TNFα*$_3$[a]	*LT-α*$_2$*β*$_1$[b]	*LT-α*$_1$*β*$_1$[b]	*Peyer's patches*	*IgA*
TNFR I[c]	−	−	−	+	Number reduced (?) disorganised	Normal
TNF-α[d]	−	+	+	+	Number near normal, disorganised	Normal
LT-α[e]	+	−	−	−	Absent	Very low
Ltβ[f]	+	+	−	−	Absent	Very low
LTβ-R[g]	+	+	+	−	?	?
LT-α plus LT-β[h]	+	−	−	−	?	?
TNF-α plus LT-β[i]	−	+	−	−	?	?
TNF-α plus LT-α[j]	−	−	−	−	Absent	Almost undetectable

[a] Soluble active homotrimers. [b] Membrane bound active heterotrimers, LT-β$_3$ homotrimers have not been reported. [c] Pasparakis *et al*, Pfeffer *et al*,[7] Neumann *et al*,[8] Le Hir *et al*,[17] Matsumoto *et al*.[18] [d] Pasparakis *et al* (this paper),[5] Körner *et al*,[9] Matsumato *et al*.[18] [e] Banks *et al*,[13] Matsumoto *et al*,[19] De Togni *et al*.[20] [f] Koni *et al*.[14] [g] Not done. [h] Not done, same effect as LT-α knockout. [i] Not done. [j] Körner *et al*,[9] Eugster *et al*,[10] Rennert *et al*.[11]

of the importance of TNF-α in the maturation of dendritic cells and their migration to lymph nodes.[4] On the other hand, in both PP and LN, as well as in spleen,[5] there was a failure of B lymphocytes to organise into primary follicles, a failure of germinal centre development and an absence of follicular dendritic cell (FDC) networks within areas of B cells.

Pasparakis *et al* believe that the primary defect in all of the secondary lymphoid tissues in their mice lies downstream of primary morphogenesis and is related more to a role of TNFα/TNFR I in the migration/organisation of B cells to form follicular structures. In particular, they favour a key role for this ligand/receptor pair in the differentiation or organisation, or both, of FDC into a network around which the follicles can develop. If correct, it is a fine point as to whether such a defect is morphogenetic (ie, failure of mesenchyme to commit to the FDC lineage during embryogenesis) or functional (failure of FDC to respond to signalling through TNFR I) and it is relevant that FDC express TNFR I and also make TNF-α.[6] It is also legitimate to question whether a reduction in PP number does not, at face value, indicate at least a partial failure of morphogenesis. It is noteworthy that initial reports on PP phenotypes in TNF$^{-/-}$ mice[5] and TNFR I$^{-/-}$ mice have been revised.[7 8] After initial reports in which it was stated that PP were absent in both gene targeted mutants, it seems now that in each case, PP were in fact present although small and difficult to identify. This conveys a clear message that in any mutation that affects the development of PP, anything less than complete serial sections of the small intestine would fail to provide convincing evidence that small anlagen have not been missed and that PP morphogenetic foci are not present in normal numbers. It is not clear from the studies on TNF-α$^{-/-}$ or TNFR I$^{-/-}$ mice whether failure to signal via the TNFR I (by TNF and/or LT-α) reduces the number of anlagen (ie, is important for the initiation of the correct number of anlagen) or whether its role lies downstream and affects the subsequent development of those anlagen. Furthermore, the analysis to date is too crude to allow a conclusion that the defects in the TNF-α$^{-/-}$ and TNFR I$^{-/-}$ mice are equivalent and therefore, that LT-α$_3$ and LT-α$_1$β$_2$ produce no PP phenotype.

A recent study on an independent TNF-α$^{-/-}$ knockout, this time performed in C57BL/6 mice, is informative on three counts.[9] Firstly, at least in spleen, areas of B cells do contain scattered cells stained by monoclonal antibody FDC-M1, indicating that FDC indeed differentiate in TNF-α$^{-/-}$ mice and that their absence is not the primary defect in PP morphogenesis in these animals (their functional state is another matter). Secondly, in this mouse strain there was only a small difference in the numbers of PP between TNF-α$^{-/-}$ and wild type mice, although morphologically they resembled the structures described by Pasparakis *et al*. This observation suggests that TNF-α is not a primary morphogenetic stimulus for the development of PP. It raises a further question: do morphogenetic events lay down a template for coordinated epithelial differentiation (follicle associated and patch villus associated) and is this at least partially under the control of TNF-α, or does subsequent lymphoid development and function shape the epithelial architecture and is this the way that TNF-α exerts its effect on PP morphology? My own response to this question is to ask which is the most unique feature of a PP. To me, it is the follicle associated epithelium and by this reasoning, my speculation is that primary organisers in PP organogenesis will create focal fields of epithelial differentiation, which will in turn induce differentiation of underlying mesenchyme. Finally, Körner *et al*[9] observed that the PP "homing receptor" MadCAM was expressed by vessels in PP. This supports the contention of Pasparakis *et al* that failure of lymphocyte entry into PP is not responsible for the PP abnormalities in TNF-α$^{-/-}$ mice. Körner *et al*[9] conclude that early morphogenesis of PP is controlled mainly by LT (see later), whereas the role of TNF-α is related more to the organisation of lymphocytes within the edifice so created. It would be of interest to know whether PP morphology in TNF-α$^{-/-}$ mice could be restored to normal by adoptive transfer of recirculating mature lymphocytes (ie, whether the defect lies in the response of stromal components of the PP or of lymphocytes to signalling via TNF-α.)

The importance of lymphotoxins in organogenesis of Peyer's patches

A more severe phenotype would be expected by disruption of genes which impinge more globally on the functions of the TNF/LT family of ligands (table 1). Targeting the TNFR I gene (TNFR II does not seem to be relevant in the context of this discussion) can block the actions of TNF-α, LT-α$_3$ and LT-α$_1$β$_2$ but signalling can still occur via membrane bound LT-α$_1$β$_2$. Peyers patches in TNFR I$^{-/-}$ mice seem to be reduced in number[8] and they have more severe disturbance of architecture than those in TNF-α$^{-/-}$ mice (Pasparakis *et al*).[8] LT-α$_3$ and/or LT-α$_2$β$_1$ may, therefore, have a role in the formation of normal PP, but they do not seem to be the primary organisers of PP anlagen. If all membrane bound LT activity is abolished (LT-α$^{-/-}$ mice), there is a striking phenotype, with agenesis of PP and all LN, disrupted splenic white pulp architecture but normal thymic development. There seems no question but that membrane bound LT is vital in the organogenesis of secondary lymphoid organs, including PP. This conclusion is borne out (almost completely) by studies in LT-β$^{-/-}$ mice, where there is selective loss of membrane associated LT. These mice lack PP and most LN but they do have mesenteric LN and variable numbers of cervical LN (albeit

with abnormal structure). As might be expected, the coup-de-grâce (elimination of both TNF-α and LT-α) also leads to agenesis of PP and all LN.[9 10]

It is interesting that lymph nodes vary in their requirements for LT-$\alpha_1\beta_2$ to induce organogenesis. A fascinating addition to this story is the use of maternally administered LTβ-R-Ig fusion protein to block membrane bound LT at various stages of gestation within the fetus.[11] Administration of fusion protein as early as nine days' gestation did not inhibit morphogenesis of mesenteric LN, whereas development of other LN was blocked even if fusion protein treatment was delayed until day 12 (brachial LN) through to day 16 (popliteal LN). In contrast, morphogenesis of PP was prevented even when treatment was given on day 18 of gestation. These results correlate with the late maturation of the gut in rodents and the appearance of PP anlagen in the late prenatal period.[12] More needs to be done to examine the precise time at which genes that encode TNF-α, LT-α, LT-β, and their receptors are transcribed in the local mesenchyme that gives rise to LN and PP.

IgA production in gene targeted mice

Mice with selectively targeted genes encoding LT-α[13 14] or LT-β[14] or with combined knockouts of the genes encoding TNF-α and LT-α[9 10] provide a unique opportunity to assess the importance of PP for IgA production (although other sites, such as bronchus associated lymphoid tissue, could also be affected). LT-$\alpha^{-/-}$ and LT-$\beta^{-/-}$ mice have very low concentrations of IgA in serum and in faeces, although serum concentrations of IgM and IgG are relatively normal. Mesenteric LN, present in LT-$\beta^{-/-}$ mice but not in LT-$\alpha^{-/-}$ mice, do not seem to confer a greater capacity to produce IgA. The presence of normal concentrations of serum IgG in these mice suggests that the defect in IgA production results from agenesis of PP rather than to a more general failure to support isotype switching. In TNF-α/LT-$\alpha^{-/-}$ double knockout mice, serum IgA and mucosal IgA plasma cells are even more profoundly depressed and there is also reduction in serum IgG concentrations.[9 10] These findings may indicate that some switching to the IgA isotype can occur at other sites (eg, the spleen) in LT-$\alpha^{-/-}$ and LT-$\beta^{-/-}$ mice.

In contrast, the structural disorganisation in PP of TNF-$\alpha^{-/-}$ mice does not lead to reduction in serum IgA concentrations.[9] The same seems to be true for TNFR I$^{-/-}$ mice, which have been reported to lack PP[8] but seem now to have small and disorganised PP (Pasparakis *et al*).

Implications for mucosal immunologists and gastroenterologists

What lessons can be learned from gene targeted mice with abnormal or absent PP? Firstly, the mutants in which PP are affected (relatively) selectively offer opportunities for exploring the roles of these organs in responses to immunising and tolerising antigens delivered orally. Secondly, the mutants with absolute agenesis of PP offer an interesting model in which to explore the permeability of the villous mucosa to antigens and macromolecules, independent of PP (and, presumably, isolated lymphoid follicles). Thirdly, how do the results in rodents relate to humans, in whom it is reported that numbers of PP increase until puberty?[15] Does this represent delayed morphogenesis de novo, or does it represent the activation of latent anlagen (perhaps by the action of cytokines such as TNF-α)? Do PP that involute in later life disappear, or can they be reactivated? Our own recent studies suggest that a range of isolated lymphoid aggregations that are present in normal human small intestine can be activated by microbial colonisation. Finally, how do these findings on early morphogenesis relate to the "lymphoid neogenesis" observed in transgenic mice when LT-α is expressed ectopically under a tissue specific promoter?[16] Is follicular hyperplasia in the small intestine, follicle formation in the stomach in atrophic gastritis or the mononuclear infiltrate in Crohn's disease a recapitulation of morphogenetic events that occur normally under control of cytokines during early development? Love them or hate them, cytokines rule.

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Commentary

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The columnar lined oesophagus: a riddle wrapped in a mystery inside an enigma

Just a few years ago, the definition and pathogenesis of Barrett's oesophagus seemed straightforward. Barrett's oesophagus was the condition in which metaplastic columnar epithelium replaced oesophageal squamous mucosa that had been damaged by exposure to refluxed gastric juice. The condition was sought primarily in patients with gastro-oesophageal reflux disease (GORD), and was identified when endoscopic examination revealed long segments of columnar epithelium extending well up the oesophagus. Biopsy specimens taken from the oesophageal columnar lining usually showed a peculiar form of intestinal metaplasia called specialised columnar epithelium or specialised intestinal metaplasia. In patients who developed adenocarcinomas in Barrett's oesophagus, the columnar epithelium surrounding the oesophageal tumour invariably contained specialised intestinal metaplasia that often exhibited dysplastic changes. Eventually, Barrett's oesophagus with specialised intestinal metaplasia became recognised as the major risk factor for adenocarcinoma of the oesophagus and oesophagogastric junction.[1]

Over the past two decades, the incidence of adenocarcinoma of the oesophagus and oesophagogastric junction has been rising dramatically in the United States and Western Europe.[2 3] Patients who have oesophagectomy for adenocarcinoma at the oesophagogastric junction often do not have endoscopically apparent Barrett's oesophagus, but rather have short, inconspicuous segments of specialised intestinal metaplasia found on histological examination of the resected specimens.[4] In 1994, investigators who were aware of this phenomenon reported the results of a study designed to estimate the frequency of metaplastic changes in the distal oesophagus.[5] In this study, consecutive patients scheduled for elective endoscopic examinations in a general endoscopy unit had biopsy specimens obtained at the squamocolumnar junction in the distal oesophagus (the Z-line) irrespective of its appearance and location. Among 142 patients who did not have endoscopically apparent Barrett's oesophagus (that is, <3 cm of the distal oesophagus lined with columnar epithelium), the investigators were surprised to find that 26 (18%) had specialised intestinal metaplasia in biopsy specimens from the Z-line.

Four similar studies, including one by Trudgill *et al* in this issue (see page 585), have been published since 1994 in peer-reviewed journals.[6–8] The results of the five studies, all of which included consecutive, unselected patients seen in general endoscopy units, are summarised in table 1. The studies show clearly that short, inconspicuous segments of specialised intestinal metaplasia can be found frequently at the squamocolumnar junction in predominantly white patient populations. This may be the only clear point that emerges from these studies however. If specialised intestinal metaplasia indeed develops as a sequela of GORD, then one might expect an association with the symptoms and signs of reflux oesophagitis. However, only one study found a significant association with GORD symptoms whereas three did not, no study found any association with endoscopic oesophagitis, and only two of the four studies that sought an association with histological oesophagitis, found it. These observations challenge the traditional notion that oesophageal columnar metaplasia develops as a consequence of GORD, although further studies that include protracted oesophageal pH monitoring will be needed before this issue can be resolved. The potential contributions of *Helicobacter pylori* infection, inflammation in gastric cardiac epithelium, advancing age, race, and sex to the development of specialised intestinal metaplasia also are unclear. One study has suggested that the pathogenesis and epidemiology of specialised intestinal metaplasia at the squamocolumnar junction may vary substantially with the location of the Z-line.[8] In this study, only white patients with GORD symptoms had specialised intestinal metaplasia found at the squamocolumnar junction when the Z-line was located within the distal oesophagus, whereas specialised intestinal metaplasia was found with similar frequency in both black and white patients irrespective of GORD symptoms when the Z-line was located precisely at the anatomical junction of oesophagus and stomach. For future studies, investigators will need to document meticulously the

TABLE 1 *Results of studies on the prevalence of specialised intestinal metaplasia (SIM) among unselected patients in general endoscopy units*

Study first author	Spechler	Johnston	Nandurkar	Chalasani	Trudgill
Year of publication	1994	1996	1997	1997	1997
Country	USA	USA	Australia	USA	UK
Number of patients	142	170	158	87	120
Prevalence of SIM at squamocolumnar junction	18%	9%	36%	18%	18%
Association of SIM with GORD symptoms	No	Yes	No	–	No
Association of SIM with endoscopic oesophagitis	No	No	No	–	No
Association of SIM with histological oesophagitis in squamous epithelium	Yes	No	Yes	–	No
Association of SIM with inflammation on columnar side of squamocolumnar junction	–	–	Yes	–	–
Association of SIM with *Helicobacter pylori* infection	–	–	No	–	No
Association of SIM with advancing age	No	No	Yes	–	Yes
Association of SIM with male sex	Yes	No	No	–	No