Timeline:

To Respond or not to Respond - A Personal Perspective of Intestinal Tolerance

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Abstract

For many years, the intestine was one of the poor relations of the immunology world, being a realm inhabited mostly by specialists and those interested in unusual phenomena. However this has changed dramatically in recent years with the realisation of how important the microbiota is in shaping immune function throughout the body and almost every major immunology institution now includes the intestine as an area of interest. One of the most important aspects of the intestinal immune system is how it discriminates carefully between harmless and harmful antigens, in particular its ability to generate active tolerance to materials such as commensal bacteria and food proteins. This phenomenon has been known for more than 100 years and it is essential for preventing inflammatory disease in the intestine, but its basis remains enigmatic. Here, I discuss the progress that has been made in understanding 'oral tolerance' during my 40 years in the field and highlight the topics that will be the focus of future research.

Introduction

The intestine has a long history as an immunological organ, with its lymphocytes and organised lymphoid tissues having been discovered many years ago, even if their functions were not fully understood at the time. When I started in the field more than 40 years ago, it was already known that there were more lymphocytes in the intestine than in any other part of the body, which of course makes complete sense when one considers its constant exposure to microbes and other external antigens. However until very recently, intestinal immunology was neglected by mainstream immunologists. The gut was held to be a dirty and complicated tissue that was difficult to work with and where experimental findings were at best phenomenology rather than precise data of immunological importance. This attitude has been transformed in recent years, in great part because of the realisation that the intestinal microbiota plays a crucial role in shaping immune function and susceptibility to disease. As a result, the intestine is now seen as a fulcrum of the immune system and there is increasing interest in exploring its unusual aspects and understanding how these might influence immunity elsewhere.

One of the most characteristic and longest known features of the intestinal immune system is its ability to develop tolerance to the wealth of harmless foreign antigens it encounters on a routine basis and yet still generate active immunity against pathogens. As well as being fundamental to understanding the pathogenesis of inflammatory bowel diseases and to creating orally active vaccines, this balancing act in the intestinal immune system is also the best physiological example of how innate immune signals are constantly being integrated to regulate adaptive immune responses. I first became fascinated by this phenomenon as a medical student in the mid 1970s when carrying out an elective project with the late Anne Ferguson, whose research interest was in coeliac disease and who was then at the forefront of the emerging field of mucosal immunology. After completing a PhD with Anne, immune regulation in the intestine has continued to be the focus of my research ever since. In this Timeline article, I will provide a personal perspective on the key discoveries that have shaped our current understanding of immunological tolerance in the intestine, often referred to simply as ‘oral tolerance’ (**Figure 2**). Strictly speaking however, this term should be used only for the local and systemic hyporesponsiveness to subsequent challenge which occurs when exogenous antigens are administered by the oral route. This is one aspect of the more general phenomenon of ‘mucosal tolerance’ that characterizes the

failure of all steady state mucosal surfaces to make effector responses against harmless materials of all kinds.

Oral tolerance – historical aspects

The first serious interest in the intestine as an immunological organ came about because of the epidemics of typhoid, cholera and other intestinal infections that were prevalent when microbiology and immunology were developing as disciplines in the late 19th century. At this time, sporadic attempts were made to vaccinate individuals orally with relevant microorganisms, some of which were partially successful. However it also became apparent from these kinds of experiments that non-living materials were ineffective in inducing active immunity in the intestine. In 1911, Wells & Osborne demonstrated that oral administration of proteins such as ovalbumin (OVA) prevented subsequent local and systemic anaphylactic reactions to these proteins in guinea pigs[1]. By 1930, it had been shown that prior feeding of contact-sensitizing agents prevented dermal hypersensitivity to these agents — the Sulzberger–Chase phenomenon[2]. Even at this early stage, the experimental tolerance that was induced by oral administration of antigens was found to be antigen specific and evidence was then obtained that tolerised lymphocytes were rendered intrinsically and permanently unresponsive[3]. However it was not until 1994 that the ability of orally administered antigens to induce systemic tolerance was demonstrated formally in humans, when feeding the novel antigen keyhole limpet haemocyanin (KLH) was found to inhibit subsequent delayed type hypersensitivity (DTH) responses after skin challenge[4].

Mechanisms of oral tolerance to protein antigens.

What became known as 'oral tolerance' was first explored in immunological detail in the 1970s and 1980s, when its scope and mechanistic basis were documented. Amongst the insights that emerged were that the oral route of antigen delivery was a particularly efficient way of inducing tolerance[5]. It also became clear that soluble antigens such as proteins were able to induce tolerance when given orally, whereas antigens in the form of particles or as part of a live organism were more likely to provoke active immunity. Even though these studies were conducted before the importance of the innate immune system, costimulation and 'danger signals' had been appreciated, they were already seen as

evidence that tolerance reflected an inability to activate the antigen presentation limb of the immune response in such a way that lymphocytes were not primed properly[5]. It was also shown that while oral tolerance could affect all aspects of the immune response, it was particularly effective at inhibiting responses that could cause damage to the host, such as T cell-mediated DTH reactions and IgE-dependent responses. Thus the idea was already forming that tolerance to harmless antigens in the intestine was a physiologically crucial phenomenon designed to prevent pathological conditions directed at, for example, food proteins[5].

The tolerance described in these early studies could be transferred to naive animals by lymphocytes and as with many other aspects of immune regulation at the time, it was proposed that this was dependent on a population of $CD8^+$ 'suppressor T cells'; oral tolerance could also be prevented by depleting such cells *in vivo*[6]. However this aspect of immunology collapsed dramatically in the late 1980s, as increasing knowledge of T cell biology and MHC restriction indicated there was no molecular basis for antigen-specific $CD8^+$ T cells to function in this way. Indeed the original observations implicating this population have never been explained satisfactorily in the intervening years, and in the 1990s, attention turned to the emerging immunoregulatory mechanisms that involved $CD4^+$ T cells.

A debate in cellular immunology at this time was whether cell intrinsic mechanisms such as clonal deletion and anergy could account for tolerance in T cells, or whether this involved extrinsic control by active inhibitory mechanisms. In the 1990s, Howard Weiner and colleagues proposed that which of these mechanisms was involved in oral tolerance might be determined by the antigen dose regime used. Whereas single high doses of protein antigen were thought to cause anergy and/or deletion of antigen-specific T cells **[Au:OK?]** , multiple feeds of lower doses were predicted to generate regulatory T (Treg) cells[7]. This was important because of the ideas that arose around this time of harnessing oral tolerance for treating immunological diseases (see below). Specifically, it was considered that active Treg cells would be more useful for this purpose. This was because of their ability to persist and to produce cytokines that would generate 'bystander' suppression by acting on effector T cells with different specificities, something which would help overcome the epitope spreading that is a feature of many autoimmune disorders. Although some evidence was presented that clonal deletion of antigen-specific T cells could be induced in oral tolerance,

this was found after feeding very large doses of antigen to full T cell receptor (TCR) transgenic mice, a system that was unlikely to represent the usual situation *in vivo*[8]. In general, it was never proven experimentally that clonal deletion and/or anergy could contribute to oral tolerance in the presence of a polyclonal T cell repertoire using physiological amounts of antigen. Indeed subsequent experiments suggest that the higher the dose of antigen fed, the better the induction of Treg cells[9].

Treg cells enter the fray.

In common with other aspects of immune regulation, most studies of oral tolerance in the last 20 years have focused on the possible role of T cells with active inhibitory properties and in particular, $CD4^+$ Treg cells. Before this subset itself was defined, regulation of T cell function in oral tolerance was proposed to be an example of the Th1/Th2 paradigm that dominated cellular immunology for some time. This was an attractive idea, as oral tolerance seemed to be particularly effective in suppressing DTH responses and it was thought that this might be due to preferential activation of Th2 cells[7]. However this could not explain why IgE production, which is a signature Th2 cell-mediated function, was also very easy to tolerize by feeding antigen. As a result, the paradigm was modified to accommodate findings that a population of transforming growth factor β (TGFβ)-secreting $CD4^+$ T cells (at the time proposed to be 'Th3' cells) was associated with oral tolerance[10]. Again these cells were never characterized in full and this work was conducted before the discovery of the transcription factor forkhead box protein P3 (FOXP3), or what we would now consider as *bona fide* Treg cells. The first evidence supporting a role for such cells in oral tolerance came from studies using surrogate markers for Treg cells, such as CD25, showing that tolerance could be transferred by $CD4^+$ T cells co-expressing these surrogate markers **[Au:OK?]** [11-14]. Control of oral tolerance by $FOXP3^+$ Treg cells was eventually shown directly in 2007[15] and has since been confirmed in different systems[16,17]. Indeed, it seems that most 'inducible' Treg cells in the steady state small intestine may be specific for food antigens[17]. The mechanism of action for these cells is usually assumed to involve production of TGFβ, but as yet there is little direct evidence for this.

The intestinal microbiota

The presence of bacteria in the normal intestine has been known from the start of microbiology as a discipline and their contribution to host metabolism and other aspects of health has been appreciated for many years. For instance, germ-free animals have very reduced immune responses and much smaller lymphoid organs[18], [19]. An obvious problem for immunologists is how the immune system could cope with this enormous burden of foreign antigen without attempting to 'reject' it. The simplistic assumption that the microbes were simply not visible to immune cells became untenable when it was shown that the large amount of secretory IgA antibodies present in the normal intestine is directed mostly at the bacteria that make up the microbiota[20]. Indeed, it was then suggested that one of the principal roles of IgA was to prevent access of commensal bacteria to the body (the concept of 'immune exclusion'[21]) and in support of this, subsequent studies have shown that a substantial proportion of commensal bacteria in the colon are coated with IgA[22]. In the mid 1990s, the ground-breaking studies of Fiona Powrie and others showed the normal immune system also contains effector T cells that could recognise the microbiota[23,24]. However these are normally kept in check by Treg cells, which were shown to act via the production of IL-10[25] or TGFβ[26], consistent with the spontaneous colitis that had been found in IL-10-deficient mice when they were first described[27].

Unlike the situation with protein antigens encountered in the gut, tolerance to the gut microbiota is compartmentalized to the intestine and the systemic immune system is essentially ignorant of its presence[28] (**Figure 2**). The explanation for this comes from earlier experiments by the group of Andrew Macpherson which showed that recognition of microbiota-derived antigens is dependent on uptake by dendritic cells (DCs) that migrate no further than the mesenteric lymph nodes (MLNs)[29]. As a result, specific immune responses to the microbiota are localised to the intestinal mucosa and involve the generation of Treg cells and secretory IgA production. Studies of the mechanisms underlying IgA class-switching and the induction of Treg cells indicated that a common factor could be the cytokine TGFβ and it has been suggested subsequently that Treg cells may themselves drive IgA production in response to the microbiota[30,31], possibly becoming follicular helper T cells (T_{FH}) in Peyer's patches[32]. Compartmentalisation of tolerance to the microbiota makes complete biological sense, as these organisms are highly pathogenic if they escape into body from the intestinal lumen and therefore it is important that protective immunity can be generated under these circumstances. There are several processes that restrict access of the microbiota to the

systemic immune system, including the physical barriers presented by the mucus layer and intact epithelium, as well as the innate immune defences that protect the epithelial barrier (see below).

Despite the intact organisms that make up the microbiota being kept at bay, one of the biggest developments in immunology in my time has been the recognition of how the microbiota shape immune responses both in the intestine and elsewhere. The first example of an individual organism having this kind of effect was the ability of Segmented Filamentous Bacteria (SFB) to drive IgA production[33] and several years later, SFB was found to be the main factor responsible for inducing T_H17 cell responses in mice[34,35]. Subsequent examples include the induction of IL-10-producing Treg cells by polysaccharide A from *Bacteroides fragilis*[36] and the ability of certain human *Clostridial spp.* to expand $FOXP3^+$ Treg cells via the local production of TGFβ and butyrate **[Au: It might be worth mentioning / citing one or two of the studies from Honda's group that have actually associated specific human microbiota strains with the induction of Treg cells and Th1 cells e.g. I'm thinking of K Atarashi et al Science. 2017 Oct 20;358(6361):359-365, Atarashi et al. Nature. 2013 Aug 8;500(7461):232-6. doi: 10.1038/nature12331.]**[37 38 39]. It is now accepted that these effects of the microbiota are not restricted to the intestine, and that microbiota at various tissue sites influence many aspects of biology throughout the body, including susceptibility to numerous diseases[40]. Furthermore, the intestine is by no means the only source of commensal micro-organisms and without doubt, these topics will be one of the main areas of research into the foreseeable future.

DCs and intestinal tolerance

Migratory DCs create a tolerogenic environment.

One of the unresolved questions about oral tolerance to protein antigens is how the phenomenon can affect both local and systemic immune responses, raising the issue of where antigen is taken up and presented to T cells. The discovery of microfold ('M') cells in Peyer's patches and their ability to transport antigen into the immune system[41] led to the assumption that this would be the route for inducing oral tolerance. Indeed, the first formal demonstration of 'suppressor T cells' in oral tolerance described these cells in Peyer's

patches[42], while both clonal deletion of antigen-specific T cells and $CD4^+$ T cells with inhibitory properties were subsequently described in the Peyer's patches of protein-fed mice[8,43]. Nevertheless a number of studies have shown that surgical or developmental removal of Peyer's patches does not alter the induction of oral tolerance to proteins (see [44] for review) and in recent years, attention has turned more to the idea that the relevant antigen is acquired by DCs in the villus lamina propria and transported by these cells to the draining MLNs where the induction of Treg cells occurs (Figure 2).

Studies by the groups of Oliver Pabst and others have confirmed that both the migration of DCs and the induction of oral tolerance are dependent on CC-chemokine receptor 7 (CCR7) and suggested that events in MLNs drive the systemic consequences of tolerance[45,46]. However it is not known how the MLNs can influence the immune system outwith the intestine. Furthermore, the earliest studies in which antigen-specific $CD4^+$ T cells could be tracked directly found that orally administered antigen was detected by T cells simultaneously throughout the immune system (that is, by both peripheral and mucosal T cells) and not only in the MLNs[44]. This systemic dissemination of antigen was consistent with work from the 1980s showing that ingested proteins are present in an immunologically relevant form in the circulation of healthy humans and mice[5,47,48]. In mice this material was shown to be tolerogenic when transferred to normal recipients and together these findings could explain the systemic consequences of oral tolerance without a specific role for the MLNs. While these apparent contradictions have not yet been explained, it seems quite possible that both processes may contribute to oral tolerance to soluble antigens, with their relative importance perhaps depending, for instance, on the dose, size and nature of the antigen.

A further issue that has never been resolved is whether the liver plays any role in the systemic consequences of oral tolerance. This is an attractive idea, as materials emanating from the intestine in the bloodstream drain directly to the liver via the portal vein and direct administration of antigen into the portal vein induces a state of systemic tolerance that appears to be rather similar to oral tolerance[49]. However older experiments in which tolerance was prevented when the portal circulation was disrupted or when Kupffer cells were depleted using the heavy metal gadolinium have proved difficult to interpret due to toxicity issues[49]. Similarly, the studies suggesting that liver sinusoidal endothelial cells (LSECs)[50] or plasmacytoid DCs[51] might act as tolerogenic antigen-presenting cells (APCs) for

oral antigens still await full explanation. This is an area which deserves revisiting with more modern and precise approaches, such as the recently described marker Clec4F that allows highly selective deletion of Kupffer cells *in vivo* [52].

While accepting these uncertainties, the current consensus is that the DCs present in the lamina propria play a crucial role in determining immune responsiveness to protein antigens in the intestine (**Figure 2**); the characterization of these cells has been a central focus of many groups in mucosal immunology in the past 10 years, including my own. In large part, this reflects the advances in flow cytometry and molecular biology that now allow small populations of cells from tissues to be analysed in depth. Nevertheless it is interesting to put this in context with the fact that the intestine was one of first tissues in which $MHCII^+$ 'dendritic cells' were identified when research into such cells was in its infancy[53]. Later work showed that expansion of DC numbers *in vivo* using the Fms-related tyrosine kinase 3 ligand (FLT3L) growth factor could enhance the induction of oral tolerance and in fact this was one of the first demonstrations that DCs could induce tolerance as well as priming effector responses in the immune system[54].

The first experiments using DCs isolated from the intestine described a population in Peyer's patches that produced IL-10 selectively[55]. This was followed by findings that $CD11c^+MHCII^+$ APCs could be isolated from the small intestinal lamina propria of antigen-fed mice and that these APCs induced tolerance to the fed antigen when transferred into recipients that had not been exposed to the antigen[56]. As with much other work at that time, the precise identification of these mucosal DCs became complicated by the realisation that they shared many markers with macrophages and it was not until very recently that it was shown using definitive markers that *bona fide* conventional DCs are required for the induction oral tolerance[57]. In the meantime, it had been shown that CD103 was expressed by most lamina propria DCs[58], although much earlier studies had inadvertently used CD103 to identify DCs migrating in lymph from the intestinal mucosa in rats[59]. The newly defined $CD103^+$ DCs in mice were found to be responsible for the uptake of orally administered antigen[60] and to preferentially promote the generation of Treg cells *in vitro* via their selective production of retinoic acid (RA) that acted in cooperation with TGFβ. For these reasons, intestinal $CD103^+$ DCs were then held to be intrinsically 'tolerogenic' DCs — and to be the major drivers of tolerance in the intestine[61-63]. More recently however, this field has been complicated by the findings that $CD103^+$ DCs in the intestine are heterogeneous in

nature; while some have the CD103$^+$CD11b$^-$SIRPα^- phenotype of the cross-presenting, IRF8- and BATF3-dependent 'cDC1' found in other tissues[64-66], the majority of CD103+ DCs in the small intestine is CD103$^+$CD11b$^+$SIRPα^+. As discussed below, these are related to the IRF4-dependent CD11b$^+$SIRPα^+ 'cDC2' found elsewhere, although they are essentially unique to the intestine in steady state conditions (reviewed in [67]). Further complexity comes from the fact that there is an additional population of CD103$^-$CD11b$^+$ DCs in the intestinal lamina propria; these also produce RA, but are less effective at inducing Treg cells *in vitro*. In the past, less rigorous gating strategies had referred to this last population with macrophages, but they are clearly distinct, by being F4/80$^-$CD64$^-$ and expressing DC-specific molecules such as FLT3 and the zinc finger transcription factor ZBTB46[68].

It is still unclear exactly which of these DC subsets is responsible for oral tolerance, although very recent results suggest that the IRF8-dependent CD103$^+$CD11b$^-$ DCs are not involved in the case of soluble antigens[57] and that this may be driven mostly by CD103$^+$CD11b$^+$ DCs in the small intestine[69]. Interestingly, there is evidence for anatomical differences for DC subset involvement, with CD103$^-$CD11b$^+$ DC appearing to be responsible for tolerance to soluble antigens in the colon[69]. Furthermore, CD103$^+$CD11b$^-$ DC seem to be the most effective subset at driving the induction of FOXP3$^+$ Treg cells *in vitro* via RA production **[Au:OK?]** and are also the only mucosal DC subset that can activate TGFβ via expression of the $\alpha_v\beta_8$ integrin[67,70,71]. Identifying the DC involved in the induction of oral tolerance is one of the important challenges for the future, as will be establishing the role of specific regulatory molecules such as IL-10, TGFβ, RA or indoleamine dioxygenase (IDO) in this process[72]. Finally, the finding that migratory DCs can transport commensal bacteria to the MLN (see above) has not yet been translated into definitive studies of which DC subset might be responsible for establishing and maintaining **[Au:OK? Or are you just referring to the induction of tolerance?]** tolerance to the microbiota; in future, more precise approaches should enable the identification of these cells and establish whether there are distinct roles for DCs and macrophages **[Au:OK? I didn't quite follow you here – is this what you are trying to get at?]** .

Acquisition of antigen by intestinal DCs.

Several mechanisms have been suggested over the years for how antigen might get access to DCs in the lamina propria, which is rather a puzzle, considering that the epithelial barrier is supposed to be impermeable to macromolecules (**Figure 3**). However as noted above, there is evidence for the presence of intact proteins in serum after antigen feeding, while some immunohistochemical studies have suggested that epithelial cells might deliver peptide products of digestion into the lamina propria[73]. Epithelial cell-derived exosomes have also been proposed to deliver tolerogenic materials from the intestinal lumen into the underlying network of immune cells in the lamina propria[74]. Work using more refined techniques has since attempted to address the issue at the cellular level, with the first intravital microscopy studies proposing that a population of 'DCs' could extend processes across the epithelium to capture antigen from the lumen[75,76]. However, it now seems that the cells involved may well have been macrophages rather than DCs, as they expressed a combination of CD11c, MHCII and CX_3C-chemokine receptor 1 (CX_3CR1), a typical macrophage pattern. Using more refined identification approaches, subsequent intravital microscopy studies have shown that a specific transport pathway associated with goblet cells may allow access of protein antigens to underlying $CD103^+$ DC in the lamina propria and that this process is necessary for oral tolerance[77]. However, other work using similar techniques has suggested that $CD103^+$ DCs may completely enter the epithelial layer and thus sample luminal contents directly[78]. This would be consistent with earlier *in vitro* work using cell lines, which showed DCs entering epithelial monolayers and forming tight junctions with the surrounding cells, thus avoiding disruption of the barrier[79]. However the role of this process in oral tolerance has not yet been demonstrated. A similar proviso applies to the finding that the conventional villus epithelium may contain a population of M cells with the potential to sample and transport antigen[80]. Lastly, an intriguing study updated the potential involvement of CX_3CR1^+ macrophages in oral tolerance, presenting evidence that these were indeed the principal cell type involved in the uptake of proteins from the lumen, but that they then transferred antigen to $CD103^+$ DCs via connexin 43-dependent gap junctions[81]. As macrophages do not migrate to MLNs in steady state conditions, it is likely that this transfer process must occur locally in the lamina propria itself.

Thus these diverse technical approaches have not yet generated a consensus on how antigen gains access to intestinal DCs and importantly, few studies have correlated events in

the mucosa with subsequent immune responses. Of equal note, current immunohistological techniques have not yet allowed the subsets of DCs and macrophages present in the intestine **[Au: should you specify 'present in the intestine' or 'involved in oral tolerance' here?]** to be delineated with sufficient precision *in situ*.

Local conditioning determines DC function.

A question of intense interest is what drives the unusual properties of intestinal DCs, such as the exclusive expression of CD103 by cDC2s in the intestine, their production of RA and their apparently tolerogenic phenotype. An obvious possibility is that these characteristics are dependent on the local tissue environment. The first evidence to support this idea came from experiments showing that human monocyte-derived DCs that were conditioned with supernatant from the Caco-2 colonic epithelial cell line could mimic the ability of isolated colonic DCs to produce IL-10 and drive the generation of Th2 cells *in vitro*. This was due to the presence of thymic stromal lymphopoietin (TSLP) in the supernatant, a mediator also expressed by colonic epithelial cells in the steady state[82]. Similar work then implicated TGFβ in the ability of epithelial cells to block inflammatory cytokine production by DCs[83].

Around the same time, attention began to focus on the role of RA, not just as a mediator of intestinal DC function, but as a factor controlling their differentiation. Exogenous RA was first shown to induce a 'mucosal' DC phenotype in mouse bone marrow-derived DCs as assessed by their ability to drive RA-dependent processes in T and B cells, such as expression of CCR9, $\alpha_4\beta_7$ and IgA class-switching[84]. This evidence that RA is important for its own production by DCs was subsequently confirmed, although the exact source of the conditioning RA is not yet clear, as both epithelial cells[85] and stromal cells[86,87] have been implicated. The primary source of retinol, from which RA is derived, is dietary vitamin A and the delivery of retinol to lamina propria DCs in the small intestine is enhanced by its high concentration in bile, reflecting storage of retinol in the liver[88]. As well as inducing its own production, RA may be responsible for other features of intestinal DCs, including their anti-inflammatory properties and ability to drive $FOXP3^+$ Treg cell differentiation *in vitro*[89,90]. Indeed recent studies propose that there is a specific precursor in the bone marrow for intestinal $CD103^+CD11b^+$ and $CD103^+CD11b^-$ DC whose development is dependent on RA[91].

One of the most unusual aspects of the intestine is the presence of substantial numbers of $CD103^+CD11b^+$ DCs (see [67] for review). These are not found in other steady state tissues, where the expression of CD103 is restricted to DCs belonging to the cross-presenting population of BATF3- and IRF8- dependent $XCR1^+$ cDC1s[92]. Within a short time of being discovered, a number of studies showed that intestinal $CD103^+CD11b^+$ DCs were related to the lineage of cDC2s that expresses SIRPα, being dependent on Notch 2, IRF4 and Krüppel-like factor 4 (KLF4) for their development. Other factors that are involved include GM-CSF, SIRPα and RA. However, most of these processes are generic to the cDC2 lineage[92] and there clearly must be something distinctive about the intestinal mucosa that accounts for the unusual properties of the cDC2s in this site. In very recent studies, we have found that CD103 expression is only one of many features that differentiate $CD103^+CD11b^+$ DCs from other $CD11b^+$ DCs[93]. These include the expression of TREM1, SiglecF, glycoprotein 2 (GP2) and CD101, as well as a distinct cassette of genes. By using these markers to interrogate CD11c-Cre-TGFβR1$^{flox/flox}$ mice, we were able to uncover a cell intrinsic and gut-specific role for TGFβ signalling in driving the local differentiation of $CD103^+CD11b^+$ DCs from a $CD103^-CD11b^+$ intermediary[93]. Further work is required to determine how TGFβ might interact with other mediators in specifying the development of these DCs and to establish its source *in vivo*. One such factor may be granulocyte-macrophage colony-stimulating factor (GM-CSF), which is needed for $CD103^+CD11b^+$ DCs to develop and one intriguing idea is that this may be produced by one of the other recently discovered populations of intestinal immune cell, the type 3 innate lymphoid cell (ILC3)[94].

Maintenance of tolerance in the mucosa

Soluble protein antigens.

Once tolerance has been induced in the MLNs or Peyer's patches, several mechanisms operate in the mucosa itself to ensure this homeostatic state is maintained. Treg cells that differentiate first in the secondary lymphoid organs exit and migrate to the lamina propria via efferent lymphatics and blood (**Figure 2**). As with other lymphocytes whose first encounter with antigen occurred in the intestinal lymphoid tissues, it was found that Treg cell homing to the small intestine requires the induction of $\alpha_4\beta_7$ integrin and CCR9

expression on these cells by RA-producing DCs[95]. In parallel, oral tolerance to protein antigens was shown to be defective in mice lacking these homing molecules[95]. An important insight into what might regulate the behaviour of Treg cells once they arrive in the small intestinal lamina propria came from studies by Oliver Pabst's group, which showed that IL-10 produced from CX_3CR1^+ lamina propria macrophages was needed to maintain local expansion of the $FOXP3^+$ Treg cell populations involved in oral tolerance to proteins[16]. Intriguingly, this study also concluded that some of the Treg cell populations that expand in the lamina propria then leave the mucosa and are responsible for the systemic consequences of oral tolerance (**Figure 2**). While this idea would be consistent with older work showing that antigen-experienced $CD4^+$ T cells are present in efferent lymph coming from the intestine[96], it remains to be proven directly.

The microbiota.

As noted above, it has been known for many years that IL-10 is also important for maintaining tolerance to microbiota in the large intestine. With the discovery that macrophages in the colonic lamina propria produce substantial amounts of IL-10 constitutively[97] and the findings that lamina propria macrophages could drive the generation of $FOXP3^+$ Treg cells *in vitro*[98,99], it seemed sensible to suggest that macrophage-derived IL-10 would be the critical factor in preventing intestinal inflammation. However, recent work has challenged this idea, by showing that whereas conditional deletion of the IL-10R in macrophages in CX_3CR1-Cre-$IL10R^{flox/flox}$ mice leads to spontaneous colitis, deletion of IL-10 itself in macrophages does not[100]. This supports earlier work showing that LysM-Cre-$Stat3^{flox/flox}$ mice (in which macrophages cannot respond to IL-10) also develop spontaneous intestinal inflammation **[Au:OK? Or 'colitis'? To answer the referee's comment]**[101]. Thus it appears that signalling via the IL-10R on macrophages is needed to maintain tolerance to the microbiota, whereas other cells, probably $CD4^+$ T cells, are responsible for producing the IL-10. A further recent idea to explain the lack of a local inflammatory response to the microbiota is that $MHCII^+$ ILC3s may induce cell death in microbiota-specific $CD4^+$ T cells, although this possibility remains to be confirmed[102]. The mechanisms governing the migration of microbiota-specific Treg cells to the colon are still not understood in any detail, although recently it was proposed that the G protein coupled receptor GPR15 might fulfil this role[103].

Intestinal disease

In the past 20 years, it has become widely accepted that when tolerance in the intestine fails, this leads to inflammatory disease. As discussed above, the first indication that this was the case following loss of tolerance to the microbiota came when it was found that *Il10*$^{-/-}$ mice developed spontaneous IBD and that this was dependent on the presence of commensal bacteria[27]. More recently, it was shown that non-functional mutations in *IL10* or its receptor pathway in humans lead to early-onset IBD[104]. Many subsequent studies in mice have confirmed that a breakdown in the processes that underpin symbiosis between the host and its microbiota can lead to IBD[40]. The first studies examining genetic susceptibility to Crohn's disease showed an association between non-functional mutations in *NOD2* and ileal Crohn's disease[105,106]. Since then, Crohn's disease has become one of the best understood inflammatory disorders at the genetic level, with most susceptibility genes coding for factors involved in innate defence mechanisms used by myeloid cells and the epithelial barrier to deal with bacteria. These include *Card9*, *Il23r*, *TNF-SF15*, *ATG16L1* that promotes autophagy in Paneth cells and the autophagy related *IRGM* gene (reviewed in [107]).

The equivalent conditions that occur when tolerance to protein antigens breaks down are coeliac disease and food allergies. Starting with seminal work in the 1990s and 2000s showing that α-gliadin-specific CD4$^+$ effector T cells could be cloned from the mucosa of untreated patients with coeliac disease but not from healthy individuals[108], evidence accumulated for gliadin-dependent inflammatory changes in coeliac intestine, involving IFNγ[109,110], IL-21[111] and type 1 interferons (IFNs)[112]. Substantial progress in understanding the immunopathogenesis of coeliac disease was also made around the same time, with the main antigenic peptides in gliadin being identified and their molecular interactions with the disease-specific HLA-DQ2 molecule being elucidated, together with the essential role of the tissue transglutaminase (tTG) in generating the peptides[113,114]. Despite this wealth of knowledge on the clinical disorder, it has never been shown directly that there is a defect in specific regulatory mechanisms such as Treg cells and there is still no fully satisfactory experimental model of coeliac disease. As a PhD student, I found that some aspects of the intestinal pathology such as crypt hyperplasia and increased numbers of intraepithelial lymphocytes could be reproduced in experimental animals by oral challenge with protein

antigen after oral tolerance had been prevented[5]. This work has been extended in the last 15 years by findings that activation of the innate immune system by IL-15 may be an important mechanism allowing tolerance to be overcome. Together with RA, IL-15 may favour the generation of effector rather than regulatory $CD4^+$ T cells and also activates cytotoxic activity by intestinal epithelial lymphocytes (IELs), leading to epithelial cell death and increased uptake of antigenic peptides[115-118]. Several studies have followed up initial work in 2003 that a non-epitope peptide within α-gliadin (p31-43) itself may trigger the release of IL-15[119,120], with the most recent suggestions being that either p31-43[121] or reovirus infection[122] may drive TLR7- and/or TLR8- mediated release of type 1 IFN and innate immunity. These new insights have considerable implications for understanding the pathogenesis of coeliac disease as well as of other human autoimmune diseases of which coeliac disease is an excellent model.

IgE-mediated food allergies are amongst the diseases that have increased most in incidence in the last 40 years and here there is more direct evidence for a breakdown in oral tolerance. For instance it has been known for some time that spontaneous remission from cow's milk allergy in children is accompanied by restoration of Treg cell numbers to normal levels[123]. Very recently, a substantial clinical trial showed that early exposure to peanut in the diet protected against the development of peanut-induced food allergy in at risk children, clearly suggesting that oral tolerance had been induced by this protocol[124]. Interestingly, it has long been known that the induction of oral tolerance to prevent IgE responses **[Au:OK?]** against protein antigens in mice requires signals derived from the microbiota[125], perhaps partly explaining how exposure to antibiotics early in life may lead to enhanced susceptibility to atopic disease[126].

Oral tolerance-based therapy

A number of animal models in the 1980s and 1990s suggested that oral tolerance might be exploited in the treatment of autoimmune disease, as feeding appropriate antigens was shown to prevent or even ameliorate established disease in experimental arthritis, type 1 diabetes, encephalomyelitis and other models of autoimmunity[7]. As well as targeting antigen-specific T cells directly, this approach was thought to have the advantage of inducing Treg cells that might inhibit the phenomenon of epitope spreading via their ability

to produce bystander suppression of other T cell clones, as already discussed above. On the basis of the promising animal work, clinical trials were carried out in the 1990s examining the use of oral tolerance in rheumatoid arthritis, multiple sclerosis, autoimmune uveitis and type 1 diabetes[127]. However none of the trials were successful and this idea has not been resurrected since. While it remains an attractive possibility, difficulties in identifying the appropriate auto-antigens and in establishing regimes that will interfere with established disease may well prevent this becoming reality for autoimmune diseases. However the recent work on peanut allergy and coeliac disease indicate there could be scope in exploiting what appears to be a natural window for tolerance induction early in life for preventing disorders caused by inappropriate responses, for example, to food proteins.

Summary

Systemic tolerance to innocuous antigens was one of the first aspects of the intestinal immune system that was identified and it is now accepted that it plays a crucial role in preventing disorders such as coeliac disease, inflammatory bowel diseases and food allergies. While we still do not understand the process in full, recent years have seen a number of important advances based on the rapid expansion of knowledge in cellular immunology. In the case of soluble antigens such as food proteins, it is now clear that the induction of tolerance requires specific populations of DCs that express CD103 and produce retinoic acid. It is likely that these DCs are derived from the villus lamina propria, where they acquire antigen by routes that are yet to be defined, before migrating in lymph to meet naïve T cells in the draining mesenteric lymph node. Retinoic acid from the DCs drives the expression of gut homing markers on T cells and induces their differentiation into $FOXP3^+$ Tregs that recirculate back to the mucosa and maintain tolerance via the production of IL-10 and/or TGFβ. Mucosal macrophages may help maintain the local survival of Tregs via the production of IL-10. The cellular events involved in tolerance to the microbiota are less clear, but the process appears to be limited to the intestine itself and IL-10 clearly plays a crucial role in preventing inflammation to these antigens. In return, one of the most important developments in the field in the past 20 years has been the realisation that the microbiota has dramatic effects on immune function throughout the body. Extending these insights at the cellular and molecular level provides ample scope for research into the foreseeable future (Box 1).

Display items:

Box 1: Future questions for the field

- How do soluble antigens induce generalised tolerance, whereas tolerance to the microbiota is restricted to the intestine?
- Are the cellular mechanisms of tolerance the same for these different forms of antigen?
- Do discrepancies between these processes relate to anatomical differences in the immune systems of the small and large intestine?
- What is the exact nature of the dendritic cells responsible for inducing tolerance and what molecular processes are involved in their interaction with T cells?
- What factor(s) drive the differentiation of the specialised dendritic cells found in the intestine?
- How do mucosal dendritic cells acquire antigen from the lumen?
- Are FoxP3^{+} regulatory T cells alone responsible for tolerance and what is their mechanism of action?
- Do MHCII expressing macrophages play a role in maintaining tolerance by presentation of cognate antigen to local CD4^{+} T cells?
- How can fundamental insights from mouse models be translated into the understanding of diseases such as coeliac disease and inflammatory bowel disease?
- Will oral tolerance be exploitable for treatment of autoimmune and inflammatory diseases?
- How does the microbiota and its products shape oral tolerance and systemic immunity and what are the consequences of antibiotic use early in life on these processes?

Figure 2: Induction and maintenance of oral tolerance

Soluble antigens such as food proteins are likely to be taken up predominantly into DCs in villus lamina propria, either directly by $CD103^+CD11b^+$ cDC2, or after phagocytic uptake of epithelial cells that have ingested antigen before dying by apoptosis. In this case, $CD103^+CD11b^-$ cDC1 will be the DC involved[71,128]. Both these subsets of DC migrate constitutively in afferent lymph to the draining mesenteric lymph node, where they meet naïve $CD4^+$ and $CD8^+$ T cells. Production of retinoic acid by the DCs during the cognate interaction with T cells induces the expression of FoxP3, together with the gut homing molecules CCR9 and $\alpha4\beta7$ integrin, leading to the generation of regulatory T cells capable of returning to the intestinal mucosa. The generation of Treg is also promoted by the presence of TGFβ, which can be converted from its latent, inactive form (iTGFβ) to the active cytokine (aTGFβ) via the action of $\alpha v\beta8$ integrin expressed selectively by $CD103^+CD11b^-$ DCs[71]. After leaving the MLN, $CD4^+$ $FoxP3^+$ Treg migrate to the lamina propria via efferent lymph and the bloodstream. Here they prevent the activation of naïve and effector T cells via the expression of CTLA-4 that removes CD80/CD86 from antigen presenting cells and by production of the cytokines IL10 and TGFβ which inhibit both APC and T cells. IL10 produced by resident $CX3CR1^{hi}$ macrophages helps maintain FoxP3 expression by $CD4^+$ Treg in the mucosa and is needed for their survival[16,99]. As these macrophages also express high levels of MHCII, they may also be able to undergo cognate interactions with specific $CD4^+$ $FoxP3^+$ Treg providing a second signal for survival, but this has not been shown directly as yet. Similarly, it has been hypothesised, but not proven, that $CD4^+$ $FoxP3^+$ Treg that have been primed in the MLN and passed through the mucosa may be involved in the systemic consequences of oral tolerance to protein antigens, requiring them to have exited the mucosa in lymph and migrated throughout the body via the bloodstream[16]. An alternative explanation for systemic tolerance is that orally administered antigens can be found in lymph and the bloodstream, gaining access to resident DCs in mesenteric and peripheral lymph nodes[47 129]. These DCs remain to be characterised, but could induce tolerance in $CD4^+$ T cells via anergy or deletion, or by generating Treg due to the lack of costimulation that characterises secondary lymphoid organs in the steady state.

Commensal microbes gain access to the immune system mainly by transcytosis via M cells in the epithelium of Peyer's patches and isolated lymphoid follicles. The antigens are then transferred to underlying DCs, probably belonging to the $CD103^{-}CD11b^{+}$ subset of cDC2. These DCs may then present antigen directly to naïve $CD4^{+}$ T cells in the PP/ILF, or after migration to the draining MLN. Although this generates $FoxP3^{+}$ Treg that can migrate to the mucosa and have similar properties to those induced by soluble antigens, the roles of RA, TGFβ and other DC subsets in these processes is unclear. An additional feature of the immune response to microbial antigens is the production of IgA antibodies. This is driven by $CD4^{+}$ follicular helper T cells that interact with B cells in germinal centres of PP/ILF, inducing a switch to IgA expression mediated by TGFβ, IL5, IL6, ICOS, CD40L and other factors. Recent evidence suggests that some of these T_{FH} may be derived from $FoxP3^{+}$ Treg that have migrated into the germinal centre[30 31]. IgA switched B cells acquire CCR9 and α4β7 integrin, exit from PP/ILF via lymph and then migrate in bloodstream to arrive in the lamina propria as plasma cells. Unlike soluble antigens, the tolerance to microbial antigens is confined to the intestine and the systemic immune system normally remains 'ignorant' of these materials[28].

Figure 3: Models of antigen delivery for the induction of oral tolerance

Antigens in the intestinal lumen can gain access to the immune system either via organised lymphoid tissues such as Peyer's patches or isolated lymphoid follicles (ILF). Microfold (M) cells in the epithelial layer of PP or ILF transport microbes and particles to dendritic cells that lie wrapped in the basal membrane of the M cell (the M cell "pocket"), or that are in the underlying subepithelial dome region. A variety of routes have been proposed for the access of antigens across the villus epithelium, although this remains a controversial topic. Intact proteins may be transported directly across or between enterocytes, while rare populations of M cells have also been described outside Peyer's patches[80]. Enterocytes may also capture and internalize antigen:antibody complexes by means of the neonatal Fc receptor (FcRn) on their surface and transport them across the epithelium by transcytosis[130]. At the basal face of the epithelium, lamina propria dendritic cells expressing FcRn and other Fc receptors pick up and internalize the complexes. Enterocytes that have apoptosed either due to senescence or after infection by a pathogen can be phagocytosed

by neighnbouring dendritic cell. $CX3CR1^{+}$ mononuclear phagocytes have been shown to acquire luminal antigen efficiently and this may involve extension of processes between the cells of the epithelium without disturbing its integrity [75]. Although these cells are likely to be macrophages that cannot present antigen directly to naïve T cells, they can then pass the antigen on to neighboring $CD103^{+}$ dendritic cells for presentation to T cells, perhaps via Connexin 43 (Cx43) -mediated gap junctions[81]. Mucus secreting goblet cells can act as a conduit for delivery of soluble antigens to lamina propria $CD103^{+}$ dendritic cells and this process appears to be important for oral tolerance[77]. $CD103^{+}$ dendritic cells themselves might also enter the epithelial layer and capture bacteria before returning to the lamina propria[131].

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Figure 1

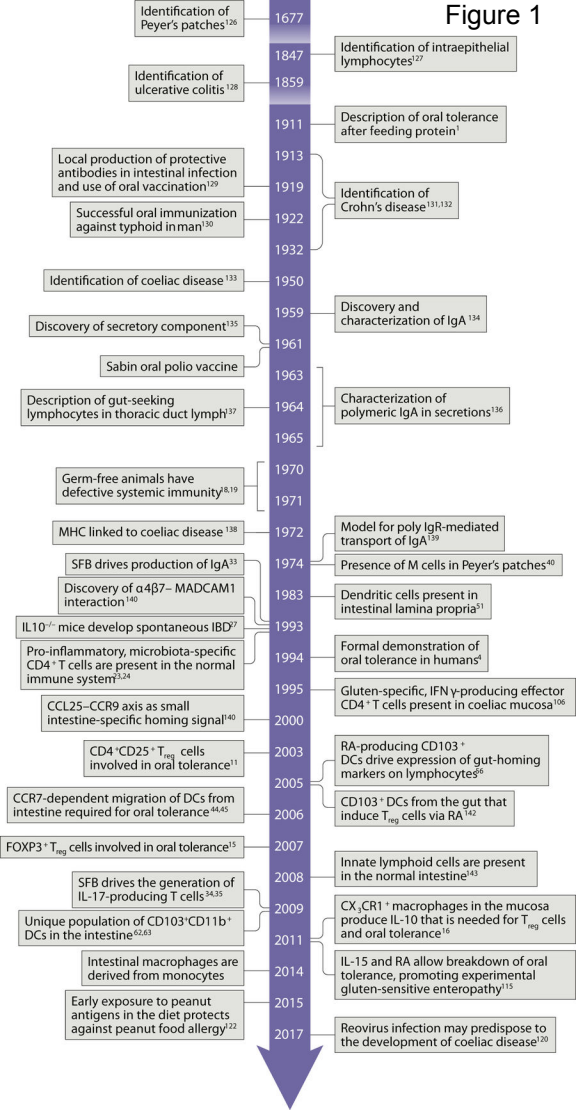

1677
Identification of Peyer's patches[126]
1847
Identification of intraepithelial lymphocytes[127]
1859
Identification of ulcerative colitis[128]
1911
Description of oral tolerance after feeding protein[1]
1913
Local production of protective antibodies in intestinal infection and use of oral vaccination[129]
1919
Identification of Crohn's disease[131,132]
1922
Successful oral immunization against typhoid in man[130]
1932
1950
Identification of coeliac disease[133]
1959
Discovery and characterization of IgA[134]
1961
Discovery of secretory component[135]
Sabin oral polio vaccine
1963
Characterization of polymeric IgA in secretions[136]
1964
Description of gut-seeking lymphocytes in thoracic duct lymph[137]
1965
1970
Germ-free animals have defective systemic immunity[8,19]
1971
1972
MHC linked to coeliac disease[138]
Model for poly IgR-mediated transport of IgA[139]
1974
SFB drives production of IgA[33]
Presence of M cells in Peyer's patches[40]
1983
Discovery of α4β7– MADCAM1 interaction[140]
Dendritic cells present in intestinal lamina propria[51]
1993
IL10−/− mice develop spontaneous IBD[27]
1994
Pro-inflammatory, microbiota-specific CD4+ T cells are present in the normal immune system[23,24]
Formal demonstration of oral tolerance in humans[4]
1995
Gluten-specific, IFN γ-producing effector CD4+ T cells present in coeliac mucosa[106]
2000
CCL25–CCR9 axis as small intestine-specific homing signal[140]
2003
CD4+CD25+ Treg cells involved in oral tolerance[11]
RA-producing CD103+ DCs drive expression of gut-homing markers on lymphocytes[66]
2005
CCR7-dependent migration of DCs from intestine required for oral tolerance[44,45]
CD103+ DCs from the gut that induce Treg cells via RA[142]
2006
2007
FOXP3+ Treg cells involved in oral tolerance[15]
2008
Innate lymphoid cells are present in the normal intestine[143]
SFB drives the generation of IL-17-producing T cells[34,35]
2009
Unique population of CD103+CD11b+ DCs in the intestine[62,63]
CX3CR1+ macrophages in the mucosa produce IL-10 that is needed for Treg cells and oral tolerance[16]
2011
Intestinal macrophages are derived from monocytes
2014
IL-15 and RA allow breakdown of oral tolerance, promoting experimental gluten-sensitive enteropathy[115]
Early exposure to peanut antigens in the diet protects against peanut food allergy[122]
2015
2017
Reovirus infection may predispose to the development of coeliac disease[120]

Figure 2

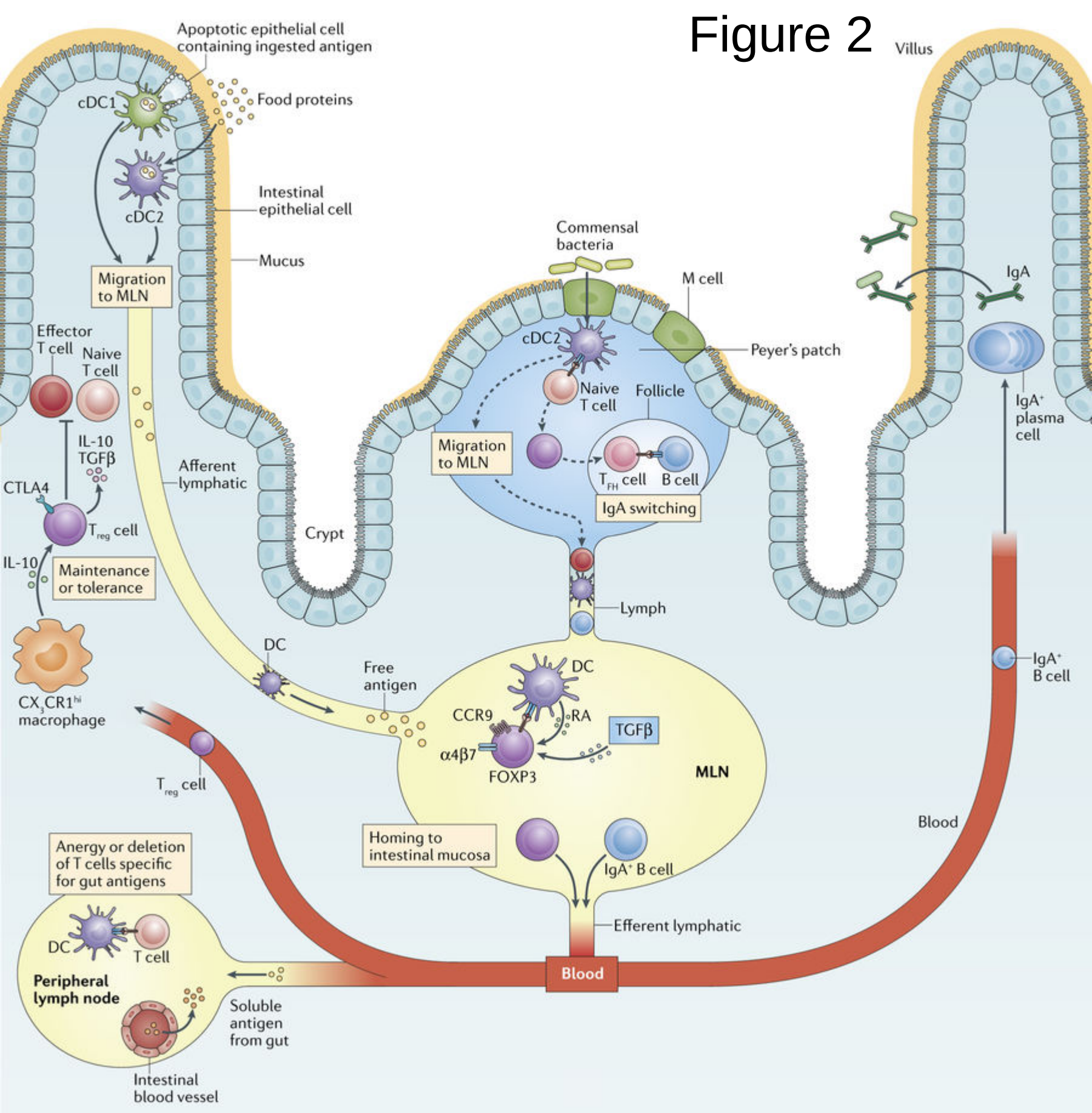
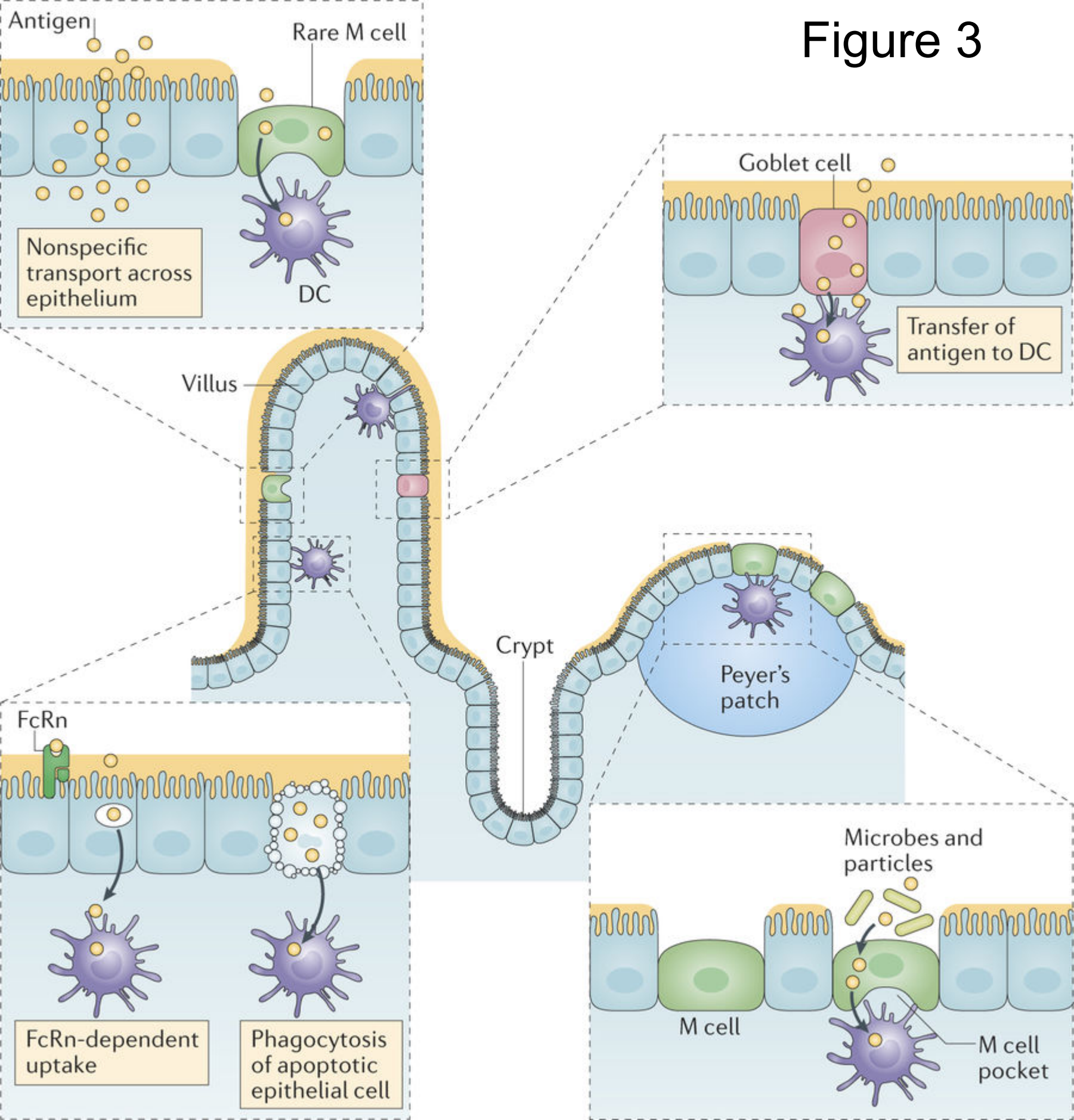

Apoptotic epithelial cell containing ingested antigen
Food proteins
cDC1
cDC2
Intestinal epithelial cell
Mucus
Migration to MLN
Effector T cell
Naive T cell
IL-10
TGFβ
CTLA4
T_{reg} cell
IL-10
Maintenance or tolerance
CX_3CR1^{hi} macrophage
Afferent lymphatic
Crypt
Commensal bacteria
M cell
cDC2
Peyer's patch
Naive T cell
Follicle
Migration to MLN
T_{FH} cell
B cell
IgA switching
Lymph
DC
Free antigen
DC
CCR9
RA
TGFβ
α4β7
FOXP3
MLN
T_{reg} cell
Homing to intestinal mucosa
IgA⁺ B cell
Efferent lymphatic
Blood
Anergy or deletion of T cells specific for gut antigens
DC
T cell
Peripheral lymph node
Soluble antigen from gut
Intestinal blood vessel
Villus
IgA
IgA⁺ plasma cell
IgA⁺ B cell
Blood

Figure 3


Antigen
Rare M cell
Nonspecific transport across epithelium
DC
Goblet cell
Transfer of antigen to DC
Villus
Crypt
Peyer's patch
FcRn
FcRn-dependent uptake
Phagocytosis of apoptotic epithelial cell
Microbes and particles
M cell
M cell pocket