



Intestinal Barrier Interactions with Specialized CD8 T Cells

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The trillions of microorganisms that reside in the gastrointestinal tract, essential for nutrient absorption, are kept under control by a single cell barrier and large amounts of immune cells. Intestinal epithelial cells (IECs) are critical in establishing an environment supporting microbial colonization and immunological tolerance. A large population of CD8⁺ T cells is in direct and constant contact with the IECs and the intraepithelial lymphocytes (IELs). Due to their location, at the interphase of the intestinal lumen and external environment and the host tissues, they seem ideally positioned to balance immune tolerance and protection to preserve the fragile intestinal barrier from invasion as well as immunopathology. IELs are a heterogeneous population, with a large innate-like contribution of unknown specificity, intercalated with antigen-specific tissue-resident memory T cells. In this review, we provide a comprehensive overview of IEL physiology and how they interact with the IECs and contribute to immune surveillance to preserve intestinal homeostasis and host-microbial relationships.

Keywords: mucosal immunology, intraepithelial lymphocytes, inflammatory bowel disease, CD8⁺ T-lymphocytes, epithelial cells

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INTRODUCTION

The intestinal epithelia are a single cell layer of large surface. Together with a mucus layer, the epithelia form a dynamic physical barrier between the host and its environment. Estimates are up to 100 trillion microorganisms, including pathogens, have made the gastrointestinal tract their home (1), which makes the intestine the largest potential port for microbial invasion. However, a proportion of the microorganisms in the intestine can contribute to the hosts' health and immunity. These commensal bacteria compete for resources with pathogenic microorganisms and provide metabolic capacity to digest food products by generating important compounds (e.g., vitamin K) or by assisting other microorganisms with supportive roles. The delicate nature of the single cell epithelial barrier, the essential function of the gastrointestinal tract to absorb nutrients and liquids, and the balance to maintain beneficial microbes, while offering protection against invasion and avoiding tissue damage, requires an effective and robust, yet tolerant, immune system.

The intestinal immune surveillance network is an integrated part of the organ, which enables it to swiftly pick up cues regarding its health status and contributes to tissue homeostasis as well as repair. Immune surveillance links rapid activation of innate immune cells to the more delayed recruitment of adaptive immune cells (2), ultimately resulting in immunological memory. Part of the innate system is the intestinal epithelial cells (IECs) themselves as well as classical innate immune cells. Mostly, macrophages, monocytes, and dendritic cells (DCs) migrate to the intestine from the bone marrow *via* blood (3). Following infection, interactions between antigen presenting cells and lymphocytes

can take place in specialized structures, unique to the intestine, such as isolated lymphoid follicles and Peyer's patches (4).

T-lymphocytes recognize foreign particles (antigens) by their surface expressed T cell receptor (TCR). With each T cell expressing a nearly unique TCR, collectively T cells can recognize nearly all foreign antigens. From the two major types of T cells found in blood and secondary lymphoid organs (SLO), CD4 expressing helper T (T_H) cells are generated in the thymus as precursors without a defined function. They recognize antigens presented in major histocompatibility complexes class II (MHCII) after processing by antigen presenting cells. T_H cells have an important orchestrating role, differentiating into effector cells with distinct supportive functions in type 1 (T_H1), type 2 (T_H2), and type 3 (T_H17) immunity and high levels of flexibility (5, 6). Specialized regulatory T cells can curtail responses and form part of a carefully balanced immune system (7). CD8 expressing cytotoxic T cells similarly derive from the thymus as naive cells. They mainly recognize antigens resulting from the target cells' transcriptional machinery and degradation of cytosolic proteins by the proteasome presented in MHCI, such as those resulting from viral infections as well as intracellular bacterial infections. Upon encountering their cognate antigen, CD8⁺ T cells differentiate into effector cells, classically thought to be part of type 1 immunity due to their high potential for interferon (IFN) γ production.

The maintenance of effector T cells is metabolically costly. Rapidly dividing cells require large amounts of energy for the production of cellular building blocks and secretion of effector molecules. These cells can potentially contribute to chronic inflammation and immunopathology. To avoid such possible danger and energy expense, the majority of effector cells undergo apoptosis after pathogen clearance, re-establishing homeostasis. Yet, some persist as memory cells, providing protection against re-infection. Memory CD8 T cells are a heterogeneous population, varying in phenotype, function, and localization (8) (**Figure 1**). This facilitates a swift and tailored response to a broad array of potential insults. In addition, the intestinal immune system has another important population of specialized CD8⁺ T-lymphocytes known as intraepithelial lymphocytes (IELs) (9). Intriguingly, IELs have characteristics of naive, effector, and memory cells require bidirectional cross-talk with IECs (10) (**Figure 1**), with one murine IEL estimated to be present for every 4–10 IECs (11, 12).

Aberrant immunity has severe consequences, especially in the intestine where a single epithelial cell layer forms the barrier between the host and a very high amount of microorganisms. Immunity against commensal bacteria can result in chronic inflammation, such as observed in inflammatory bowel diseases (IBDs). In this review, we focus on CD8 expressing T cells, particularly IELs, which, located in the very top layer of the intestinal barrier, are ideally positioned to monitor the intestinal microbiota. They may contribute to modulating immunity toward microbes as well as immunopathology, and are involved in tissue homeostasis and epithelial repair. We will discuss some of the properties of IELs and speculate on their role in the intestinal immune surveillance network.

Conventional CD8 T Cells

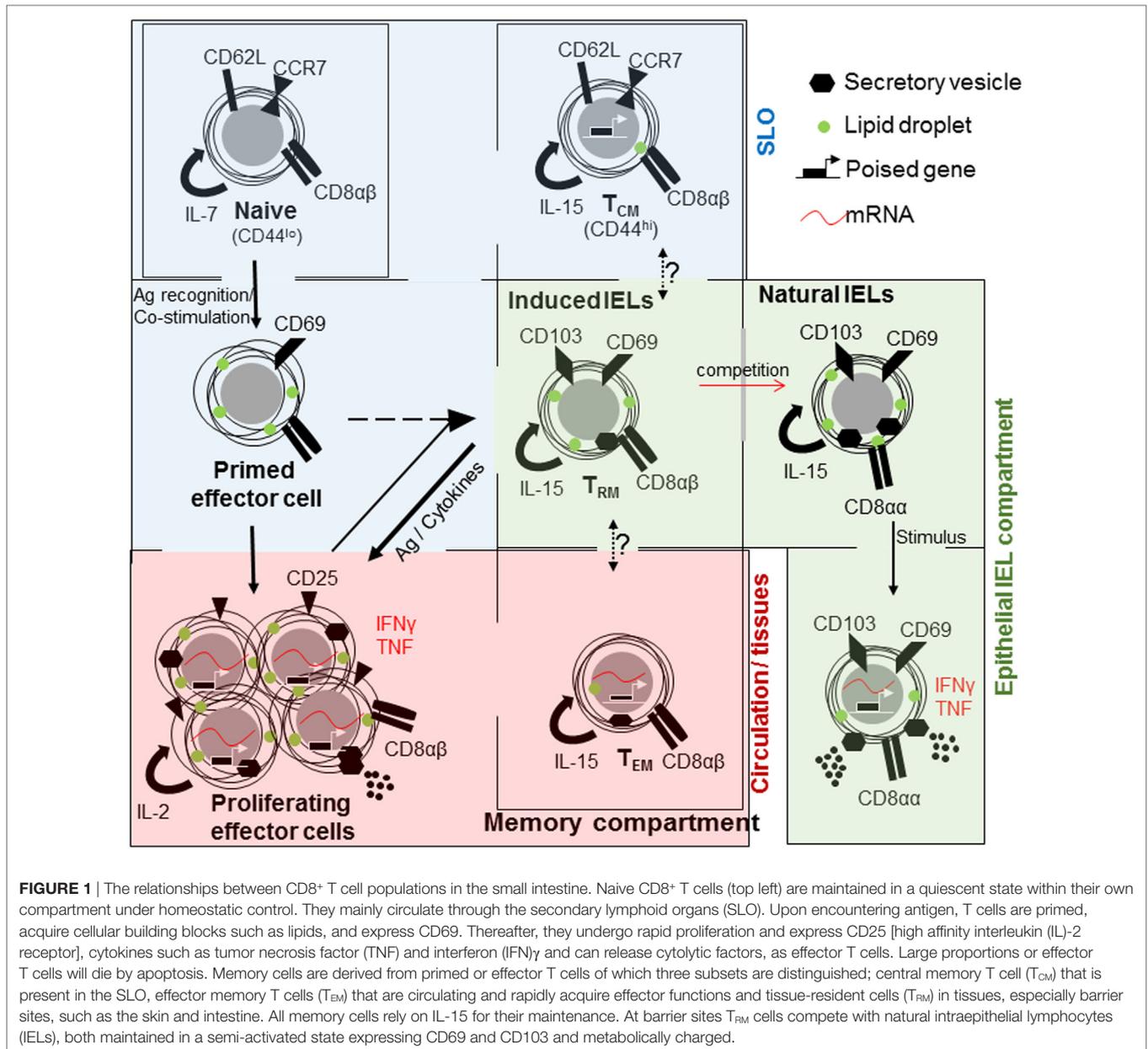
The initiation of an adaptive immune response requires several myeloid and lymphoid cell types. These cells need to be brought together and act in a strictly orchestrated manner in time and space to license immune cell activation (13). Critical interactions are those between antigen presenting cells, especially DCs and T cells (14). In order to become fully activated, naive T cells require signaling through TCR (signal 1) as well as costimulatory receptors (signal 2), such as CD28 and CD40. Additional cues (signal 3) provide inflammatory context and involve cytokines and chemokines (15, 16).

During the initiation phase, naive CD8⁺ T cells rapidly proliferate and differentiate into cytotoxic T-lymphocyte effector cells thereby gaining the ability to kill target cells by releasing perforin and granzymes, and secrete large amounts of cytokines, such as tumor necrosis factor (TNF) and IFNs (17) (**Figure 1**). The rapid proliferation during the expansion phase ensures that a limited number of precursor cells can counter infectious agents. Effector cells migrate to most tissues in the body to ensure the removal of all infected cells and pathogens (18). However, such a response cannot be sustained and proximally 95% of effector cells die in a contraction phase upon pathogen clearance (19).

A limited number of cells develop into memory cells, returning to a state of quiescence with slow cell turn over and effector molecule transcription. Despite this they are able to rapidly reactivate, proliferate, and express effector molecules upon reencounter with a similar pathogen (18, 20–23). How memory T cells develop remains incompletely understood. There are different signals influencing T cells upon and after encountering their cognate antigen that influence the size and quality of the T cell memory pool (8).

Three subtypes of memory T cells are recognized, they are; effector memory (T_{EM}) T cells, central memory (T_{CM}) T cells (18, 24, 25), and tissue-resident memory (T_{RM}) cells expressing CD69 and CD103 (26–29) (**Figure 1**). Differences in cell localization, recall ability, and effector functions provide intersecting levels of protection against re-infection (30). Memory cells found circulating through blood, lymph, and SLO are referred to as T_{CM} cells and express CD62L and CCR7, which enable entry in lymphoid organs and circulation (31–34). Those cells primarily found in non-lymphoid tissues are T_{EM} cells (18, 22).

Although migration of T cells is a pillar of successful immune defense, experiments using defined tissue grafts from ganglia, skin, and intestine as well as the use of parabiosis have defined a residential population of memory T cells (27, 35–37). At epithelial barrier sites such as the skin, lungs, reproductive organs, and gastrointestinal tract, a unique memory population is found; T_{RM} cells. These cells share characteristics with T_{EM} cells, expressing CD44 and low levels of CD62L (**Figure 1**). They are found at the initial site of infection, providing very regional immune surveillance and protection against re-infection (35, 38, 39), and do not recirculate (40). The discovery of T_{RM} cells and subsequent detailed analysis have resulted in a paradigm shift that most memory T cells are an integral part of non-lymphoid tissues (41). But, these cells did not settle on empty ground to fill a previously non-existent niche. T_{RM} cells compete, successfully, with innate or



innate-like lymphocytes, which are already present at the original site of infection (39).

Intraepithelial Lymphocytes

A variety of innate or innate-like lymphoid cell types reside in tissues, including natural killer (NK) cells, innate lymphoid cells, and T cells expressing the $\gamma\delta$ TCR chains ($\gamma\delta$ T cells), homodimers of CD8 α or a semi-invariant TCR $\alpha\beta$ such as NKT cells, and mucosal associated invariant T cells. The top layer of the epithelia, in murine and human intestine as well as murine skin, contains large populations of such innate-like T cells within the IEL population.

Intestinal IELs express the prototypical tissue-resident integrin CD103 (integrin αE), with which they interact with IECs (10), as well as C-Type lectin and early activation marker CD69, and the

NK cell inhibitory receptor 2B4 (CD244) (42). Antibody staining for CD8 α , CD69, and CD103 in lymphocytes sourced from the intestinal intraepithelial fraction provides a homogenous cell population (42). However, IELs can be divided into subsets based on their activation mechanism and on the antigens, which they may recognize. Induced or adaptive IELs are derived from conventional CD8 $\alpha\beta$ T cells, which recognize non-self antigens in the context of MHC I. They home to the intestinal barrier upon encountering their cognate antigen in the intestine as T_{RM} cells (9, 43). Induced IELs accumulate with age (44), replacing natural IELs (39).

Natural or innate-like IELs also originate in the thymus where they acquire homing factors and identity upon selection on self-antigens and seed the intestine as a precursor population

(45–51). They express CD8 $\alpha\alpha$ homodimers, in contrast to conventional CD8 $\alpha\beta$ T cells (52) (**Figure 1**). They express either the conventional $\alpha\beta$ TCR or the non-conventional $\gamma\delta$ TCR. In the small intestine around 60% of all IELs express TCR $\gamma\delta$, in marked contrast to SLO in which $\gamma\delta$ T cell represent less than 1% (53). In humans, natural IELs predominantly express TCRV δ 1 with a contribution from TCRV δ 3, the majority of which express CD8 (54, 55). In contrast to murine cells, human $\gamma\delta$ T cells may process and present antigen (56).

Contrary to T cells found in SLO and in line with T_{RM} cells, IELs do not circulate through blood and lymph and are tissue-resident (57). IELs seem to respond to a broad range of inflammatory cues, but the precise identity of these signals remains unknown. They modulate epithelial cell homeostasis and local immune responses by targeting other immune cells, viruses, and bacteria (9, 58, 59). The majority of IELs hold cytoplasmic granules containing large amounts of granzymes, cytokines, and chemotactic factors (42, 43, 60–62). At the first sight, the cytotoxic properties of IELs suggest, they can cause damage to the epithelial barrier by powerfully attacking infected cells, particularly IECs (63). However, IELs are well adapted to the intestinal environment in order to survive and perform their functions in protecting the delicate epithelial layer. In recent years, several studies of IELs have revealed distinct characteristics regarding their maintenance, activation, and contribution to the host immune response to preserve a healthy epithelial barrier.

Maintaining IELs

Intraepithelial lymphocytes develop pre-birth, occupy the epithelia before microbial colonization, and play an important role in immune protection during early life (64). It remained debatable for a considerable time if natural IELs take up residence at the intestinal epithelia as precursor naive-like cells or as antigen-experienced memory-like CD8⁺ T cells poised for activation or reactivation. The later would suggest that a priming step may be required, post-thymic development in the SLO, before seeding in IEL compartment. Transcriptional analysis, comparing IELs harvested under non-inflammatory conditions with memory CD8⁺ T cells, revealed paradoxical findings of their activation status (42, 43). IELs constitutively express transcripts of genes associated with activated cytotoxic T cells [granzyme A, granzyme B, serglycin, Fas ligand (FasL), and CCL5]. Yet, at the same time, IELs highly express transcripts of genes involved in immune regulation. These include cytotoxic T-lymphocyte associated protein 4, Ly49E-G, the NK cell inhibitory receptor Ig superfamily-related gp49B, and programmed cell death 1 (42). Factors involved in microbe-toxicity, such as regenerating islet-derived protein 3 gamma (Reg3 γ), are readily detected in IELs under steady state conditions (65). In addition, several transcripts have been translated, and proteins are present and stored in secretory vesicles, e.g., granzymes (66). IELs home are retained in a poised activation state in mice lacking most secondary organs (67, 68), suggesting priming in secondary organs for natural IELs is not essential and the IEL activation status may be maintained by factors in the local epithelial environment.

Natural IELs are present in axenic mice. However, reduced numbers (of induced IELs) and decreased cytotoxicity of IELs

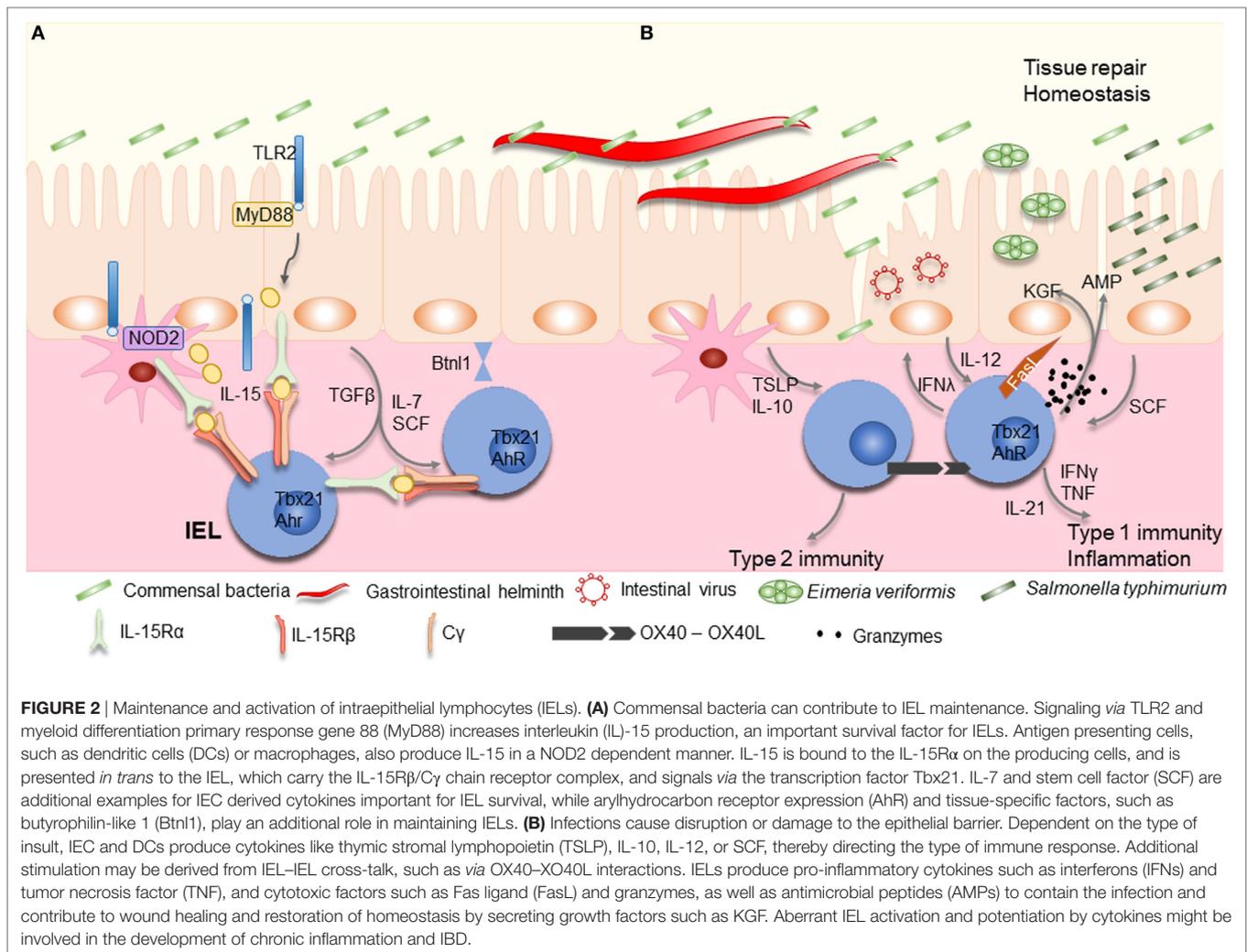
from germ-free mice indicate that signals from the microbiota or other environmental stimuli are required to maintain intestinal CD8⁺ T cells and their function (51, 69, 70). The ligand activated transcription factor, arylhydrocarbon receptor is critical for IEL maintenance (59, 71). Of interest, the provision of ligands can be achieved *via* food intake, especially green vegetables, and may also be obtained from the microbiota (59, 72). Curiously, 30–50% of IELs from conventional standard pathogen-free mice express the marker Thy1 (CD90), but those found in axenic mice do not. Colonization of germ-free mice results in the generation of Thy1-expressing IELs (69), but as yet no functional differences have been attributed to the expression of Thy1.

Intraepithelial lymphocyte maintenance and activation also critically relies on interactions between IECs and microorganisms. Myeloid differentiation primary response gene 88 (MyD88), the adapter protein used by many toll-like receptors (TLRs), interleukin (IL)-1R, and IL-18R activate the transcription factor nuclear factor- κ B, is required for IEL maintenance *via* the production of IL-15 (65, 73) (**Figure 2**). IL-15 production signals *via* the type 1 transcription factor, Tbox expressed in T cells (Tbet) to maintain IEL precursors (51). TLR2 may be at least one of the pattern recognition receptors involved in IEL maintenance, *via* IL-15 induction, its absence resulting in marked reduction of intestinal IELs (74, 75). Although IL-15 may be induced by microorganisms, they may not be essential for its production as axenic mice have reportedly higher levels of *Il15* transcripts, and no differences in numbers of natural IELs were observed (51). Nucleotide-binding oligomerization domain-containing protein (NOD)2, an intracellular sensor for microbial products has also been shown to be important for IEL maintenance (76). IELs in NOD2-deficient mice show reduced proliferation and increased levels of apoptosis. Once again, NOD2 signaling, *via* recognition of gut microbiota, results in IL-15 production (**Figure 2**). Of interest, NOD2 is able to tune the signaling of TLR2 dose dependently (77). Although these results have been achieved using whole body knock outs for MyD88, TLR2, or NOD2, and therefore, the exact role of IECs remains to be determined, they indicate that microorganisms may play an important role in IEL maintenance.

IEL Activation Status

Due to the positioning of IELs just underneath the single epithelial layer and their potential involvement in modulating intestinal pathology, the activation status of IELs is intensively studied. Transcriptional data of IELs foretells puzzling semi-activation of IELs that could enable them to deal with a broad range of pathologies quickly, with reduced requirement for immediate energy absorption and new gene expression (78). Unlike conventional CD8⁺ T cells, IELs express high levels of *Tnfsf6* transcripts during steady state (42, 43), but do not express the encoding FasL protein on their surface until additional activation takes place (61). Despite their poised state and effector-like or T_{EM} cell characteristics, IELs do not contain transcripts for cytokines, which they secrete during conditions of inflammation (43). This suggests IELs require additional cues to initiate part of their effector function capacity.

Understanding the activation properties of IELs is essential to gain insight into mechanisms of local immunity and events



associated with tolerance, chronic inflammation, and immunopathology. Intestinal mucosa resected from patients with IBD (79) or celiac disease (80) contains increased numbers of activated T cells, a hallmark of intestinal inflammatory disorders (81). Yet, in a chemically induced colitis model, dextran sulfate sodium, 2,4,6-trinitrobenzene sulfonic acid (TNBS) or T cell transfer colitis, and IELs were found to be protective (60, 76, 82–84). This raises questions regarding the role of IELs in the intestinal immune network, whether they can, at least in part, contribute to chronic inflammation and pathology, or if they have a more tolerogenic or regulatory role. Furthermore, although cytotoxicity and microbicidal activity are an important part of IEL activity, it is not clear if their potential to produce cytokines and chemokines can be tailored to the level or identity the microbial threat.

Transcriptional data also suggested that IELs are metabolically prepared for swift action. IELs, compared with memory CD8⁺ T cells, contain increased levels of mRNA for metabolic enzymes, especially those involved in the generation of fatty acids and cholesterol esters (42, 43). In line with the expression of CD69, IELs seem arrested in a semi-activated state. Yet, in stark contrast

to effector cells, IELs survive for a considerable period of time. For example, murine skin IELs are generated only during embryogenesis, but can be found throughout adult life and into old age. The skin and intestinal epithelia are lipid-rich, but availability of other nutrients may be limited (85). This may explain why skin T_{RM} cells appear to use mitochondrial β -oxidation of exogenous lipids, mediated by intracellular transport proteins, including fatty-acid-binding protein-4 and -5, supporting their longevity and protective function (78). Similarly, natural IELs highly express surface molecules involved in lipid uptake, such as apolipoprotein E and low-density lipoprotein receptor (42). The increased presence of receptors and enzymes involved in lipid metabolism in IELs compared with conventional T cells suggests that altered metabolic processes may be involved in maintaining their poised activation status. However, it remains to be determined if the increase in lipid metabolism sets IELs apart or if it reflects their semi-activated status, since recently activated conventional T cells utilize the same pathways (86). Lipids are also required for the differentiation of CD8⁺ memory T cells, the formation of which requires metabolic reprogramming

characterized by enhanced mitochondrial fatty-acid oxidation (87). Although, cell-intrinsic lipolysis is implicated in memory cell formation, suggesting the acquisition of fatty acids from the external environment is not critical, these lipids may have been obtained during the initial priming stage. These data imply that the metabolism of IELs reflects, in part, that of recently activated T cells and that lipolysis may be inhibited in IELs to arrest them in a poised activation status, thereby preventing progression to a quiescent memory status.

Role for the TCR in IEL Activation

The understanding of the role of the TCR on IEL development, differentiation, homing, and activation has long been hampered by the absence of a known selective or activating ligand. Recent work has identified tissue-specific ligands expressed in the thymus, driving the development and homing of either murine skin IELs or intestinal TCR $\gamma\delta$ IELs, not peptide-MHC or lipid-CD1 complexes, but the butyrophilin-like molecules skint-1 and butyrophilin-like 1 (Btl1), respectively (88–90). The role of the TCR $\alpha\beta$ expressed on natural CD8 α IELs remains unknown (91). The collective data from these studies strongly suggest that the TCR $\gamma\delta$ is required for both thymic selection and imprinting of IEL identity as well as their maintenance in specific tissue niches. However, butyrophilins, part of the immunoglobulin superfamily [for detailed review see Rhodes et al. (92)], are, unlike MHC or CD1 molecules, not known to present ligands. Thus, it remains unclear if IELs are stimulated *via* their TCR or if they sense other cues, such as inflammatory, tissue damage or cell stress factors provided by IECs or accessory cells (93), or are under the influence of metabolic alterations as a result of cell damage or bacterial growth in the microenvironment which can enhance T_H cell subset differentiation (94).

Agonist-driven positive selection of IELs in the thymus suggests that mature IELs at the epithelial barriers could subsequently be activated by specific TCR ligands. IEL TCR activation may be achieved by cell surface receptors, such as non-classical MHC molecules (95, 96). The parameters required for an agonist to activate IELs upon conditions of inflammation or tissue damage exclude constitutively expressed surface molecules, such as skint-1 and Btl1, unless a gradient reaching a critical activation threshold can be achieved. If an agonist able to activate IELs exists, it does not preclude direct IEL activation by microbial products such as observed for $\gamma\delta$ T cells in both man and mouse (97–99). Such direct activation is commonly observed in conventional CD8⁺ T cells that have been pre-selected in the thymus and successfully primed and expanded in the periphery. Reactivation of memory CD8⁺ T cells is readily achieved by cytokines and TLR ligands, resulting in secretion of IFN γ from polyclonal T cells that bridge innate and adaptive immunity (100). Similarly, peripheral $\gamma\delta$ T cells can be directly activated by TLR ligands and combinations of cytokines (99, 101).

Administration of anti-CD3 ϵ antibodies, which directly stimulate the TCR signaling complex thereby bypassing TCR-specific ligation, has often been used as a proxy to stimulate IELs in mice. However, its systemic activity, due to indiscriminate total T cell activation in all tissues, results in “cytokine release syndrome,” increasing serum levels of IL-2, TNF, and IFN γ , and

leading to intestinal phenotypes, such as diarrhea (102, 103). The small intestines from mice treated with anti-CD3 show increased epithelial ion transport, altered spontaneous muscle activity, and reduced IEC viability (104). The effect of anti-CD3 is rapid, with DNA fragmentation observed after 30 min in the areas most enriched with IELs, followed within hours by IEC shedding into the lumen (105). Similar effects on IEC viability were observed upon administration of anti-TCR $\gamma\delta$ antibodies, but not with those stimulating TCR $\alpha\beta$ (106). The effect on IEC shedding, however, was fully dependent on TNF receptor signaling and may not necessarily depend on IEL activation since conventional T cells can also secrete large amounts of TNF.

Following anti-CD3 stimulation, poised IELs acquire aspects of fully activated effector T cells with higher expression of CD44, Ly-6C, OX40, FasL, and CD25 and reduced expression of CD45RB protein, accompanied by expression of cytotoxic mediators as well as cytokine transcripts (61, 107). Effects of anti-CD3 on IEC viability appear to correlate well with the cytotoxic capacity of IELs, especially since release of granzyme B is observed upon anti-CD3 stimulation (62). However, DNA fragmentation is independent of the pore forming protein perforin (62). This suggests that IECs are non-specifically targeted by their proximity to activated T cells or by their susceptibility to soluble mediators. The accumulative data postulate that *in vivo* activation of IELs can at least in part be achieved *via* TCR ligation. IEL activity can have a major impact on intestinal physiology, altering electrolyte balance and IEC viability. However, their potential to damage IECs markedly contrast with the requirement to maintain an intact single cell intestinal barrier to efficiently protect the host and questions if TCR stimulation accurately recapitulates the physiological role of IELs. IEC–IEL bidirectional interactions are instrumental to maintain IELs, but it remains unknown if IECs directly contribute to IEL activation and, if they do, what the identities of the activating cues are?

IEL Activation by Microbes

Commensal bacteria can invade tissues when opportunity arises. Such opportunities occur upon initial microbial invasion of new-borns before species-specific adaptive immunity has fully developed or when the host is immune compromised (108). Since, activating IELs may not require antigen processing or rely on presentation by MHC-like molecules, it remains possible that IELs recognize molecular patterns generated by bacterial non-peptide antigens or conserved unprocessed protein antigens produced by bacteria or released by epithelial cells upon damage or cell stress (109).

Invasion of pathogens or tissue damage could create the conditions for commensal microorganisms to invade the intestinal tissues. Innate immunity relies on the detection of highly conserved pathogen-associated molecular patterns (110). Receptors involved in the detection of invasion will respond to the microbial components present in both pathogen and commensal microorganisms. But it has become clear that not all microorganisms evoke a similar response. Indicators of viability, such as the presence of prokaryotic mRNA invoke a much stronger immune response (111). The balance of immunity and tolerance at the epithelial interphase is also illustrated by the production of IgA,

the predominant antibody isotype critical at mucosal sites (112). IgA mainly coats pathogenic bacteria, which can confer colitis in axenic mice (113). IgA is a poor activator of the immune system; in line with the idea that strong immunity at mucosal sites is best avoided. IELs have been implicated in coordinating IgA response. TCR δ -deficient mice, harboring reduced IEL numbers, show reduced IgA levels in serum, saliva, and fecal samples. TCR δ -deficient mice also produce much lower levels of IgA antibodies upon oral immunization (114).

Role for IECs in IEL Activation; Co-stimulation

A requirement for co-stimuli or T_H cell help is linked with the need for clonal expansion and differentiation, creating a significant delay in adaptive immunity. Immune surveillance by tissue-resident lymphocytes requires a swift response without prior cell expansion or differentiation, observed for $\gamma\delta$ T cells and memory T cells, such as T_{RM} cells (27, 115). This is in line with the hypothesis that the IEL response primarily serves to contain a potential threat, not necessarily resulting in microbe eradication or the establishment of immunological memory, thereby limiting microbial or toxin dissemination and keeping the single cell barrier intact by avoiding intestinal pathology.

Homing of IELs to the small intestine seems consistent with oligoclonal activation by commonly encountered antigens. The poised activation status of IELs could ensure their rapid activation without the need for an array of instructive signals. IELs seem not to depend on signal 2, required for conventional T cell activation and protective immunity (116). CD28 as well as additional co-receptors, such as CD2 and CD5, appear reduced or absent from IELs (43, 117–119). Furthermore, expression of MHC molecules or the costimulatory B7 proteins on at least keratinocytes is not required for activation of skin IELs (97). However, since the triggering of IELs could contribute to immunopathology, their activation is likely controlled on several levels, such as by signals derived from inflammation or tissue damage. The absence of a requirement for classic costimulatory signals for IEL activation suggests that close interactions with IECs play a prominent role.

OX40 (CD134, TNFRSF4) is expressed by activated T cells controlling cell expansion (120), including IELs (121). Its expression correlates well with T cell activity observed in patients with IBD, active celiac disease, Crohn's disease (CD), and ulcerative colitis (UC) (122, 123). *In vitro* activation of IELs with anti-CD3 ϵ antibodies results in the expression of both OX40 and its ligand (OX40L) (121). Of note, OX40L is not expressed upon activation of conventional T cells. Additional ligation of OX40 seems to boost IEL activity and reduce the secretion of IL-10. This suggests that accumulation of IELs at sites of inflammation may alter their potential and that such co-stimulation may not necessarily depend on OX40L expression by IECs or myeloid cells (Figure 2).

Skin and intestinal IELs express the junctional adhesion-like molecule-1 (JAML-1), which provides co-stimulation upon ligation with the coxsackie-adenovirus receptor (CAR) (124, 125). JAML signaling results in cytokine production from skin IELs and may provide additional context for full IEL activation, presumably

as response to infection or tissue damage. However, the latter requires its expression to be regulated upon insult or microbial invasion, which remains to be determined. Furthermore, its ligation by neutrophil-derived soluble JAML compromises intestinal barrier integrity and reduces wound repair through decreased IEC proliferation (126). Thus, the role of JAML—CAR in barrier defense remains to be clarified.

Role for IECs in IEL Activation; Cytokines

Intestinal IELs can express receptors for TNF, leukemia inhibitory factor, thymic stromal lymphopoietin (TSLP), stem cell factor (SCF; c-Kit ligand), transforming growth factor (TGF) β , IL-12, IL-15, and IL-21 (43, 127). TGF β , most likely derived from IECs upon microbial stimulation, is required to maintain natural CD8 $\alpha\alpha$ IELs and to induce CD103 expression. The absence of TGF β or its receptor results in markedly reduced numbers of IELs, while over expression increased the IEL population (50). How TGF β influences IEL activity remains unknown.

Interleukin-15 plays a central role in maintenance of natural IELs and emphasizes the close interactions between IECs and IELs (128–131). IL-15 is presented in *trans* to IELs by epithelial cells, in the thymus, skin, and the intestine, which express both the IL-15R α and IL-15 (132). IL-15R signaling induces the expression of anti-apoptotic molecules, Bcl-2 and Bcl-xL by IELs (133). The production of IL-15 is regulated, at least in part, by contact with microbial components. MyD88- and TLR2-derived signals are required for IEL maintenance *via* the induction of IL-15 production (65, 73, 75). T_{RM} cells are similarly dependent on IL-15-mediated signals (134), whereby high levels of IL-15 can TCR-independently trigger CD8⁺ T cells to become cytotoxic (135, 136). Upon IEC damage, IL-15 production increases (65), as observed during celiac disease, and correlates strongly with IEL activity (133, 137). IL-15 stimulation of IELs results in increased IFN γ and TNF production, granzyme-dependent cytotoxicity, NK receptor expression, and increased survival (138). Of note, the increase in IL-15 production in conjunction with additional cues, such as retinoic acid can stimulate DCs, thereby inducing the secretion of pro-inflammatory factors and indirectly activate IELs. IL-15 induces the secretion of IL-21 by IELs, observed in celiac disease, which may be part of a self-sustaining feed-forward loop as observed in Th17 cells, enhancing IEL activation and cytotoxicity (139, 140).

Another important cytokine involved in T cell homeostasis is IL-7 (141). It is secreted by non-hematopoietic cells, especially thymic and IECs, with enhanced expression observed upon tissue damage (142–144). IEL development requires IL-7R signaling in the thymus, but local IL-7 expression by IECs can restore the $\gamma\delta$ IEL subset, not other $\gamma\delta$ T cell subsets, suggesting extrathymic development or maturation of $\gamma\delta$ IELs may take place in the intestinal compartment (51, 142, 145). The use of acute IL-7 reporter mice indicates that production of IFN γ by T cells, such as IELs, can modulate the level of IL-7 and IL-15 produced by IECs, thereby regulating IEC homeostasis, absorptive function as well as the composition of the microbiota (144, 146). Vice versa, IEC derived IL-7 can regulate IEL survival and proliferation, particularly induced CD8 $\alpha\beta$ IELs (147). Overexpression of IL-7 results in lymphoid expansion and colitis (148).

Intestinal epithelial cells show a basal production of TSLP that is important for host protection during helminth infections (149). Similar to IL-15, TSLP receptor stimulation of CD8⁺ T cells enhances expression of Bcl-2 (150), and may play a role in IEL survival. TSLP seems to enhance type 2-mediated immunity (Figure 2). Upon TSLP encounter, IECs and DCs produce IL-10 and reduce IL-12 production, thereby reducing type 1 immunity (151). IELs are known to be able to secrete IFN γ and loss of intestinal integrity results in IEC-produced IL-12 (152), the prototypical driver of type 1 immunity. In the absence of TSLP, mice are more susceptible to colitis and have increased levels of IFN γ producing cells. *Salmonella typhimurium* infection increases the expression of SCF produced by IECs (153). Its receptor, c-Kit, is expressed by IELs (127). The absence of SCF results in marked reduction of IEL numbers in mice, while its presence seems to play a role in IEL activation (154). Whether SCF and TSLP act as instructive cues initiating divergent IEL activation profiles remains to be investigated.

Containing Invasive Microbes

An ascending bacterial load exists from duodenum to jejunum and ileum, accumulating in very high numbers in the cecum and colon. Of note, IEL numbers are descending from duodenum to ileum, with few found in the colon (4). The causality of this striking inverted relationship remains unknown. IELs can produce antimicrobial factors and tissue repair factors in response to bacteria that penetrate the intestinal epithelium (60). IELs play an important role in the regulation and differentiation of epithelial cells at the base of the crypts (58, 155). IELs thereby help to preserve the integrity of damaged epithelial surface by providing the localized delivery of an epithelial cell growth factor (60). The mucosal protection afforded by IELs is of critical importance particularly during the first hours after bacterial exposure (156), in line with the hypothesis that IELs function to contain microbes upon invasion and initiate barrier repair thereby reducing immunopathology. With respect to pathogenic infections, in the majority studied, IELs offer protection against a wide variety of intestinal species, including *Eimeria vermiformis* (64, 157, 158), *Toxoplasma* (159, 160), *Encephalitozoon cuniculi* (161), Norovirus (107), and *Salmonella* (159, 160). Interestingly, infections with at least the pathogens *Salmonella* and *Toxoplasma* have indicated that IELs migrate to the site of infection and into the lateral intra-intestinal space (160), possibly initiating IEL-IEL co-stimulation (121). Collective release of preformed antimicrobial peptides (65, 156, 162) could directly contribute to microorganism containment and clearance (163).

Upon intestinal infectious challenges, protection and pathology are a result of the interplay between the microbes, the IELs, conventional T cells, and other immune cells. During *Eimeria* infection, IELs' production of IFN γ and TNF is instrumental in protective immunity, and expression of junctional molecules to preserve epithelial barrier integrity (158). However, elevated IFN γ and TNF levels in the intestinal mucosa also contribute to the pro-inflammatory cascade involved in barrier disruption and pathology (164). $\gamma\delta$ IELs are able to reduce pathology and their absence exaggerates mucosal injury upon *Eimeria vermiformis* infection (157). The absence of $\alpha\beta$ T cells results in

reduced capacity to clear the parasite, in part compensated by the adoptive transfer of CD4⁺ T cells. In the absence of $\gamma\delta$ IELs, *Salmonella* or *Toxoplasma* infection result in increased microbe transmigration due to reduced epithelial barrier integrity. This increased transmigration leads to increased immunity mediated by conventional T cells (159). This indicates that IELs are not ultimately responsible for microbial clearance, but can modulate the initial response and recruitment of immune cells in order to moderate the risk of immunopathology.

Intraepithelial lymphocytes can contribute to viral immunity. Viral control depends largely on conventional T cells but, at least, $\alpha\beta$ IELs take part in viral clearance in the mucosa (165–167). Intestinal viral challenge, such as the non-enveloped RNA virus norovirus (MNV) present in many laboratory animal facilities, results in infection of IEC and myeloid cells (168). The infection can be controlled by IFNs, particularly IFN λ (169). Of note, the IFN λ (IFN type III) response seems to be operating particularly at epithelia barriers. This indicates that barrier immunity is kept local, to avoid systemic responses in which type I IFNs play a dominant role (170, 171). IELs, upon stimulation with plate-bound anti-CD3, can transcribe IFN genes, type I, II, and III, and the supernatant of *in vitro* activated IELs reduces viral infection (107, 172). *In vivo* anti-CD3 administration as well as culture supernatant from activated IELs results in IFN type I/III receptor-dependent expression of IFN responsive genes in intestinal IECs. Administration of anti-CD3 antibodies before intestinal viral challenge with murine norovirus can reduce viral load (107). However, due to the polyclonal stimulation of all T-lymphocytes, it remains unclear what contribution IELs provide and which properties may be uniquely attributed to them. It remains unknown if IELs are stimulated upon intestinal viral invasion and if so, how such invasion would enable the activation of IELs.

Besides microbicidal and cytotoxic activity, IELs produce cytokines and chemokines. Some chemokine transcripts are already present in IELs under steady state conditions, such as CCL5 and XCL1, but not those encoding for cytokines, such as IFN γ and TNF (43). This suggests that the recruitment of additional immune cells and release of these powerful cytokines and other chemoattractants such as CXCL1, CXCL2, and CXCL9 (65), might be delayed compared with cytotoxicity. The clearance of pathogens and the instigation of immunological memory involve careful orchestration of various cellular components. At epithelial sites, pathogen containment by innate-like T-lymphocytes may precede recruitment of myeloid cells and subsequent involvement of the adoptive arm of the immune response.

IELs in IBDs

The initiation of an immune response is not taken lightly, especially at the intestinal barrier. When immune activation does take place, pathogen clearance, pathology, and the need to maintain tissue integrity and its repair are offset. The consequences of microbial invasion or aberrant immunity can be severe (81). IBD can affect any part of the intestine and present with extra-intestinal manifestations. Despite advances in IBD understanding, the cause(s) and mechanism(s) remain unknown and disease incidence is increasing with changes in the environment and life style likely

making a contribution (173). Disease occurrence and severity are further compounded by diet, dehydration, and antibiotic use (174) as well as age (175).

There are indications that IBD results from alterations in innate immunity resulting in excessive adaptive immune activation (176, 177). IBD seems to result from immunogenetic predispositions and environmental factors, especially a dysregulated response to microorganisms (178). The presence of micro-infections or patches of affected intestinal tissue, associated with bacterial presence, next to seemingly unaffected tissue suggest localized immune activation or inefficient immune resolution (179). Several immunologic and histopathologic features of IBD, such as the presence of activated T cells secreting IFN γ and IL-17 and immunopathology (180), can be explained as a defect in mucosal immune regulation and as a consequence of persistent mucosal T cell activation (181). Several studies have shown that IL-12 production is increased in inflammatory lesions of patients with CD (182). In line with activated T cells, UC patients have an increased level of IL-7 and IL-15, which may perpetrate additional T cell activation (183, 184).

There is limited data for a role of IELs in IBD, either disease enhancing or reducing. Accumulation of $\gamma\delta$ IELs in inflamed areas of IBD patients have been reported, and associated with increased levels of IL-15 and TSLP (133, 151). Although identifying lamina propria migrated $\gamma\delta$ T cells and $\gamma\delta$ IELs is difficult, these cells constitutively produce IFN γ in patients suffering from either CD or UC (185). It remains to be determined if reduced numbers of IELs contribute to increased susceptibility to inflammation or are a consequence of ongoing inflammation, such as due to an increase in $\alpha\beta$ effector T cells or activation-related cell death. Murine studies have demonstrated potential protective roles for $\gamma\delta$ IELs in intestinal inflammation as well as IBD models (59, 82, 186), but others have suggested that IEL expansion and activation can exacerbate the progression of colitis (187, 188). Lesions in IBD are mainly found in areas of reduced IEL density and highest load of bacteria, the colon, and ileum (178). The numbers of IELs correlate inversely with disease severity, and IEL numbers are restored to levels observed in healthy controls upon treatment with anti-TNF (189). This indicates vulnerability at those sites with altered immune surveillance and intense bacteria–IEC interactions, in line with the presence of adherent-invasive *Escherichia coli* at IBD lesions (179).

Loss-of-function mutations of NOD2 are strongly associated with CD (190–193). This correlates with the loss of IELs seen in mice deficient for NOD2 (76), but detailed insights are lacking and NOD2 plays an important role in other cell populations such as DCs, able to influence type 1 and type 3 immunity (194). Reduced proportions of $\gamma\delta$ IELs at the intestinal mucosa of CD patients suggested a protective role for these cells (189, 195, 196). IELs show increased activity at inflammatory sites in IBD patients, secreting IFN γ and TNF (189). Furthermore, IELs may enhance the production of IFN γ in the human colon (197). Nevertheless, murine IELs have been shown to be able to reduce the production of IFN γ production by conventional CD4 T cells, indicating their capacity to reduce

type 1 immunity (198). In line with IELs forming an important part of the first line of defense is the increased susceptibility to infection of CD patients, which have reduced IEL numbers, with the intracellular parasite microsporidia (199). Unfortunately, the data on a role for IELs in preventing or reducing susceptibility to IBD remain inconclusive. The majority of IELs are found in the small intestine, a site not easily accessible, and IELs as a population of human T-lymphocytes are not well defined with respect to their identity and location. Often $\gamma\delta$ T cells, such as those found in the circulation, are used as a proxy for IELs. However, murine studies and data on human TCR δ -chain usage have shown IELs to be very different from $\gamma\delta$ T cells found in other tissues [(55, 59) #1587].

CONCLUSION

Intraepithelial lymphocytes are an integral part of the epithelial barrier. They do not exist as isolated cells monitoring the front line, but have close bidirectional interactions with IECs and possibly other immune cells. This close interaction enables the reception and provision of signals at very close range to maintain epithelial integrity. This interaction may be crucial in initiating local repair and containing low level of microbial invasion. Localized cues of potentially low dose would enable the activation of poised IELs without necessarily alerting the adaptive immune system. The single cell epithelial barrier is under constant threat of assault. Containing such threats locally with minimal immune activation is of great benefit to limit immunopathology and maintain optimal nutrient uptake.

How IELs, displaying many characteristics of effector T cells, are maintained in a poised state remains poorly understood. Differences identified in metabolic pathways may reflect their partial activation status or indicate differential metabolic wiring of IELs. The cues that enable full IEL activation remain ill-defined and the activity of IELs, the existence of different modes of action, are unknown. With technological advantages, such as multicolor flow cytometry and microscopy new players at the mucosal sites have been identified. This may now help us to unravel the complex multiplayer immune surveillance network of the mucosal immune response with real potential to discover novel targets for therapies to alleviate or even cure the different forms of IBD and celiac disease.

AUTHOR CONTRIBUTIONS

All authors wrote the review and contributed to the figures.

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