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Impaired selection of IgA and intestinal dysbiosis associated with PD-1-deficiency

Mikako Maruya, Shimpei Kawamoto, Lucia M. Kato and Sidonia Fagarasan*
Laboratory for Mucosal Immunity; Research Center for Allergy and Immunology; RIKEN Yokohama Institute; Yokohama, Japan

A major function of immunoglobulin A (IgA) is to maintain balanced bacterial communities in the gut. We have previously shown that diversification of IgA upon somatic hypermutation (SHM) is critical for IgA function yet the principles governing the selection of IgA in the gut have remained elusive. Here we discuss recent progress in understanding this process as revealed by our studies in mice that lack the inhibitory co-receptor programmed cell death–1 (PD-1). We found that PD-1 affects the dynamics of germinal center (GC) B cells by controlling the number and the nature of T helper cells in the Peyer's patches (PPs). Deregulation of the T cell compartment impacts the selection of IgA plasma cells leading to gut dysbiosis. When the PD-1-dependent checkpoint is missing, gut bacteria go beyond the mucosal barrier and induce systemic GCs that can generate antibodies with auto-reactive properties.

Keywords: germinal center, gut, IgA, microbiota, PD-1, T_{FH}

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*Correspondence to: Sidonia Fagarasan; Email: sidonia-f@rcai.riken.jp

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Introduction

The gut is colonized by trillions of bacteria that perform vital metabolic and defensive functions for the host. These bacteria, generally called commensals, coevolved with mammals and for this reason they are integral in digestive and immune system function and homeostasis. Besides serving as natural defense against invading pathogens, commensals have important contribution to nutrient processing, generation of immunomodulatory products (vitamins, short-chain fatty acids, etc.) and to the development of the immune system. Conversely, the immune system maintains diverse and healthy bacterial communities in the gut, which is facilitated by innate and adaptive mechanisms, the latter represented by dynamic production of immunoglobulin A (IgA).[1]

A conspicuous response that follows the gut bacterial colonization is the production of IgA by the B cells located in the gut-associated lymphoid tissues (GALT).[2] IgA, the most abundantly produced antibody isotype in mammals, is secreted into the gut lumen [also called secretory (S)IgA] as dimeric molecules linked by a "joining" chain and wrapped within a secretory component also called polymeric Ig receptor (pIgR) (that mediates export of polymeric Ig to mucosal surfaces). The protective role of SIgA was elucidated in the context of mucosal infections, where it was shown that IgA acts as a first line of defense by preventing attachment and limiting the access of microorganisms to or beneath the epithelium—a process known as immune exclusion.[3]

However, it is increasingly clear that IgA was selected and maintained throughout the evolution not just to stop pathogen entry through the epithelium but rather to maintain the complex interplay between commensals, epithelium and immune system. Recent studies revealed that mice completely devoid of B cells or SIgA (e.g., μMT, $IgA^{-/-}$, $AID^{-/-}$, $pIgR^{-/-}$ mice) showed changes in microbial ecology in the gut, impaired epithelial barrier function, increased serum titers of antibodies specific for commensal bacteria, food antigens or self-antigens.[1,4]

Furthermore, diversification of IgA repertoire by somatic hypermutation

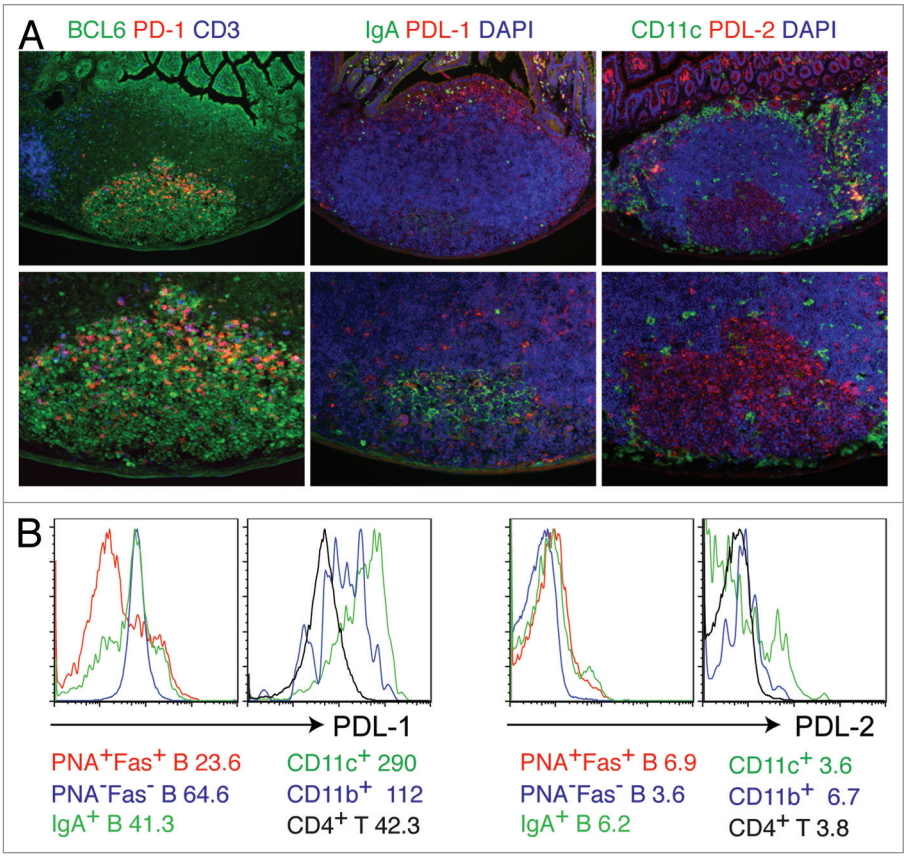

Figure 1. PD-1 and PD-1 ligands expression in Peyer's patches. (**A**) Sections of the PPs from WT mice as indicated color of lettering below. Original magnification: upper panels, 10 ×; lower panels, 20 ×. Note the high expression of PD-1 on T cells and PD-L2 expression by B cells located in the GCs (area defined by high expression of BCL-6). (**B**) Representative histograms of PP cells stained for the surface markers indicated. Numbers below the histograms indicate the geometric mean fluorescence intensity of PD-L1 and PD-L2 in the corresponding color-coded subset of cells.

(SHM) appears critical to maintain intestinal homeostasis. Indeed, studies on mice lacking the activation-induced cytidine deaminase (AID) or expressing the mutant AID (AID^{G23S}) that supports the class switch recombination (CSR) but not the SHM process have demonstrated that mutated-presumably selected high-affinity IgAs are required to control the balance of bacterial community and epithelial integrity.[5,6]

SHM takes place mostly in germinal centers (GC), specialized microenvironments, in the presence of AID and T cells, named T follicular helper (T_{FH}) cells.[7,8] T_{FH} cells are defined as T cells expressing high levels of the chemokine receptor CXCR5 and inhibitory co-receptor programmed cell death-1 (PD-1).[9] While the role of CXCR5 on T_{FH} is clear (it allows their migration in response to CXCL13 chemokine produced by follicular dendritic cells (FDCs) at the core of the B cell follicles) the function of PD-1 was obscure. In fact, the very high expression of PD-1, which is characteristic of anergic[10] and senescent[11] T cells, is at odds with the efficient helper activities of T_{FH} cells.

We sought to elucidate the role of PD-1 in GC biology. Our quest was fueled by several observations made during years by Honjo and his colleagues at Kyoto University, namely that: (1) PD-1 plays a critical role in maintenance of tolerance as $PD\text{-}1^{-/-}$ mice develop species-specific autoimmune diseases,[10] (2) the incidence of diseases in $PD\text{-}1^{-/-}$ mice varies largely among mouse colonies[10,12] and (3) $PD\text{-}1^{-/-}$ mice crossed on AID-deficient background do not develop diseases.[12]

All these remarkable observations suggest that autoreactive antibodies in PD-1-deficient mice may arise after AID-induced genetic alterations in GCs, which are driven by stimulation from the gut microflora.[13]

PD-1 and PD-1 Ligands

PD-1 is an inhibitory receptor found on the surface of activated T cells and some B cells that plays a critical role in shutting down ineffective immune responses and maintenance of tolerance.[14] PD-1 belongs to the CD28-CTLA4 Ig superfamily and interacts with two ligands: PD-L1 and PD-L2. Hematopoietic and non-hematopoietic cells in lymphoid as well as non-lymphoid organs widely and constitutively express PD-L1. By contrast, PD-L2 is more restrictively expressed on dendritic cells (DC) and macrophage subsets, GC B cells and memory B cells.[15]

In the gut, particularly in the organized structures called Peyer's patches (PPs), the highest expression of PD-1 is found on T cells localized mainly in the GC (predominantly the FDC-rich zone, called light zone of GC, where B cell selection is thought to take place) (**Fig. 1A**, left panels).[16] The PD-L1 is most abundantly expressed by DCs and macrophages located in subepithelial dome, T cell zone and GCs of the PPs. Curiously, the PD-L1 expression seems downregulated on GC B cells, but re-induced on IgA B cells and further upregulated in plasma cells (**Fig. 1A** middle panels, **B** and data not shown). PD-L2 is expressed by a subset of DCs located in T cell zone of PPs, but in contrast to PD-L1, it is also expressed by GC B cells and IgA B cells located predominantly in the light zone of GCs (**Fig. 1A** right panels **and B**). Thus, PD-1 on T cells would be engaged by PD-L1 or PD-L2, depending on the location, activation and differentiation stage of the interacting cells. In the GCs, it seems that the PD-1 on T_{FH} cells is mostly engaged by PD-L2 expressed on GC B cells, and this interaction would probably modulate in situ the GC reaction. The PD-1 interaction with PD-L1 on DCs takes place before T cells becoming T_{FH} cells and this engagement would be important to control the size of T cell compartment

and probably the phenotype and repertoire of the PP T cells.

PD-1 Regulates Bacterial Communities in the Gut

We set to evaluate the gut microbiota in PD-1$^{-/-}$ mice. Classical microbiological methods revealed an equivalent number of culturable bacteria in the small intestine of WT and PD-1$^{-/-}$ mice. However, PD-1$^{-/-}$ mice had a severe reduction (more than 90%) in the number of anaerobic bacteria. The total numbers of "healthy" bacteria such as *Bifidobacterium*[17] and *Bacteroidaceae* were undetectable or markedly reduced in PD-1$^{-/-}$ mice. By contrast, the *Enterobacteriaceae*, which are minor representatives in the small intestine of WT mice, were significantly increased in PD-1$^{-/-}$ mice (**Fig. 2A**). These results were confirmed and extended by culture-independent analyses of amplicons generated by primers directed against variable region 2 and 3 of bacterial 16S rRNA genes. There were clear differences in gut microbial ecology associated with the mouse genotype. At the Phylum level, PD-1$^{-/-}$ mice had increased percentages of Firmicutes, Proteobacteria and TM7 and a decreased frequency of the Bacteroidetes and Deferribacteres compared with WT mice (**Fig. 2B**). Analyses of the family-level distribution revealed that PD-1 deficiency associated with an increase in *Erysipelotrichaceae* of the Firmicutes, *Prevotellaceae* of the Bacteroidetes, *Alcaligenaceae* of the Proteobacteria and TM7 *genera incertae sedis* (data not shown).

Interestingly, some of the bacteria increased in PD-1 deficiency are already reported to be associated with several pathological conditions. Alcaligenes is an indigenous opportunistic bacteria residing in the organized structures such as PPs[18] that was shown to promote systemic inflammation in mice lacking innate lymphoid cells.[19] Moreover, expansion of certain species of Proteobacteria (i.e., *E. coli*) was linked with intestinal inflammation and colitis-associated colorectal cancer,[20] while expansion of *Prevotellaceae* and TM7 were shown to be involved in systemic auto-inflammatory[21] and metabolic disorders associated with inflammasome-deficiencies.[22]

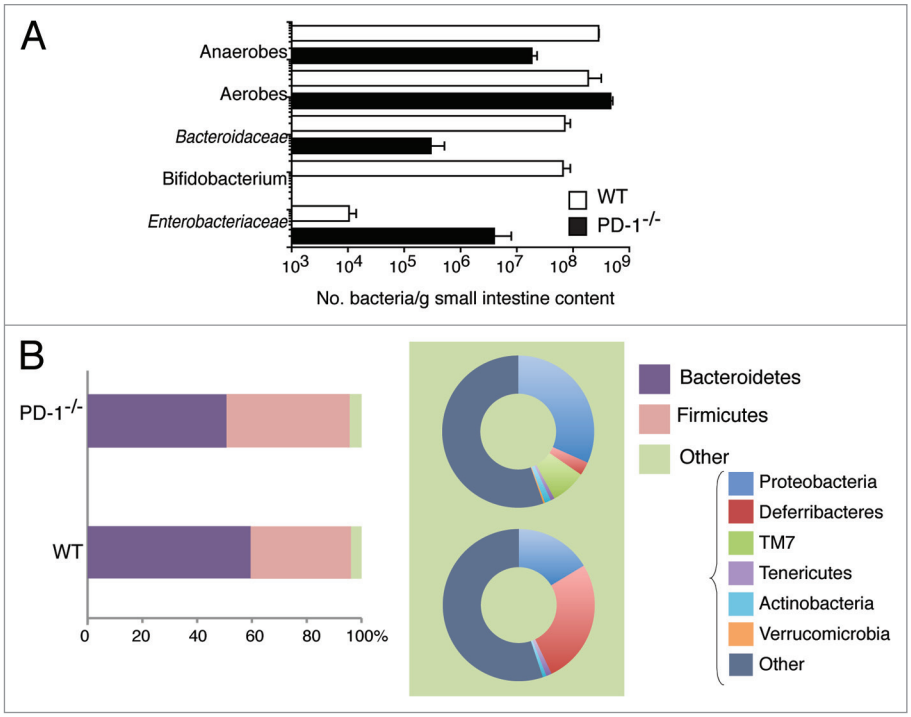

Figure 2. Microbial structure in the gut of WT and PD-1-deficient mice. (**A**) Culture-dependent analyses of gut microbiota. Contents of the entire small intestine from three mice of each genotype (two month old and kept in specific-pathogen free conditions) were pooled and bacteria were identified using standard microbiological methods. Note the absence of *Bifidobacteria* and the increased in *Enterobacteriaceae* in PD-1$^{-/-}$ mice. (**B**) Culture-independent analyses of gut microbiota. Phylogenetic classification of 16S rRNA frequencies in the ceccal contents from WT and PD-1$^{-/-}$ mice. Minor phyla are represented in doughnut charts.

PD-1 Deficiency Impact on Quality of IgAs in Gut

An important function of intestinal IgA is to maintain a highly diverse and balanced bacterial community in the gut and as such to prevent the expansion of certain bacterial groups that could cause excessive activation of the immune system. The IgA regulatory function is partly achieved through bacterial coating/shielding. Indeed, in the absence of IgA, such as in AID$^{-/-}$ mice, we observed expansion of segmented filamentous bacteria (SFB) that attached to the epithelial cells and induced generalized hyperplasia of the immune system.[5,23] Gut dysbiosis manifested with a skew toward Firmicutes over Bacteroidetes and expansion of Proteobacteria was also observed in AID^{G23S} mice capable to undergo CSR (and hence with normal levels of IgAs) but defective in SHM.[6]

Thus, we inquired whether PD-1 deficiency impacts on IgA compartment in the gut. At first glance, there were no differences in the frequencies and numbers of IgA plasma cells in the lamina propria (LP) between WT and PD-1$^{-/-}$ mice. Nevertheless, in-depth analyses revealed that the IgAs secreted into the gut lumen of PD-1$^{-/-}$ mice had reduced bacteria-binding capacity, as the proportion of bacteria coated with IgA was considerably reduced in PD-1$^{-/-}$ mice compared with WT mice. The observed bacteria-coating reduction could be due to poor quality of IgAs or alternatively (but not mutually exclusive) to different structures of bacterial communities in the gut of PD-1$^{-/-}$ mice. We have obtained supporting evidence for the former possibility (the second remains to be further tested).

Both WT and PD-1$^{-/-}$ mice had a highly diverse, polyclonal IgA repertoire with most (> 85%) of the IgH sequences having SHM and high ratios of replacement (R) to silent (S) mutations in

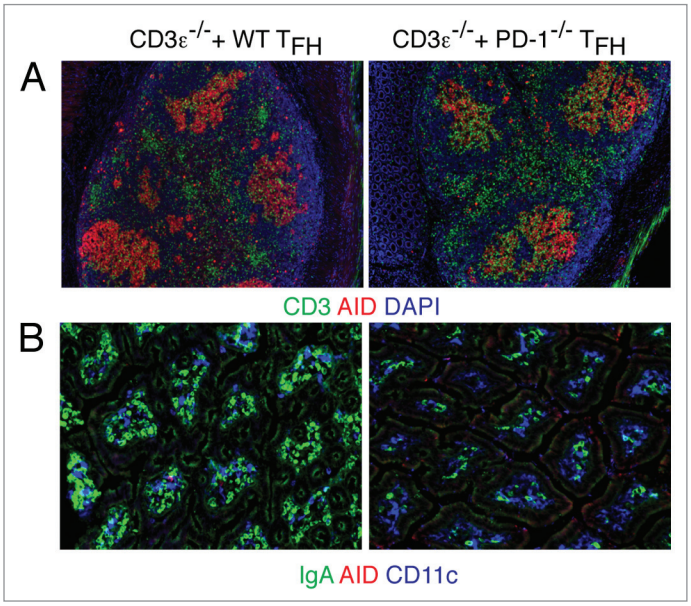

Figure 3. T_{FH} cells from PD-$1^{-/-}$ mice expanded in PPs but failed to support generation of IgA plasma cells upon their transfer into CD3$\varepsilon^{-/-}$ mice. (**A**) Sections of the PPs and (**B**) small intestine stained as indicated from the CD3$\varepsilon^{-/-}$mice 3 mo after reconstitution with T_{FH} cells from WT and PD-$1^{-/-}$ mice. Original magnification: panels in (**A**) 5 ×; panels in (**B**) 20 ×.

complementarity-determining regions (CDR) compared with those in framework regions (FWR) as signs of antigen-mediated selection. However, the affinity maturation was lower in IgA-producing cells isolated from LP of PD-$1^{-/-}$ mice. Thus, the reduced bacteria coating appears to be due to reduced affinity maturation of the IgA responses in PD-$1^{-/-}$ mice. Therefore, PD-1 plays a role in regulation of antibody diversification that impact on symbiotic relationships between host and commensal bacteria in the gut.

PD-1 Regulates Selection of IgA in Germinal Centers of Peyer's Patches

As most of the mutated IgAs present in LP are generated in PP GCs, we wished to know how PD-1 deficiency impacts on the GC reaction in gut. Of note, two characteristics distinguished GC in PPs from those induced upon immunization in peripheral lymph nodes (pLNs) namely: (1) PP GCs are constantly induced by bacteria and thus are non-synchronized GCs triggered by various and perhaps variable antigens and (2) most of the AID-expressing B cells in GC of PPs switch to IgA while the preferred isotype by pLNs or spleen GC B cells is IgG.[2,4] The latter aspect depends on the availability of TGF-β1, an IgA-cytokine abundantly secreted and presented in active form among others by PP FDCs conditioned by bacterial and metabolic products, such as LPS and retinoic acid.[24]

The frequency and absolute number of IgA^+ cells were higher in PPs of PD-$1^{-/-}$ mice than in WT mice. Yet, the frequency of clonally related sequences (with identical V_H-D_H-J_H and junctions) was reduced in PD-$1^{-/-}$ mice compared with WT mice, possibly indicating impaired clonal expansion of IgA^+ B cells in GCs of PD-$1^{-/-}$ mice. Indeed, the classical BrdU incorporation pulse-chase study revealed an increased turnover of GC in PD-$1^{-/-}$ mice with a quicker passage through the PPs which likely associates with inappropriate selection. In these non-synchronized gut GCs we could not observe significant differences in affinity maturation of GC IgA between WT and PD-$1^{-/-}$ mice. However, what we did observe was reduced affinity maturation of IgAs in LP, enhanced turnover of IgA-plasmablasts in LP which resulted from a quicker inflow from the GCs and enhanced cell death in LP of PD-$1^{-/-}$ mice. These observations indicate that the PD-1 deficiency affects the selection of IgAs in the gut. Thus, unlike in the pLN GCs, the elimination of inappropriate B cells generated in PP GCs takes place not only in situ but also after their arrival as still proliferating plasmablasts into the LP, in both WT and PD-$1^{-/-}$ mice. Together, these findings suggest that gut is equipped with multiple T cell- and antigen-limiting checkpoints to regulate IgA antibody responses.

Expansion of T_{FH} Cells with Reduced IL-21 Production in PPs of PD-$1^{-/-}$ Mice

We next asked what could be the cause of defective IgA selection in GCs of PD-$1^{-/-}$ mice and found that PD-$1^{-/-}$ mice have significantly more $CD4^+$ T cells than WT mice. Expanded PP T cells in PD-$1^{-/-}$ mice included $CXCR5^{hi}ICOS^{hi}$ T_{FH} and $CXCR5^+ICOS^{int}$ T cells called $preT_{FH}$ cells.[25] Furthermore, several differences distinguished these activated T cells in PPs of PD-$1^{-/-}$ mice from those in WT mice. The expression of BCL6, the key protein that shapes the pre-GC dynamics and induces the T_{FH} program[26] was higher in T cells from PD-$1^{-/-}$ mice; while IRF4, another transcription factor required for the STAT3-dependent production of IL-21, was reduced in both T_{FH} cells and $preT_{FH}$ cells from PD-$1^{-/-}$ mice. Indeed, the IL-21 production by CXCR5-expressing T cells was significantly reduced in PD-$1^{-/-}$ mice. IL-21 was shown to be important for GC formation and function, since its absence affects B cell proliferation, differentiation into memory B cells and immunoglobulin production.[27,28] In contrast to IL-21, the staining with antibody detecting the active form of TGF-β1 revealed that in PD-$1^{-/-}$ mice especially the $CXCR5^{hi}ICOS^{hi}$ T_{FH} and $CXCR5^-ICOS^{low}$ T cells expressed even higher levels of TFG-β1 than their counterparts in WT mice. The reduced IL-21 production and abundant presence of active TGF-β1 may explain the reduced clonal expansion of IgA^+ B cells in GC of PD-$1^{-/-}$ mice.

All these observations raise the following possibilities: (1) excessive number of T_{FH} cells critically impacts the IgA selection in PP GCs and (2) the T_{FH} cells in PD-$1^{-/-}$ mice might also be generated from precursor cells of a different nature.

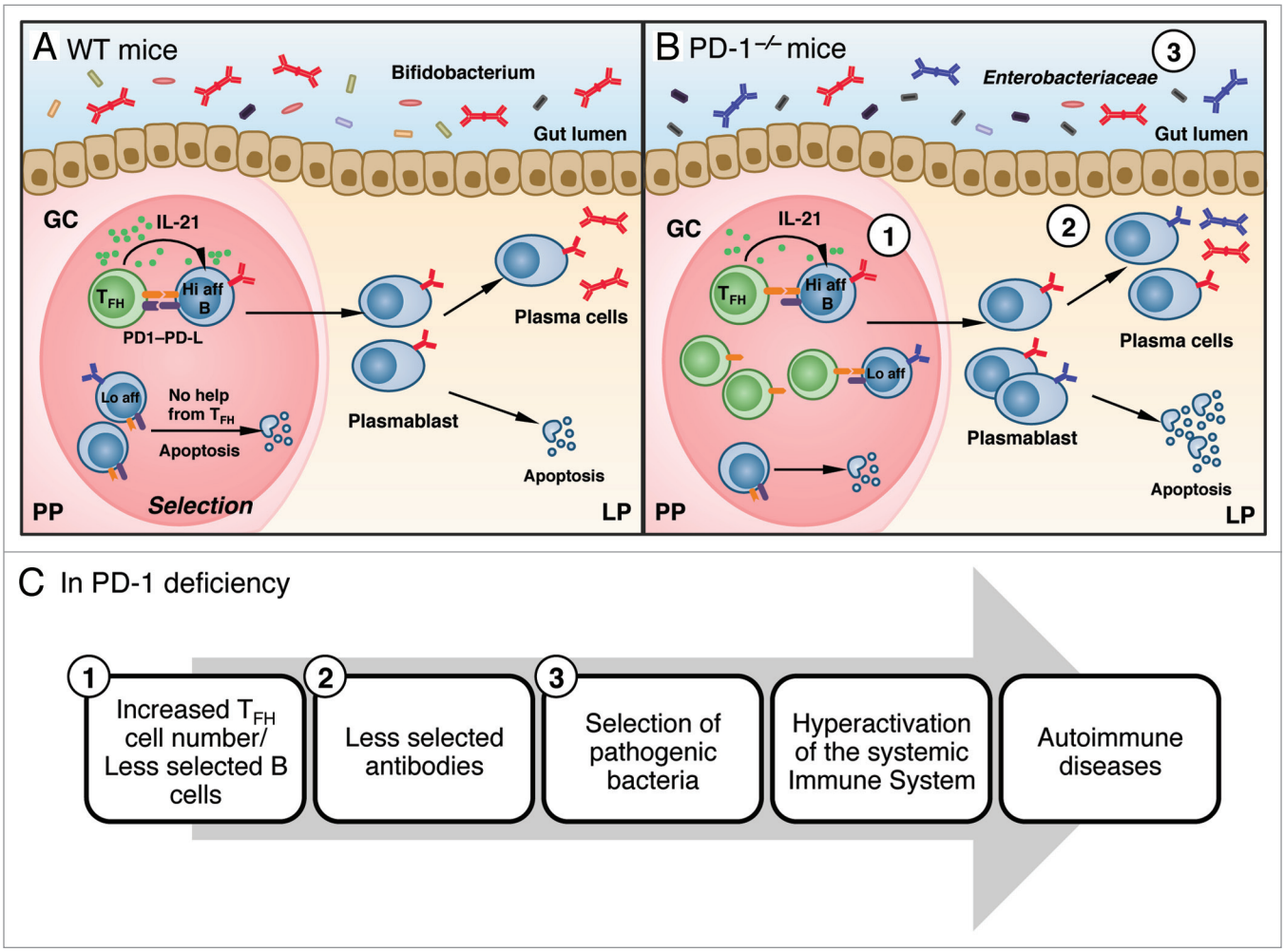

Figure 4. Schematic representation of IgA selection within and outside the GCs. Discrimination of GC IgA B cells with different affinities for gut antigens requires B cell receptor (BCR) engagement and competition for T_{FH} cell help. (**A**) In WT mice, the limited number of T_{FH} cells implies that only the B cells capable to present more antigen (high affinity) will receive T_{FH} help, with B cells capturing less antigen (low affinity) or those losing the integrity of the BCR undergoing apoptosis. (**B**) In PD-$1^{-/-}$ mice, both the high and low affinity B cells will receive help from the numerous T_{FH} present within the GCs. In both cases, the IgA B cells generated in the PP GCs migrate out to the mesenteric lymph node (MLN) where they further proliferate and differentiate into plasmablasts, which via the thoracic duct and blood will reach LP. In addition to selection in the GCs, IgAs appear to undergo a second, likely commensal-driven selection within the LP. In this way, the proliferating plasmablasts are reselected to fit the geographical distribution of bacteria along the intestine. (We do not exclude the possibility that IgAs are undergoing selection also at their first post-GC station, in MLN). (**C**) The possible sequential events leading to generation of auto-reactive antibodies in PD-$1^{-/-}$ mice.

To evaluate the impact of T_{FH} cell numbers for selection of IgA^+ B cells in GC and generation of IgA plasma cells in the LP, we transferred T_{FH} cells isolated from PPs of WT and PD-$1^{-/-}$ mice into T cell-deficient (CD3$\varepsilon^{-/-}$) mice. As expected, the T_{FH} cells isolated from PD-$1^{-/-}$ mice vigorously expanded and outnumbered the WT T_{FH} by a factor of two (**Fig. 3A**). Yet, the PD-1-deficient T_{FH} cells generated significantly less IgA plasma cells in the LP (**Fig. 3B**). These results strongly indicate that increased help from T cells interferes with selection of B cells in GCs, as initially proposed by Cyster and colleagues and reinforced by the Nussenzweigs' group.[29,30] This is even more evident in PPs, where the continuous availability of essentially non-limiting amounts of bacterial antigens displayed on the surface of FDCs implies that the competition for the T_{FH} help plays a more important role in the selection of B cells in GC of PPs.

The precursors of T_{FH} cells might be also different in PD-$1^{-/-}$ mice. For instance, the T_{FH} cells in PPs of PD-$1^{-/-}$ mice might be generated by a unique and unconventional way from the $Foxp3^+$ T cells that were shown by us to convert into T_{FH} cells in PPs.[31] Indeed, the transfer of $Foxp3^+$ T cells into CD3$\varepsilon^{-/-}$ mice generated T_{FH} cells as well as IgA plasma cells regardless of whether they were isolated from WT or PD-1-deficient mice. Interestingly, the frequency of ex-Foxp3– T_{FH} cells that produced IL-21 was similar between CD3$\varepsilon^{-/-}$ mice that received PD-1 sufficient or deficient $Foxp3^+$ T cells, yet less than that observed in mice injected with T_{FH} cells from WT mice. Further, the affinity maturation of IgAs generated with the help of ex-Foxp3 T_{FH} cells was reduced compared with that from "genuine" T_{FH} cells. Together all these results strongly suggest that: (1) a significant

fraction of IgA in PD-1$^{-/-}$ mice might be generated with the help of unconventional T_{FH} cells generated from Foxp3^{+} T cells (which might be induced in the gut—iTreg and capable to present high amounts of active TGF-β1) and (2) the quality of IgA may depend on the number as well as the nature of T cells present in GCs.

Dysbiosis in PD-1$^{-/-}$ Mice Causes Excessive Activation of the Whole Body Immune System

Based on our observations that PD-1$^{-/-}$ mice have altered gut microbiota and defective IgA selection and on previous reports demonstrating that PD-1$^{-/-}$ deficiency leads to the development of autoimmune diseases, we predicted that dysbiosis in PD-1$^{-/-}$ mice would impair the gut barrier and induce excessive activation with inflammatory features of the systemic immune system.

Indeed, we found a significant increase in numbers of CD4^{+} T cells, GC B cells and T_{FH} cells not only in PPs but also in pLNs and spleen of PD-1$^{-/-}$ mice. Along with B and T cell hyperplasia we found that serum from PD-1$^{-/-}$ mice contained antibodies specific for components of commensal bacteria, clearly indicating a breach in the normal mucosal-systemic compartmentalization.[32] Administration of broad-spectrum antibiotics led to normalization of the activation phenotype of PD-1$^{-/-}$ mice.

Of note, not only the number but also the features of CD4^{+} T cells differed in PD-1$^{-/-}$ mice compared with WT mice. PD-1-deficient CD4^{+} T cells were prone to secrete more IFN-γ but less IL-21 compared with control WT T cells, indicating that they might be of a different nature and/or functional properties. Indeed, in addition to commensal-reactive antibodies, sera from PD-1$^{-/-}$ mice contain polyreactive IgG antibodies that recognized host tissues (e.g., gastric parietal cell, data not shown), thus confirming previous observations. Most likely these cross-reactive Igs were generated in pLN or spleen GCs set in motion by bacteria species (or their products) originating in gut.

Together these findings strongly indicate that the skewed gut microbial communities and the associated "leaky" gut barrier leads to generalized activation of the immune system and drive the expansion of self-reactive B and T cells and production of auto-antibodies.

Conclusions

Multiple studies demonstrated that PD-1-PD-L interaction is critical in maintaining the balance between stimulatory and inhibitory signals regulating immune responses. The engagement of PD-1 by its ligands inhibits the activation and effector function of T cells and induces peripheral tolerance. That PD-1 affects the survival of B cells in pLN GCs and the formation and affinity of long-lived plasma cells was shown by the Shlomchiks' group.[15] We revealed that in gut, PD-1 is an essential component of the program that regulates IgA selection required for the maintenance of fit microbiota. Defects in IgA regulation by perturbing microbial composition and gut microenvironment have the potential to increase autoimmune susceptibility. In humans, IgA-deficiency associates with high incidence of autoimmune diseases, gastrointestinal and respiratory infections.[33,34] In several mouse models of autoimmune diseases, the development of the disease required intestinal bacteria and expansion of specific subsets of T helper cells (i.e., SFB and Th17).[35] These findings suggest that the maintenance of appropriate composition and density of microbiota by dynamic IgA production in the gut confers resistance against autoimmunity in peripheral compartments (**Fig. 4**). Our analysis of PD-1$^{-/-}$ mice opens new perspectives for studies aimed at understanding the contribution of gut microbiota to the development of autoimmune diseases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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