Addendum

Intestinal IgA as a modulator of the gut microbiota

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ABSTRACT
Accumulating evidence suggests that dysbiosis plays a role in the pathogenesis of intestinal diseases including inflammatory bowel disease (IBD) as well as extra-intestinal disorders. As a modulator of the intestinal microbiota, we isolated a mouse monoclonal IgA antibody (clone W27) with high affinities for multiple commensal bacteria, but not for beneficial bacteria such as Lactobacillus casei (L. casei). Via specific recognition of an epitope in serine hydroxymethyltransferase (SHMT), a bacterial metabolic enzyme, W27 IgA selectively inhibited the in vitro growth of bound bacteria, including Escherichia coli (E. coli), while having no effect on unbound beneficial bacteria such as L. casei. By modulating the gut microbiota in vivo, oral administration of W27 IgA effectively prevented development of colitis in several mouse models. Here we discuss how intestinal IgA modulates the gut microbiota through recognition of SHMT.

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Introduction
Dysbiosis of gut microbiota disrupts intestinal homeostasis and causes intestinal diseases including inflammatory bowel disease (IBD) such as Crohn’s disease and ulcerative colitis (UC), as well as extra-intestinal disorders including allergies, asthma, metabolic syndrome, cardiovascular disease, and obesity. Therefore, restoration of gut microbiota symbiosis is key to preventing and treating many diseases. One promising agent capable of shaping the gut microbiota community is intestinal IgA. Recently, we isolated a high-affinity poly-reactive IgA (clone W27) from mouse intestinal lamina propria cells, and identified it as a useful gut commensal modulator. In standard mouse models of colitis and IgA disorders, oral treatment of W27 prevented pathological colon phenotypes by improving the composition of gut microbiota. Here we discuss the physiologic significance of recognition of a specific epitope of a bacterial metabolic enzyme by W27, as well as the importance of the properties of intestinal IgA in maintaining gut homeostasis.

A high-affinity and poly-reactive W27 IgA targets a bacterial metabolic enzyme and affects bacterial cell growth
Intestinal IgA is important for control of commensal microbiota in the gut, but it remains unknown how IgA recognizes and shapes this microbial community. To answer these questions, we generated IgA-producing hybridomas derived from lamina propria mononuclear cells of small intestine from immunized wild-type mice. We selected the IgA clone W27 because it exhibited the strongest ability to bind multiple commensal bacteria while binding weakly or not at all to beneficial bacteria such as Lactobacillus casei and Bifidobacterium bifidum. To explore how W27 distinguishes these bacterial species, we determined the epitope of E. coli at the amino-acid level. W27 recognized the sequence EEHI in the N-terminal region of E. coli serine hydroxymethyltransferase (SHMT) in a highly specific manner, thereby suppressing the growth of E. coli. SHMT, which is structurally well conserved from bacteria to mammals, catalyzes the reversible, simultaneous conversion of L-serine to glycine and tetrahydrofolate (THF) to...
5,10-methylenetetrahydrofolate. SHMT-deficient E. coli exhibit retarded growth. In the previous studies including comprehensive proteomic analysis, SHMT was detected in the periplasm fraction in E. coli. It suggests that IgA can recognize SHMT on the surface of E. coli cells. The crystal structure of SHMT has shown that the N-terminal domain of SHMT covering the epitope of W27 is important for the formation of dimer. The dimer formation of SHMT is believed to be the functional entity. Therefore, we speculate that the binding of W27 IgA to the N-terminal epitope of SHMT may disrupt the functional dimer formation of SHMT, resulting in growth suppression of E. coli via inhibition of SHMT enzymatic activity. The further study is necessary to prove this hypothesis.

When we aligned the amino-acid sequences in the N-terminal regions of SHMT from various species, we identified a series of conserved and distinct regions (Table 1). The 4 underlined amino acids are the epitope by which W27 selectively recognizes its target bacteria (Table 1). To determine the physiologic importance of the EEHI sequence in SHMT, we searched for bacterial species that have the EEHI sequence in SHMT; the results of this analysis showed that EEHI-containing bacteria were mostly Gammaproteobacteria and Betaproteobacteria species, including numerous pathogens (Table 1). Gammaproteobacteria and Betaproteobacteria, whether pathogenic or not, are facultative anaerobes and are present in a wide range of habitats, from aquatic environments to the human intestinal tract. Therefore, they are generally not considered to be beneficial partners in the human gut. We speculate that W27 preferentially recognizes Gammaproteobacteria and Betaproteobacteria through the EEHI sequence of SHMT, thereby helping to maintain a healthy gut environment.

To determine whether the EEHI sequence was of general importance, we generated IgA-producing hybridomas from several genetically different mice, including unimmunized C57BL/6 and BALB/c wild-type mice, unimmunized C57BL/6 AIDG23S mice lacking only high-affinity IgA due to a somatic hypermutation defect, and ovalbumin-immunized C57BL/6 wild-type mice. Because all these mice were kept under specific pathogen–free (SPF) conditions, they were never exposed to the pathogens listed in Table 1. Indeed, the relative abundance of Gammaproteobacteria and Betaproteobacteria in murine feces is extremely low (<0.05% for Gammaproteobacteria and <0.5% for Betaproteobacteria) under the SPF conditions in our animal facility. Unexpectedly, however, most of the isolated IgA clones (42 of 44) recognized the peptide containing the EEHI sequence with varying affinities (Fig. 1). This finding suggests that intestinal IgA-producing cells were preferentially selected to react to the EEHI sequence in mice, even though bacteria expressing the epitope are underrepresented in their gut microbiota. We assume that the EEHI sequence of SHMT is critical for bacterial...
selection by intestinal IgA, at least in mice. However, the signal that stimulates this biased IgA selection in vivo remains to be determined.

**IgA deficiency and low-affinity IgA cause a variety of gut pathological phenotypes in mice**

As we reported previously, both activation-induced cytidine deaminase (AID) mutant mice, AID<sup>−/−</sup> mice lacking all IgA and AID<sup>G23S</sup> mutant mice lacking high-affinity IgAs, suffer from lymphoproliferative disorders such as germinal center (GC) hyperplasia in the small intestine. We hypothesized that low-affinity IgA in AID<sup>G23S</sup> mice was responsible for this phenotype. However, the affinities of monoclonal IgA against the EEHI sequence did not differ significantly between wild-type and AID<sup>G23S</sup> mice (Fig. 1). To determine whether IgA generated by AID<sup>G23S</sup> mice had lower affinity for *E. coli*, which has the EEHI sequence in SHMT, we extracted polyclonal IgA from the gut contents of wild-type and AID<sup>G23S</sup> mice and compared the binding abilities for *E. coli* by ELISA. Indeed, polyclonal IgA of AID<sup>G23S</sup> mice bound *E. coli* with lower affinity than that of wild-type mice (Fig. 2).

**Figure 2.** Relative binding ability of polyclonal intestinal IgA from gut contents of mice. ELISA with serially diluted polyclonal intestinal IgA of mice was performed against *E. coli*. Gut contents (from duodenum to rectum) were collected from wild type, AID<sup>G23S</sup>, and AID<sup>−/−</sup> mice (3 mice per genotype). The filtered gut contents were precipitated with ammonium sulfate and applied to a PD-10 column (GE Healthcare) to replace the buffer with PBS. Data are from a single experiment. For AID<sup>−/−</sup> mice, serially diluted suspension of gut contents in PBS were applied. O.D.: optical density.

We next investigated the histology of these mutant mice and found that they exhibited significant pathological tissue damage, not only in the small intestine but also in the colon (Fig. 3a and b). Even at younger ages, the colon mucosa of AID<sup>G23S</sup> mice exhibited diffuse crypt atrophic damage (Fig. 3a). In AID<sup>−/−</sup> mice, huge lymphoid accumulations were evident at younger ages, and in addition some of old AID<sup>−/−</sup> mice exhibited lymphoid polyposis–like lesions in the colon in which most of the crypts were lost, resulting in a dramatic reduction in the number of Alcian blue–positive goblet cells (Fig. 3a). The level of lipocalin 2 in feces, a biomarker of early-phase intestinal inflammation, was significantly elevated in aged AID<sup>G23S</sup> and AID<sup>−/−</sup> mice relative to age-matched wild-type mice (Fig. 3b). These findings suggest that

**Figure 3.** Absence of high-affinity IgA in gut induces a pathological colonic phenotype in mice. (a) Representative colonic sections stained with hematoxylin and eosin (HE) and Alcian blue (AB) with Kernechtrot Stain Solution. Scale bars, 200 μm. (b) Fecal lipocalin 2 levels were measured at various ages as a biomarker of intestinal inflammation. Black circles; 9–29-week-old mice; open circles, mice over 30 weeks old mice. Bars indicate medians. **P < 0.01, ***P < 0.001 vs. WT (wild-type mice).
lymphoproliferative disorders may predispose the colon to inflammatory conditions, resulting in tissue damage, in these mutant mice.

Interestingly, these colon phenotypes were apparent only in BALB/c, but not in C57BL/6, background mutant mice, although GC hyperplasia was observed in mutant mice of both backgrounds. Accordingly, we hypothesized that distinct microbial composition due to differences in genetic background might be responsible for colon phenotypes in BALB/c mutant mice. High-throughput 16S rRNA gene sequencing from the feces of co-housed mice revealed a significant difference between wild-type and AID^{G23S} mice (all OTUs; \( P < 0.05 \)) (Fig. 4a). A difference between BALB/c and C57BL/6 backgrounds was also evident (all OTUs; \( P < 0.05 \)) (Fig. 4b), regardless of the genotype (WT only OTUs and G23S only OTUs; \( P < 0.05 \)) (Fig. 4c and d). These findings suggest that, in AID^{G23S} mice, low-affinity IgA and genetic background affect gut microbial composition, resulting in apparent colon phenotypes in BALB/c AID^{G23S} mice. Further studies are required to determine the impact of genetic background on gut microbiota composition and inflammatory tone in the colon.

Based on the observations described above, we predicted that the relative abundance of

**Figure 4.** The gut microbiota difference in genotyping as well as genetic background. Principal components analysis (PCoA) based on pyrosequencing data of bacterial 16S rRNA gene in feces. The results were plotted according to genotype [wild-type (WT) vs. AID^{G23S} (G23S)] (a) and strain [C57BL/6 (B6) vs. BALB/c] (b). The results of PCoA shown by strain in each genotype (c: wild-type only, d: G23S only). Four wild-type and 6 AID^{G23S} mice of the B6 background and 3 wild-type and 3 AID^{G23S} mice of the BALB/c background were included. Mice of each genetic background were cohoused. Fecal samples were obtained twice, at the ages of 8 and 20 weeks.
Gammaproteobacteria and Betaproteobacteria might increase in feces of AIDG23S mice in comparison with feces of wild-type mice. However, the relative abundance of Gammaproteobacteria and Betaproteobacteria was too low (<0.5% for Betaproteobacteria and <0.05% for Gammaproteobacteria) in the feces of AIDG23S mice raised under SPF conditions, and we were unable to measure a significant difference. At present, our data suggest that low-affinity IgA in AIDG23S mice caused dysbiosis, although the responsible bacterial species could not be specified. Further studies with mice kept under non-SPF conditions or transplanted with human feces will be necessary to identify the bacteria responsible for the phenotypes of AID mutant mice.

**W27 IgA, an efficient gut microbiota modulator, improved the colon phenotypes in AID mutant mice**

According to the high-affinity and poly-reactive properties of W27 against possible “pathobiont” commensals, we predicted that oral supplementation of W27 could prevent the immune hyperactivation and colon pathology in BALB/c AIDG23S and AID−/− mice. As we reported recently, GC hyperplasia improved significantly in W27-treated mutant mice in comparison with untreated AIDG23S and AID−/− mice, indicating that high-affinity IgA is required for prevention of immune hyperactivation (Fig. 5a).

In addition to the disappearance of colon crypt damage in W27-treated AIDG23S mice, the percentages of colon Foxp3+ Treg cells within the CD4+ cell population were significantly elevated in W27-treated AIDG23S mice and AID−/− mice (Fig. 5b), suggesting that oral W27 treatment improved their gut environments and established an anti-inflammatory condition. Consistent with this, oral treatment with W27 increased the relative abundance of bacterial species known as Treg inducers, such as Lachnospiraceae and Ruminococcaceae, in the feces of W27-treated AIDG23S mice. The fact that W27 oral supplementation significantly improved a variety of gut phenotypes observed in murine models with IgA disorders (AIDG23S and AID−/− mice) has encouraged us to move ahead to clinical intervention.

**Figure 5.** W27 oral treatment improved GC hyperplasia in Peyer’s patches (PPs) and increased the abundance of Foxp3+ CD4+ cells in the colon. (a) Total number of GC B cells (B220+ PNAhigh) in PPs from mice. W27 was orally administered to AIDG23S mice via drinking water at concentration of 25 μg/ml for 4 weeks. W27 (30 μg, twice a week for 4 weeks) was orally administered by gavage in AID−/− mice. (Data were modified from.4) (b) Percentage of Foxp3+ cells among CD3+CD4+ colonic cells. W27 was orally administered to AIDG23S mice and AID−/− mice via drinking water at concentration of 25 μg/ml for 4 weeks. All data are expressed as medians ± range. **P < 0.01. *P < 0.05. Statistical analysis was performed by 2-sided Mann–Whitney test.**
## Perspectives

A wide range of IgAs exists in the gut lumen, and each IgA must recognize its target independently. Our recent observations supported others’ findings that intestinal IgA selectively binds harmful bacteria to eliminate them from the gut microbiota. On the other hand, several reports demonstrated that IgA binding could facilitate gut colonization by bound bacteria, rather than suppressing their growth. These results do not necessarily contradict our findings, because the IgA repertoire is so huge that it must contain a broad range of clones with different functions. However, we assume that the majority of intestinal IgA suppresses bound bacteria rather than aiding their growth, because more than 90% of independent IgA clones that we isolated from small intestine could recognize the EEHI sequence and suppress bacteria harboring it (Fig. 1).

We propose that high-affinity and poly-reactive intestinal IgA such as W27 shapes the gut microbiota to maintain its diversity by suppressing (not killing) fast-growing bacterial species such as Proteobacteria that harbor the EEHI sequence in SHMT. Thus, high-affinity and poly-reactive IgA may have potential not only for maintaining gut homeostasis, but also for combating antibiotic-resistant pathogens by suppressing their growth.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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