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INTERACTIONS OF COMMENSAL GUT MICROBES WITH SUBSETS OF B- AND T-CELLS IN THE MURINE HOST*

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SUMMARY

Although mechanisms operative in the induction and maintenance of specific, adaptive immunity, including 'cognate' B/T interactions, have been extensively studied and defined, we still know little about the mechanisms operative in developing and maintaining B- and T-cell dependent 'natural' immunity. Particularly, we are still rather ignorant concerning gut microbial/gut or systemic APC, T cell and B cell interactions that lead to lymphoid cell mediated 'natural' immunity: Specific or broadly reactive, activation via TCR and BCR and/or via other receptors such as the TLR series, and whether T/B interactions are operative at this level? Here we will address: 1) the general role of gut microbes in the development and maintenance of the intestinal, humoral immune system; 2) the general role of gut microbes in the development of B1 cell mediated, 'natural' gut IgA and the dependence of these B1 cells on bystander T cell help; 3) the relative contributions of B1 vs. B2 cells to gut 'natural' and specific IgA responses; 4) the role for particular 'normal' gut microbes in the initiation of inflammatory bowel diseases (IBD) in mice with a dysregulated immune system; and 5) the possible roles of gut microbes in facilitating oral tolerance, a mechanism likely operative in forestalling or ameliorating IBD. A central theme of this paper is to attempt to define the specificities of activated, functional $CD4^+$ T cells in the gut for Ags of particular, usually benign gut microbes. We will also consider the still-unresolved issue of whether the contributions of B1-derived IgA in the gut to the 'natural' Ab pool are Ag-selected and driven to proliferation/differentiation or whether the main stimuli are not via BCRs but rather other receptors (TLRs, etc.). The main experimental approach has been to use antigen-free, germ-free, or gnotobiotic (mono- or oligo-associated with precisely known bacterial species) mice.

INTRODUCTION

This overview aims to address the interactions of normally benign members of the gut microbiota with B-cells and T-cells of the mammalian host. We

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will mostly consider those host cells located in inductive sites for development of gut mucosal immunity — Peyer's patches (PP), solitary follicles (SF) — and effector sites in the gut-lamina propria (LP), inter-epithelial leukocyte (IEL) spaces. Although mechanisms operative in the induction and maintenance of specific, adaptive immunity, including 'cognate' B/T interactions, have been extensively studied and defined, we still know little about the mechanisms operative in developing and maintaining 'systemic' or mucosal lymphoid cell-dependent 'natural' immunity. Our main experimental approach has been to use antigen-free, germ-free, and gnotobiotic (mono- or oligo-associated with precisely known bacterial species) mice. For a comprehensive review of this field see *Cebra* et al. (1999). The intent of this overview is not to offer specific, practical suggestions for developing effective mucosal vaccines for particular nosocomial or opportunistic pathogenic bacteria that infect via mucosal surface. Rather, we aim to inform vaccinologists concerning how the gut mucosal immune system generally responds to enteric microbes and raise appreciation for the role of innocuous gut colonisers in possibly ameliorating particular infections via stimulation of the 'natural' gut mucosal immune system, i.e. the possible use of probiotic microbes as a complement to specific immunisation.

FINDINGS AND CONCLUSIONS

The role of benign gut microbiota in the development and maintenance of the humoral immune system; effects of gut IgA on the colonising bacteria

The main basis for implicating the 'normal' gut microbiota in the development and maintenance of gut IgA production comes from comparing antigen-free or germ-free mice with conventionally reared mice. Adult conventional mice have a plethora of IgA plasmablasts in the gut LP. A majority of all the productive plasmablasts in the entire body is found in the gut. Neonates show a lag in the outgrowth of these cells until weaning. There is a paucity of IgA plasmablasts in gut LP of adult antigen-free or germ-free mice. Thus, normal colonisation of the gut with benign, commensal microbes is accompanied by the rapid rise in IgA plasmablasts in the gut LP.

Since PP have been implicated as inductive sites for the generation of IgA-committed B cells (*Craig* and *Cebra*, 1971), we decided to assess the effects of mono-associating germ-free mice with a benign mouse commensal, *Morganella morganii* (Gram-negative rod) on activating germinal centre reactions (GCRs) in PP. Such GCRs generate IgA committed, specific B cells in immunocompetent mice. While conventionally reared mice contain PP exhibiting chronic GCRs, the PPs of germ-free mice are quiescent and lack B-blasts. Our findings (*Shroff* et al., 1995) were: (a) that GCRs in PP waxed and waned over 10-28 days post-colonisation and remained quiescent up to 314 days, even though the gut bacterial load was $>10^8$ CFU/g; (b) in order to relate the GCRs stimulated by *M. morganii* colonisation to both 'natural' and specific IgA production in the gut, we used a tissue fragment culture assay that permits quantisation of IgA produced in PPs and in each segment of gut (duodenum, jejunum, ileum, caecum, large intestine) following microbial colonisation (see Figure 1). This assay avoids enzymatic degradation of IgA in the intact gut,

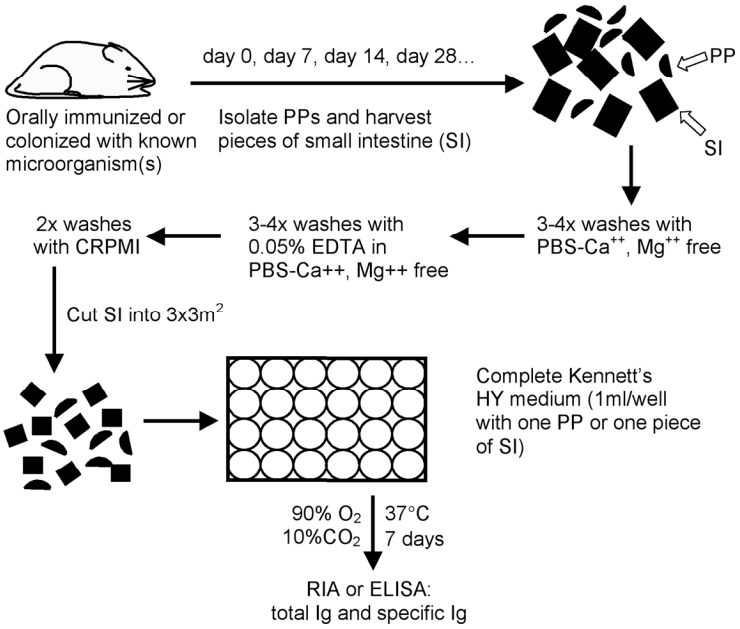

Figure 1: Assessment of humoral immune responses in GALT (gut associated lymphoid tissues) by organ fragment cultures.

variable dilution of IgA by gut fluids, and aggregation of different IgA molecules with mucin (see *Logan* et al., 1991). Our findings were that total ('natural') IgA production in the gut and PPs rose from very low levels in tissue fragment cultures from germ-free mice to about 20-25% of that from conventionally-reared mice within 14 days and remained at that level for >54 days, although GCRs in PPs had disappeared. Further, the microbial specific IgA Abs also rose to about 5% of the total IgA being produced over the same time period. As expected, plasma cells giving ELISPOTS vs. bacterial Ags also rose and these persisted for >314 days despite the cessation of GCRs in PPs; (c) the chronically colonising *M. morganii* became 'coated' with IgA, as detected by FACS analysis, by day 14 and this coating persisted >314 days without any apparent effect on the persistence of the microbe in the gut; (d) *M. morganii* that had translocated were cleared from both the spleen (58 days) and MLN (208 days), although they continued to persist in gut lumen in high numbers (>10^8 CFU/g). Thus, the chronically present GCRs in conventionally reared mammals cannot simply be accounted for by persistence of the bacteria. Likely, as will be supported below, the specific IgA Abs provide a shield to exclude bacterial products of a particular coloniser and consecutive exposure to 'novel' colonising bacteria is required to maintain the chronic GCRs in PP. Further, coating of gut bacteria by IgA did not seem to compromise their retention in the gut, at least when they were the sole colonisers. Of course, specific IgA Abs vs. microbial antigens responsible for attachment to and translocation across the gut epithelium, especially of frank and opportunistic enteric pathogens, may interfere with these processes. Likely most 'natural' and specific IgA Abs reactive with surface Ags of all gut bacteria may have little qualitative effects

on persistence of bacteria in the gut. Quantitative effects on bacterial persistence *vis a vis* other microbial competitor have yet to be accurately evaluated in gnotobiotic hosts.

Another benign gut colonising bacteria is 'segmented filamentous bacteria' (SFB). SFB colonisers of mammals were first comprehensively studied by *Davis* and *Savage* (1974) and their relationship to members of the genus Clostridia was shown using comparative 16S RNA analyses by *Snel* et al. (1995). These SFB are obligate anaerobes, Gram-positive, not-cultivatable, spore forming, segmented, gut bacteria which are major colonisers of the mammalian gut from weaning to puberty. Their ability to persist in the terminal ileum depends on use of a 'holdfast' segment to attach to the brush border of epithelial cells (ECs). Colonisation of formerly germ-free mice with SFB results in a rise in 'natural' IgA to about 2/3 of the level found in conventionally reared mice. This is the greatest rise induced by mono-association observed using any one of six other gut-colonising bacteria (*Talham* et al., 1999). However, only about 1% of this total IgA can be shown to be specifically reactive with the Ags in SFB sonicates (*Talham* et al., 1999). The GCRs in PP also wax and wane after SFB colonisation, however, the GCRs in PPs can be re-activated by super-colonisation with another gut commensal, *M. morganii*, at day 113. Subsequently, within 10 days specific IgA Abs can be detected reactive with *M. morganii* and these persists >80 days after secondary colonisation. Thus, the chronic presence of GCRs in the PPs of conventionally reared mice is likely due to continuous exposure to novel and ever changing members of the gut microbiota.

Each of seven different gut colonising bacteria, when used to mono-associate germ-free mice, resulted in its own characteristic level of total IgA production at steady state and its own characteristic proportions of demonstrably specific IgA Abs (1-15%) (*Bos* et al., 2001).

Several other principles have emerged from using SFB to mono-associate mice: (a) the immune responsiveness of both nursing dam and pups can determine the numbers and sites of colonisation of SFB (*Jiang* et al., 2001). By crossing immunocompetent mice with severe-combined immunodeficient (SCID) mice, and then back-crossing the F_1 mice to male or female parental SCID mice, we obtained four groups of germ-free offspring: Immunocompetent (+/-) pups with either immunocompetent (+/-) or SCID (-/-) mothers and SCID pups with either immunocompetent (+/-) or SCID (-/-) mothers. Pups with immunocompetent mothers showed a delay in gut colonisation with SFB, compared with pups from SCID mothers. If the pups were SCID (-/-), the eventual SFB colonisers persisted in the small intestine for the length of the experiment (70 days post partum). But if the pups were themselves immunocompetent (+/-), they cleared the SFB from small intestine 30-35 days post partum. The immunocompetent dams also prolonged the time to activate gut production of IgA by pups (if +/-, immunocompetent) compared with immunocompetent pups of SCID mothers. We suspect that suckled maternal and actively produced neonatal IgA Abs can forestall or prevent colonisation of small intestine by blocking the essential 'holdfast'/EC brush border interactions; (b) the diet can dramatically influence the level of gut colonisation by a single species of bacteria. SFB was used to mono-associate immunocompetent germ-free mice. After 20-30 days of colonisation and ingestion of conventional chow, groups of mice were split and half of each group was switched to a

chemically defined, elemental diet (*Pleasants* et al., 1986). SFB colonisation of the gut was monitored as well as IgA output in the various parts of gut. Within 5-13 days after switching to a chemically defined diet, almost all SFB disappeared from all sections of gut. Within 13 days, IgA output, relative to that of litter mates continuing on a conventional diet, dropped to about 1/3 and remained at that relative level for 55 days, when the experiment was terminated (*Thurnheer* et al., unpublished). Thus, some gut colonising bacteria are unable to survive alone in the presence of a chemically defined diet. The IgA levels rapidly dropped after switching to a chemically defined diet, either as a result of removal of SFB or because of the change in diet. Nevertheless, these observations suggest at least two subsets of IgA plasmablasts in the gut, differing in turnover time.

These latter findings, made using mono-associated hosts, may be extrapolated to attempts to orally vaccinate premature and full term human neonates vs. nosocomial or opportunistic pathogens. Neonates born of mothers effectively exposed to such pathogens may offer passive protection to their offspring via specific IgA Abs in milk but also forestall effective active immunisation with mucosal vaccines. Artificial diet formulae may be inadequate for the outgrowth of a potential probiotic gut microbe that may ameliorate such nosocomial or opportunistic infection.

The role of B1- vs. B2- cell subsets in gut mucosal IgA production; the dependence of IgA production by B1 cells on bystander T cell 'help' and the specific reactivity of $CD4^+$ T cells locally with gut microbial Ags

In a number of mammals, B cells can be divided into B1 and B2 cell subsets: B1 cells generally are surface IgM^{high}/IgD^{low} and many are $CD5^+$ and $Mac1^+$; B2 cells are surface IgM^{low}/IgD^{high} and are negative for CD5 and Mac1. In the adult mouse most B1 cells are localised in peritoneal and pleural cavities. These cells exhibit local self-renewal and do not depend upon replenishment from bone marrow stem cells. The B1 cells exhibit multi-reactivity with a variety of bacterial and autoantigens and both B1-derived IgM and IgA express germ-line encoded V-genes with few point mutations and no evidence of 'affinity maturation' (*Bos* et al., 1996). B1 cells are not found in PPs or peripheral lymph nodes (PLN). They account for the majority of 'natural' IgM in the circulation but their contribution to gut IgA, although demonstrated, has until recently not been quantitatively defined in physiologically 'normal' mice.

Our first indication that B1 cells might benefit from or require bystander T cell 'help' came from efforts to determine whether B1-derived gut IgA could be stimulated vs. intestinal murine rotavirus (EDIM strain), and neutralise/clear this multi-determinant Ag without T cells (*Kushnir* et al., 2001). SCID mice become chronically infected with rotavirus in intestinal ECs, and shed viral Ag in their faeces. We found that transfer of unfractionated peritoneal cavity cells, B1 cells plus $CD4^+$ T cells, or $CD4^+$ T cells alone would result in cessation of viral shedding, but B1 cells alone did not. Transfer of peritoneal cavity cells to infected SCID mice resulted in viral specific IgA production in the gut but transfer of B1 cells alone did not. However, transfer of both types of cells resulted in appreciable expression of 'natural' IgA in the gut. Examination of cells from recipient mice 8-10 weeks after cell transfer to infected SCID mice showed, surprisingly, that transferred, FACS-purified B1 cells also resulted in the appearance of appreciable $CD4^+$ T cells in peritoneal cavity and gut LP. Fi-

nally, experiments using congenic IgA allotype-different donors and exchange of B1 cells added to B1-depleted peritoneal cavity cells, showed that the allotype of specific, anti-viral IgA Abs was almost exclusively that of the non-B1 cell donor (*i.e.*, likely the product of B2 cells). Thus, we concluded that B1 cells could not produce effective IgA anti-rotavirus Abs, and suspected that their production of 'natural' IgA might depend on the outgrowth of $CD4^+$ T cells, contaminating the FACS-purified B1 cells. Since we transferred only 2 x 10^5 FACS-sorted cells, we reckoned that the purification could only have left about one to two thousand contaminating T cells, but that these expanded extensively over 8-10 weeks *in vivo*. Since we suspected that our cell inoculum size was 'borderline' with respect to contaminating T cell content, we transferred this dose of FACS-purified T cells into a large number of conventionally reared SCID recipients (*Jiang* et al., submitted). We found expression of gut IgA in some but not all recipients, and the level of IgA production after 8 weeks was nicely correlated with the occurrence and extent of $CD4^+$ T cell outgrowth in peritoneal cavity and spleen. Although B1 cells have been found not to require or benefit from Ag-specific, cognate, $CD4^+$ T cell 'help', we suspected that they might benefit from bystander T cell 'help', possibly developed locally in gut LP in response to microbial Ags.

To address these issues we prepared B1 cells at greater purity by treatment of donor peritoneal cavity cells with anti-Thy1 plus complement, and then sorting for B1 cells and against $CD4^+$ T cells. Such further purified B1 cells, upon transfer to conventionally reared SCID mouse hosts, showed repopulation of the recipient mice with B1 cells but not T cells. Addition of graded doses of FACS-purified $CD4^+$ T cells (0-2 × 10^5) to these B1 cells (2 × 10^5) resulted in increasing expression of 'natural' gut IgA in conventionally reared SCID mice, 8-10 weeks after transfer. A role for gut microbial Ags in this phenomenon was shown by cell transfer into conventionally reared and germ-free SCID mice: Neither B1 cells nor unfractionated peritoneal cavity cells gave rise to appreciable gut IgA production in germ-free mice, although the peritoneal cavity cells resulted in a robust (app. 80% of intact, conventionally reared BALB/c mice) gut IgA production in conventionally reared mice (*Jiang* et al., submitted for publication).

To test whether $CD4^+$ T cells of a specificity irrelevant to that of B1 cells could provide the bystander 'help' necessary for gut IgA production and whether activation/stimulation of these T cells was necessary, we used monoclonal DO11.10 T cells (ovalbumin peptide/class II^d specific transgenic mice crossed onto a RAG-2 -/- background). If the DO11.10 T cells were activated *in vivo* in the donors by giving OVA in drinking water, they facilitated gut IgA production upon co-transfer with B1 cells in conventionally reared but not in germ-free SCID mouse recipients. This observation suggested that 'activated' $CD4^+$ T cells could provide bystander 'help' but that B1 cells also needed some sort of gut microbial stimulation. If the DO11.10 T cells were taken from quiescent donors (no OVA given to donors), they would facilitate gut 'natural' IgA expression by B1 cells in conventionally reared SCID mice only if OVA was administered via drinking water to recipients (*Jiang* et al., submitted).

To determine whether mono-associated SCID mice could provide the necessary $CD4^+$ and/or B1 cell stimulation for gut IgA production, we transferred $CD4^+$ T cells plus B1 cells from conventionally reared donors into *Bacteriodes distasonis* colonised recipients. Neither 2 × 10^5 B1 cells alone nor B1

cells with either 2×10^3 or 2×10^5 $CD4^+$ T cells resulted in appreciable gut 'natural' IgA production, but transfer of unfractionated peritoneal cavity cells did. We think it likely that B2 cells in peritoneal cavity, benefiting from the 'help' of accompanying T cells, accounted for the IgA production. However, if the co-transferred $CD4^+$ T cells were from SFB-mono-associated donors, they did provide the 'help' required by B1 cells from conventionally reared donors to produce 'natural' IgA in the guts of SFB mono-associated SCID recipients (*Jiang* et al., submitted).

We conclude that: (a) B1 cells benefit from 'bystander' $CD4^+$ T cells in the gut in order to develop into IgA plasma cells (IL-5, IL-6, IL-10, TGFβ, etc., LKs); (b) the $CD4^+$ T cells must be locally activated by specific Ag in their gut locale — these Ags normally appear to be microbial Ags. The specificities of the T cells are likely unrelated to those of the B1 cells benefiting from 'bystander' help; (c) although the presence of colonising gut microbes seems to be required for the B1 to plasmablast transition in the gut, we have no evidence of Ag-specific selection and stimulation of these B1 cells. Possible 'activating' molecules include microbial Ags, microbial mitogens (LPS, peptidoglycan, CpG, etc.) and the activation may be via BCRs, toll-like receptors, or both.

Finally, the strong effect of gut microbial stimulation on T cell outgrowth can be shown by comparing fluorochrome (CFSE)-labelled $CD4^+$ or CD8+ T cells into conventionally reared vs. germ-free SCID mice. 'Homeostatic proliferation' generally leads to the rapid expansion and populating of lymphoid tissues in conventionally reared SCID mice but transfer of T cells into germ-free SCID mice dramatically diminishes their outgrowth, as judged by only minimal decrease in fluorescence intensity of the transferred cells (*Surh* et al., unpublished).

In an effort to estimate the relative contributions of B1 vs. B2 cells to 'natural' IgA and to specific anti-microbial IgA Abs we designed a nearly physiologically normal model (*Thurnheer* et al., 2003): Using germ-free newborn pups of the Igh^a allotype, we suppressed the dissemination of B cells from the bone marrow by giving bi-weekly injections (10 total injections) of anti-IgM^a beginning at day 1 post partum. On day 3 we injected 2×10^6 peritoneal cavity cells, containing about 0.8×10^6 B1 cells, from an adult C.B17 Igh^b congenic donor. After 8 weeks, we tested for 'balanced' chimerism and then mono-associated such mice with SFB, *M. morganii*, or *B. distasonis*. Examination of peritoneal cavity cells at 8 weeks showed that most germ-free mice were balanced chimeras, with almost all B1 cells from the donor and almost all (80-85%) B2 cells from the recipient. These mice were also balanced chimeras functionally, as about half of the circulating IgM was donor derived and half from host cells over a period of 70 days after colonisation with either of the three gut microbes. Following bacterial colonisation, the GCRs waxed and waned in PPs, indicating a local B2 cell response. Periodic analysis of gut tissues over 70 days following colonisation indicated that <10% of the 'natural' or bacteria-specific IgA was derived from the B1 cell donor. Thus, in these neonatal, germ-free, chimeric mice, with no known impairment of their T cell system, most of the intestinal IgA seems to be of B2 cell origin. As shown above, at least some of the gut IgA is reactive with normal members of the gut microbiota. *Macpherson* et al. (2000) have shown that anti-microbial IgA can be stimulated in TCR (-/-) mice, with no functional T cells. Since we have shown that B1 cells

seem to require 'bystander' $CD4^+$ T cell 'help', what may be the origin of the anti-microbial gut IgA Ab that arises in TCR (-/-) mice? We have shown that oral infection with reovirus, an Ag with repeating determinants, of TCR (-/-) mice results in some anti-viral IgA Ab (*Zuercher* et al., unpublished). Unlike the response in W/T mice, this Ab is insufficient to clear the virus. However, the infection does result in a vestigial GCR in PPs, likely due to limited proliferation of B2 cells. We suggest that this modest B2 cell expansion, in the absence of a cognate T cell interaction, does not result in affinity maturation of B2 cells that develop few if any progeny with mutated, expressed Ig V-genes except that B2 responses may occur in GCRs while B1 responses may not. Both responses require bystander 'help', provided by otherwise activated $CD4^+$ T cells or dendritic cells (either interdigitating or follicular). Consideration of these possibilities may be relevant to raising protective Abs vs. many microbial Ags expressed by nosocomial and opportunistic mucosal pathogens. Possibly, combination of subunit vaccine and polyclonal stimuli-particularly those that interact with both B- and T cells via toll-like receptors-may provide an effective combination of 'natural' and specific immune responses.

The role of particular members of the gut microbiota and host T cells in the initiation, development, and control of inflammatory bowel disease (IBD): The possible relationship of controlling (Trl) T cells to those mediating oral tolerance

In the past 10 years, a number of animal models for human IBD have been developed. Most of these utilise conventionally reared rats or mice, which exhibit some sort of dysregulation or imbalance of their immune systems. One well-studied mouse model involves the transfer of $CD4^+$, $CD45RB^{high}$ T cells into conventionally reared SCID mice. The recipients usually exhibit a wasting disease and develop classic symptoms of ulcerative colitis (UC) in 10-14 weeks (*Morrissey* et al., 1993; *Powrie* et al., 1993). We have found that such pathologic consequences do not develop in germ-free mice, which exhibit no wasting or symptoms of UC (*Jiang* et al., 2002). In an effort to identify a microbial provocateur, we mono-associated germ-free SCID mice with one of five gut colonising, benign bacterial species, before transfer of 'naive' $CD4^+$ T cells. $CD4^+CD45RB^{high}$ T cells have been defined as naïve or inexperienced T cells, also expressing high level of CD62L, and lack expression of other activation markers, such as CD69 and CD44, which are prevalent in the PP of germ-free mice (*Morrissey* et al., 1993; *Powrie* et al., 1993; *Talham* et al., 1999). No wasting disease or IBD symptoms developed in these mice. However, a sixth mono-associate, *Helicobacter muridarum*, did result in an accelerated development of wasting, and UC (at 4-5 weeks) upon colonisation of SCID mice prior to transfer of 'naive' T cells (*Jiang* et al., 2002). *H. muridarum* was first described as a benign commensal, living in colonic crypts of healthy, conventional, immunocompetent mice (*Phillips* and *Lee*, 1983). The developing disease was accompanied by extensive outgrowth of *H. muridarum* in colonic crypts and caecum, a severe inflammatory response in the colon with trans-mural cell infiltration, and severe loss of weight. All mono-associating, gut colonising bacteria we tried stimulate outgrowth of transferred $CD4^+$, $CD45RB^{high}$ T cells in formerly germ-free SCID mice. However, *H. muridarum* results in the development of an imbalance of activated macrophage and of INF-γ producing T cells in the gut

lamina propria of colonised, recipient SCID mice. Thus far, the specificities of $CD4^+$ T cells that initiate experimental IBD have not been defined in any animal model for IBD.

Generally, co-transfer of $CD4^+$, $CD45RB^{low}$, $CD25^+$ T cells (subset including Tr1 cells) from conventionally reared donors, along with $CD4^+$, $CD45RB^{high}$ T cells ('naïve' T cells) from conventionally reared donors into conventionally-reared SCID mice forestalls or ameliorates the development of IBD initiated by the latter (*Morrissey* et al., 1993; *Powrie* et al., 1993). The specificities of these regulatory Tr1 cells, has long been an issue. We find that Tr1 cells derived from conventionally reared donors, have no effect on the development of IBD when given along with the initiator ($CD4^+$, $CD45RB^{high}$) T cells from germ-free or conventionally reared donors. However, if the Tr1 cells are taken from immunocompetent, *H. muridarum* mono-associated mice and transferred to germ-free SCID recipients prior to colonisation with *H. muridarum*, they effectively block development of IBD initiated by 'naïve' T cells. This observation suggests a specificity of the effective Tr1 cells for microbial Ags.

These findings suggest that normally innocuous members of the gut microbiota, such as *H. muridarum*, can act as an initiator of IBD in mice with a dysregulated immune system, since immunocompetent mice with functional regulation elements, do not develop IBD upon mono-association with *H. muridarum*. It is not surprising that $CD4^+$,$CD45RB^{low}$, $CD25^+$ T cells from such mice can control the development of IBD in dysregulated SCID mice.

Possible roles of gut microbes in facilitating oral tolerance

Finally, we believe that the cellular mechanisms for mediating the forestalling or amelioration of IBD are similar to those mediating acquired oral tolerance. A typical scheme for the experimental initiation of oral tolerance to a dietary, protein Ag is to give the Ag (ovalbumin, conalbumin) orally and then the fed mice, along with unfed controls, are primed parenterally with the same Ag in adjuvant.

We find that germ-free mice are refractory to the development of oral tolerance, as judged by their failure to show a diminished response of their peripheral T cells *in vitro* upon feeding a dietary Ag, relative to unfed control mice. Mono-association of germ-free mice with the normal murine *E. coli* (Schaedler's *E. coli*), results in susceptibility to the expression of oral tolerance. Thus, gut colonisation with at least some benign, commensal microbes somehow facilitates the development of oral tolerance (*Boiko* et al., unpublished).

Thus our above observations in 3 and 4 above support the stimulation of gut $CD4^+$ T cells to either initiate IBD or down regulate development of IBD and systemic immune responses to orally administered protein Ags. It is still unclear whether microbial products act via TCR and/or the toll-like receptors of T cells. Clearly, development of effective oral vaccine formulation will require selective stimulation of protective T cells and Abs and circumvent the down regulatory effects of Tr1 cells.

GENERAL SUMMARY

Microbial colonisers of the mammalian gut play a role in the development and maintenance of the intestinal mucosal immune system. The host makes a humoral mucosal response to each benign gut bacterial species used to

colonise adult GF mice. This response is characterised by transient GCR in PPs and the increased production of IgA in the gut. Each bacterial species used for mono-association has a characteristic stimulation of steady state levels of gut IgA production and ratio of specific IgA Abs to 'natural' IgA. In many cases, the level of 'natural' IgA is appreciably higher than demonstrably specific IgA.

Both B1- and B2-cells contribute to 'natural' IgA production in the gut. The B1 cells require 'bystander' $CD4^+$ T cell 'help' to develop IgA production in intestine. At least some of these gut T cells seem to be specific for Ags associated with particular gut microbes and require local colonisation to be functionally 'activated'. It is still unclear whether B1 cells can be specifically selected and activated by particular Ags or whether they are stimulated by microbial products via TLRs rather than BCRs. Under near physiologically normal conditions, B2 rather than B1 cells appear to account for most of the 'natural' and anti-microbial specific IgA produced in the gut. B2 cells may produce anti-microbial IgA Abs without a requirement for T cell help via vestigial GCRs and possibly without the accumulation of cells with V-gene mutations that often lead to affinity maturation.

We describe a mouse model for human IBD that has a dysregulated immune system and an identifiable bacterial provocateur, the normally benign *H. muridarum*. Naïve $CD4^+$ T cells, introduced into formerly germ-free SCID mice, mono-associated with *H. muridarum*, result in development of a wasting disease and ulcerative colitis. This disease appears to be initiated by activated $CD4^+$ T cells, which infiltrate the large intestine, produce IFN-γ and activate terminal effector macrophage. The specificities of the initiator T cells are unknown. However, regulatory $CD4^+$, $CD25^+$, $CD45RB^{low}$ T cells (Tr1 cells) can forestall or ameliorate the wasting and progression of the IBD symptoms. These seem to require specific, microbial Ag stimulation and may act in a 'bystander' fashion to down-regulate the development of the initiator $CD4^+$ T cells. Such Tr1-type T cells also appear to play a role in oral tolerance and also be stimulated by colonisation of the gut with enteric microbes. Oral tolerance — systemic hypo-responsiveness of T cells following ingestion of protein Ags — is absent or diminished in germ-free mice, but can be facilitated by colonisation with *E. coli*. Since the tolerance is elicited by feeding a dietary Ag (ovalbumin, conalbumin), it is likely that any Tr1-cells elicited by colonisation with *E. coli* act in a 'bystander' fashion.

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