

'Hygienic' Lymphocytes Convey Increased Cancer Risk

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Abstract: Risk of developing inflammation-associated cancers has increased in industrialized countries during the past 30 years. One possible explanation is societal hygiene practices with use of antibiotics and Caesarian births that provide too few early life exposures of beneficial microbes. Building upon a 'hygiene hypothesis' model whereby prior microbial exposures lead to beneficial changes in CD4+ lymphocytes, here we use an adoptive cell transfer model and find that too few prior microbe exposures alternatively result in increased inflammation-associated cancer growth in susceptible recipient mice. Specifically, purified CD4+ lymphocytes collected from 'restricted flora' donors increases multiplicity and features of malignancy in intestinal polyps of recipient *Apc*^{Min/+} mice, coincident with increased inflammatory cell infiltrates and instability of the intestinal microbiota. We conclude that while a competent immune system serves to maintain intestinal homeostasis and good health, under hygienic rearing conditions CD4+ lymphocytes instead exacerbate inflammation-associated tumorigenesis, subsequently contributing to more frequent cancers in industrialized societies.

Keywords: Hygiene, *Apc*^{Min/+}, cancer, inflammation, microbiome.

INTRODUCTION

Routine environmental exposures to microbes and microbial products are increasingly understood to affect risk of chronic inflammatory diseases later in life [1-9]. Microbial exposures are abundant in the natural environment, but are greatly reduced with hygienic practices and antibiotic usage that are widespread in modern lifestyles. Microbial exposures represent important background stimulation for normal immune system development, such that limited microbe exposures in early life result in persistent overreaction to stimuli later in life [10]. This concept that early-life microbial exposures and their connection with immune over-reactions later in life has been referred to as the "hygiene hypothesis" [10-16]. In this 'hygiene' model, too few exposures and insufficient CD4 cell priming leads to uncontrolled inflammatory responses and chronic inflammation. Immune over-reactions resulting in chronic inflammation have also been implicated in causation of cancers in the colon and other sites in humans [16].

Studies in lymphocyte-deficient mice using adoptive transfer techniques have shown that CD4+

lymphocytes significantly modulate inflammation in the lower bowel [17-22] and throughout the body [16, 23-26]. While intact CD4+ cell populations protect from cancer and other pathology, prior studies using adoptive transfer of CD4+ lymphocytes in Rag-deficient mouse models of inflammatory bowel disease (IBD) have dissected mechanisms involving gut microbiota and counter-regulation of inflammation. Such studies have revealed an interleukin-10 (IL-10)-dependent suppression of colitis-associated colon cancer [21, 27, 28]. This showed explicitly that inhibition of enteric inflammation is pivotal in intestinal tumorigenesis [16, 21, 27, 28]. We have previously tested roles for T cells using adoptive cell transfer in sporadic CRC in C57BL/6 mice heterozygous for a mutation in the *Apc* gene (*Apc*^{Min/+}) [23, 28], which are genetically prone to intestinal polyps that mimic early stages of sporadic CRC in humans [29, 30]. Although risk for sporadic colorectal cancer (CRC) is reduced by non-steroidal anti-inflammatory drug (NSAID) usage in humans, intestinal polyps without overt inflammation are less clearly associated with inflammation than is IBD-CRC. We now know, that despite a lack of overt inflammation, there were higher systemic levels of TNF- α , IL-6 and IL-17 in *Apc*^{Min/+} mice with intestinal polyposis [16] matching findings in colon cancer in humans [31]. More recently, it has become clear from studies using other model systems that intestinal microbiota and inflammation are inextricably linked with risk of developing colon cancer [32-35].

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Prior work in our laboratory [24, 36, 37] and others [38, 39] supports a model in which enteric infections early in life may ultimately suppress IBD and cancer by modulating T cell responses, consistent with the observations of the “hygiene hypothesis” by Belkaid and Rouse [11]. Specifically, we showed that the beneficial cancer-suppressing effects of microbial infections are dependent on Interleukin (IL)-10 [27, 28, 36, 37] a cytokine that also provides suppressive and feedback inhibitory effects on allergies and autoimmune responses [12]. Early life exposures to microbial products have been well studied regarding the etiologies of allergies and asthma. It follows that reduced or delayed exposures to microbiota or their products in childhood might hinder normal immune functions in adult life. Although the ‘hygiene hypothesis’ has been considered in depth for etiology of autoimmune diseases [10, 13], few studies other than our own [14-16, 24, 36, 37] have addressed these concepts as they may relate to cancer development in bowel or extra-intestinal sites.

In humans, the risk of developing CRC is lower in countries that have less stringent hygiene practices [40, 41]. To test this concept of whether this may be due to T cells that fail to protect from intestinal pathology, we applied a T cell transfer animal model using over-reactive “hygienic” lymphocytes in adenoma-prone *Apc^{Min/+}* model. CD4⁺ lymphocytes were isolated from restricted flora *wt* C57BL/6 mice and then injected at dosage of 3×10^5 cells per mouse into mice with additional accumulated gut microbial diversity: co-housed littermate *Apc^{Min/+}* and *wt* recipient animals. We found that cells collected from the uninfected “hygienic” donor mice not only failed to provide protection against intestinal tumor development, but rather increased intestinal tumor burden commensurate with destabilizing changes in the host gut microbiome.

RESULTS

Apc^{Min/+} Mice are Genetically at Increased Risk for Intestinal Polyps

In humans, the risk of developing CRC is lower in countries that have less stringent hygiene practices [40, 41]. To test whether roles for lymphocytes in sporadic CRC, as they may conform to the aforementioned paradigm of autoimmunity, we examined C57BL/6 mice heterozygous for a mutation in the *Apc* gene (*Apc^{Min/+}*), making these mice genetically prone to intestinal polyps that mimic early

stages of sporadic CRC in humans [29, 30]. Here we examined polyposis in 5-mos-old *Apc^{Min/+}* and *wt* littermate mice (Figure 1). Despite a lack of overt intestinal inflammation, *Apc^{Min/+}* mice are prone to intestinal polyposis matching important aspects of colon cancer in humans [31]. This provides a framework to test in immunologically-intact animals whether ability of CD4⁺ T cells to suppress cancer may be more dependent on the prior microbial exposures of the lymphocyte donor rather than that of the recipient animals [16].

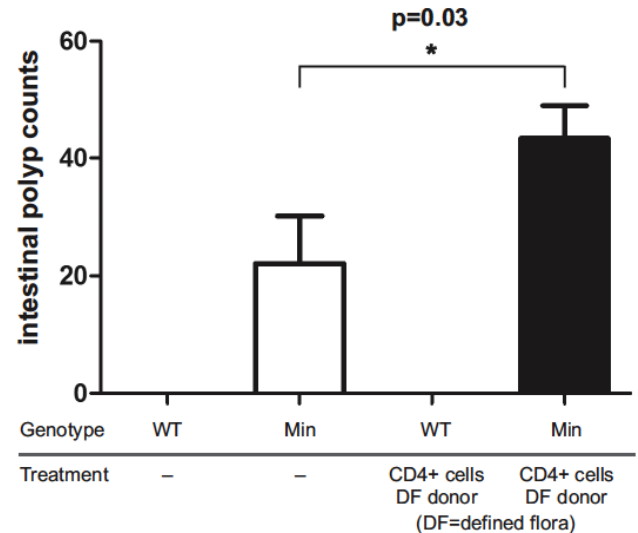


Figure 1: The adoptive transfer of “hygienic” CD4⁺ cells increased *Apc^{Min/+}* (Min) prior to mouse polypogenesis. The y-axis depicts the mean ± SEM of intestinal polyp counts. **p* < 0.05.

Gut Microbiome is More Divergent in *Apc^{Min/+}* mice than in Co-Housed *wildtype* (*wt*) Littermates

It was previously shown that dysregulated inflammatory responses may destabilize the gut microbiome and contribute to colon cancer [32-35]. To test the roles of ‘hygienic’ CD4⁺ cells in this putative destabilizing process, we first performed on mouse stool a microbiome analysis using high-throughput sequencing of the V4 region of the 16S gene using an Illumina HiSeq platform. After quality filtering, we recovered an average of 26,879 reads per sample from 58 samples collected from 18 animals, including 12 harboring the *Apc^{Min/+}* mutation and 6 littermates with a *wt* genotype. We clustered these sequences into 1703 operational taxonomic units for further analysis as previously described [42]. We then compared the compositional variance of baseline sequence data collected from *wt* mice and among *Apc^{Min/+}* mice. Interestingly, we found that although the alpha diversity was not significantly different between these groups,

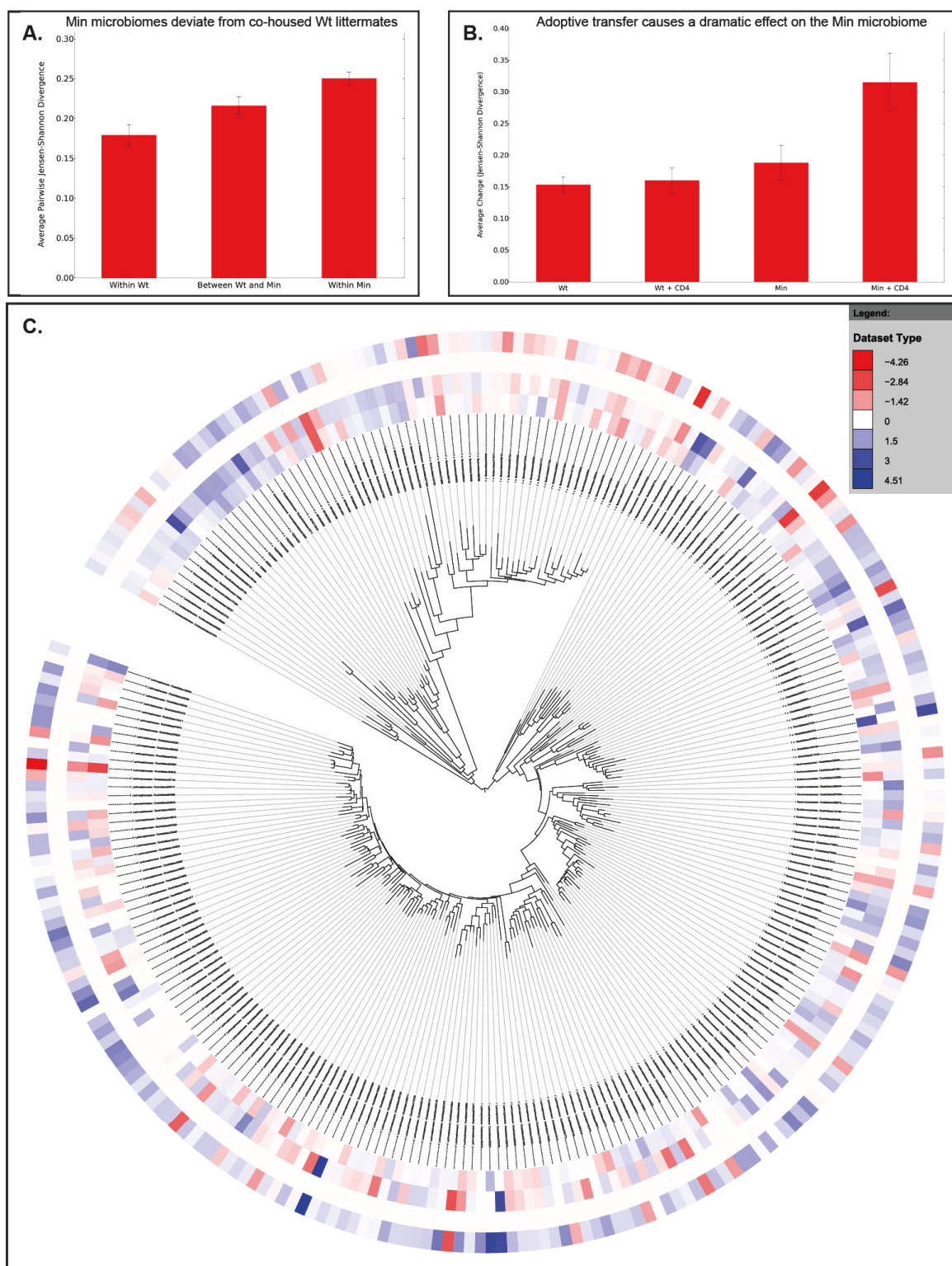


Figure 2: The $Apc^{Min/+}$ (Min) prior to mouse microbiomes are more divergent than *wild type* (wt) littermates when examining the V4 region of the 16S gene using an Illumina platform. **(A)** The average pairwise Jensen Shannon Divergence (JSD) among $Apc^{Min/+}$ mice is significantly greater than among wt mice or even between $Apc^{Min/+}$ mice and wt mice. $Apc^{Min/+}$ mice also deviate from the metacommunity significantly more than do wt mice. **(B)** The microbiome of $Apc^{Min/+}$ mice is dramatically changed after adoptive transfer of 'hygienic' CD4+ cells, while wt mice experience little change. **(C)** Display of operational taxonomic unit (OTU)-level events reveal what is driving high level change shown in bar plots **(A & B)** above. The plot shows the log-fold differences (abundance) of each OTU, with the innermost ring as the untreated $Apc^{Min/+}$, 2nd ring is $Apc^{Min/+}$ after lymphocyte transfer, 3rd ring is Untreated wt, outermost ring is wt mouse microbiome after CD4+ lymphocyte transfer. Values are plotted as color intensities red (decreased) or blue (increased) and range from 4.5 to -4.5 log-fold changes, when compared with untreated wt mice.

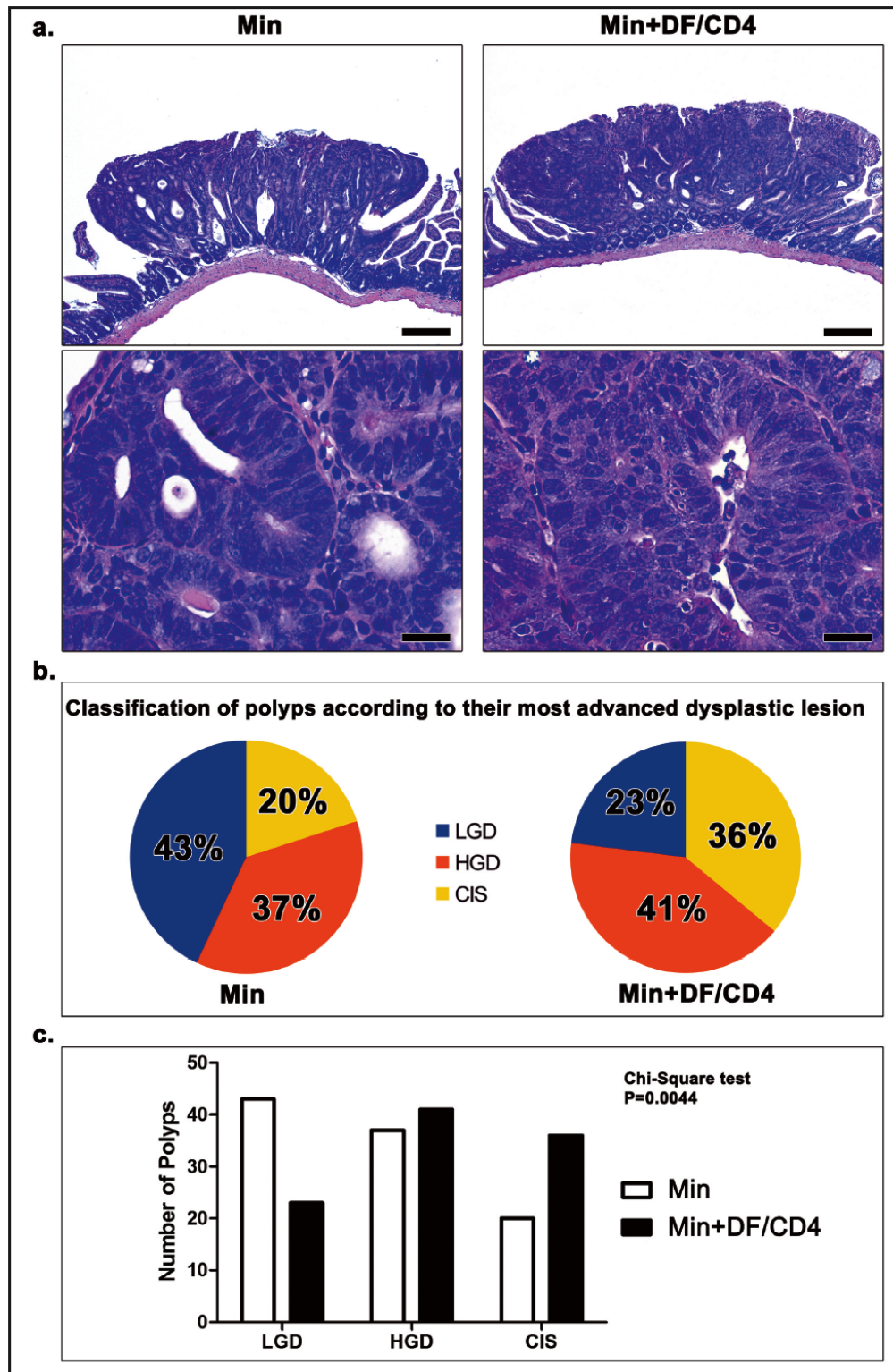


Figure 3: Effects of “hygienic” CD4⁺ cell transfer on *Apc*^{Min/+} (Min) prior to mouse polyp histopathology. (a) Side by side comparison of typical small intestinal polyps of *Apc*^{Min/+} controls (left panel) and *Apc*^{Min/+} “hygienic” cell recipients (right panel). The most advanced glandular dysplasia/adenoma lesion found in each polyp is shown below in higher magnification. While the sham-treated control mouse polyp contains LGD and HGD lesions, the polyp of its age-matched “hygienic” lymphocyte-treated counterpart contains CIS characterized by increased glandular shape and size irregularities, epithelial pseudostratification, cellular atypia, nuclear size and pleomorphism, abnormal mitotic figures and apoptosis. (b) The occurrence of CIS is higher in the *Apc*^{Min/+} mouse polyps of “hygienic” CD4⁺ cell recipient mice compared to their sham-treated controls and (c) reaches statistical significance. Hematoxylin and Eosin, Scale bars: a-upper panel=250 μ m; a-lower panel=50 μ m. Numbers on the y axis of bar graph correspond to the mean $\pm$ SEM of polyps classified according to their most advanced dysplasia/adenoma lesion.

the beta-diversity (or divergence within a group) was higher even in the unmanipulated *Apc*^{Min/+} group. Specifically, the average Jensen-Shannon Divergence (JSD) among *Apc*^{Min/+} mice (0.24) was higher than among co-housed *wt* littermates (0.18, $p = 2.55 \times 10^{-5}$, Mann-Whitney U-test). The *Apc*^{Min/+} microbiome is more variable across individuals, consistent with reduced regulation of the microbial population under the influence of the *Apc*^{Min/+} mutation (Figure 2a). That this observation persists despite coprophagia among co-housed *wt* littermates that were reared together suggests that the *Apc*^{Min/+} mutation may play a role in regulating the composition of the microbiome, potentially mediated by cell-based immunity.

Transfer of CD4+ Lymphocytes Collected from 'Hygienic' Restricted Flora Donor Mice Rapidly Increases Inflammatory Cell Infiltrates and Intestinal Tumorigenesis

In immune competent animals, whole CD4+ T cells potently suppress inflammation, and their ability to do so was previously shown to be more dependent on the prior microbial exposures of the lymphocyte donor rather than that of the recipient animals [16]. To test this concept of whether T cells may fail to protect from intestinal pathology under more "hygienic" conditions in the adenoma-prone *Apc*^{Min/+} model, we applied a T cell transfer model. CD4+ lymphocytes were isolated from restricted flora source [hygienic] C57BL/6 mice and then injected at dosage of 3×10^5 cells per mouse into littermate *Apc*^{Min/+} and *wt* littermate recipient animals. We found that cells collected from "hygienic" donor mice not only failed to provide protection against intestinal tumor development, but rather increased intestinal tumor burden in *Apc*^{Min/+} mice when compared with sham-dosed controls (Figure 1).

Furthermore, the adenomatous polyps of mice that received "hygienic" lymphocytes were in a more advanced stage of the adenoma to adenocarcinoma progression compared to their sham-treated counterparts. Based on previously described histomorphological criteria [21, 43, 44] focal lesions of dysplasia/adenoma within polyps were identified as low-grade dysplasia (LGD) or high-grade dysplasia (HGD) and carcinoma in situ (CIS, intraepithelial neoplasia) (Figure 3a). The classification of small intestinal polyps according to the most advanced lesion they contained showed that "hygienic" T cell recipient mice had significantly more polyps bearing CIS compared to control mice (Figure 3b and 3c). Lymphocytes, macrophages, neutrophils, plasmacytes and mast cells in the intestinal adenomas of both groups of *Apc*^{Min/+} mice followed the typical polyp-associated inflammatory cell topographical distribution pattern [23, 44]. However, the inflammatory cell accumulation was more pronounced in the polyps of "hygienic" cell recipients. We have previously shown that neutrophils enhance intestinal tumorigenesis and that their accumulation in cancer-prone epithelia is influenced by CD4+ cell subsets [23, 44, 45]. We next quantitatively assessed MPO+ granulocytes (neutrophils) in the polyps of *Apc*^{Min/+} mice. We found that tumor-associated MPO+ cells were significantly more in the mice treated with "hygienic" CD4+ lymphocytes when compared to the sham-dosed controls (Figure 4).

Gut Microbiome is More Divergent in *APC*^{Min/+} Mice After Adoptive Transfer of 'Hygienic' Pro-Inflammatory CD4+ Lymphocytes

Finally, to test whether the 'hygienic' CD4+ cells lead to increased microbial divergence that may

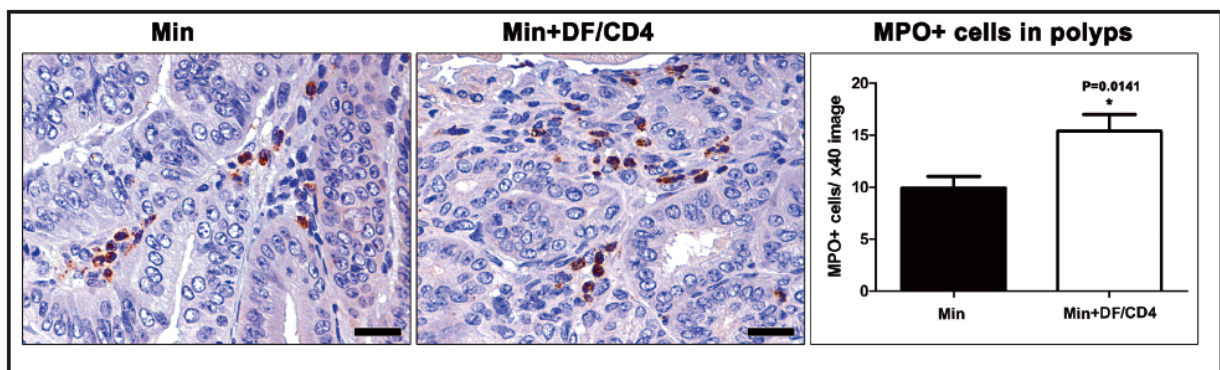


Figure 4: Tumor-associated MPO+ cells in *Apc*^{Min/+} (Min) prior to mouse polyps. The polyps of "hygienic" cell-treated mice contain significantly more MPO+ cells (neutrophils) compared to the polyps of sham-treated control mice. IHC: DAB chromogen, hematoxylin counterstain. Scale bars=25 μ m. Numbers on the y-axis of bar graph correspond to the mean $\pm$ SEM of immunohistochemically positive cell counts. * $p < 0.05$.

contribute to cancer risk, we examined stool of cell transfer recipients of lymphocytes from syngeneic C57BL/6 *wt* cell donors of ultra-hygienic restricted flora health status into these *Apc*^{Min/+} or *wt* mice housed under conventional conditions. In the absence of this intervention, mice experienced modest ecological drift equivalent to a Jensen Shannon Divergence (JSD) of approximately 0.17 in both *Apc*^{Min/+} and their *wt* littermate mice. *wt* mice subjected to lymphocyte transfer experienced a similar level of change in their microbiome (0.16, ns). However, *Apc*^{Min/+} mice subjected to lymphocyte transfer experienced a radical change in their microbiome of approximately double (0.31) the background rate of change in untreated controls ($p = 0.05$, Mann-Whitney U-test in Jensen-Shannon Divergence between paired time points in each animal) (Figure 2b). Operational taxonomic unit (OTU)-level microbial events were found to be driving the higher level changes in diversity (Figure 2c). The radical change in microbiome after transplant of 'hygienic' lymphocytes coincided with increased gut inflammatory index (Figure 4), and also features of intestinal adenoma multiplicity (Figure 1) and malignancy (Figure 3). This further implicates the immune system in the diversity of the *Apc*^{Min/+} microbiome and predilection to cancer.

DISCUSSION

To test this concept of whether T cells arising under "hygienic" conditions may fail to protect from intestinal pathology, we applied a T cell transfer model in the adenoma-prone *Apc*^{Min/+} model. Under normal conditions, whole CD4+ T cells prevent intestinal pathology [16, 20]. However, we found that cells collected from the restricted flora "hygienic" donor mice not only failed to provide protection against intestinal tumor development, but rather increased intestinal tumor burden. Adenomatous polyps of mice that received "hygienic" lymphocytes were in a more advanced stage of adenocarcinoma development, coincident with an increased inflammatory cell index within adenomas. Taken together, these observations connect the immune system, hygienic rearing, and diverse immune-mediated diseases including allergies, autoimmune disease, and cancer [16].

The radical change in microbiome after transplant of 'hygienic' lymphocytes coincided with an increased gut inflammatory index and intestinal adenoma multiplicity and malignancy, further implicating the immune system in the diversity of the *Apc*^{Min/+} microbiome and predilection to cancer. Indeed, inflammation-associated

gut microbial ecology instability has previously been linked with opportunistic pathogenic infections and colon cancer [34]. It is noteworthy that the *Apc*^{Min/+} microbiome was found to be more variable across untreated individuals, when compared to *wt* littermates, consistent with reduced regulation of the microbial population under the influence of the *Apc*^{Min/+} mutation. That this observation persists despite coprophagia when co-housed with *wt* litter mates that were reared together suggests that the *Apc*^{Min/+} mutation plays a role in regulating the composition of the microbiome, potentially mediated by cell-based immunity.

In summary, it has been well established in humans and in mice that chronic inflammation increases the risk of CRC [21, 23, 25, 31, 36, 37, 45, 46]. It is paradoxical, then, that the risk for developing CRC is actually lower in countries that have less stringent hygiene practices with fewer exposures to potentially pathogenic organisms [40, 41], such as in North America. This paradox is explainable using cell transfer assays reveal that "hygienic" CD4+ cells may under some circumstances serve to promote carcinogenesis and increase cancer risk. This may be due in part to inability of "hygienic" source lymphocytes to suppress Th-17 inflammation [16], leading to gut microbiome instability that directly or indirectly influences cancer growth. Ultimately, in aging or genetically susceptible hosts this immune dysregulation leads to aberrant wound healing and ultimately contributes to cancer growth.

MATERIALS AND METHODS

Experimental Animals

All animals were housed in AAALAC accredited facilities and maintained according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Massachusetts Institute of Technology. C57BL/6 strain mice of defined flora health status were obtained from Taconic Farms (Germantown, NY) to provide 'hygienic' CD4+ cell donors for experimental animals. C57BL/6 background *Apc*^{Min/+} mice on a C57BL/6J background were originally obtained from Jackson Labs (Bar Harbor, ME), then rederived by embryo transfer into Taconic microbial status recipient mice, and then bred in-house under standard conditions as (heterozygous X *wt*) crosses to provide *Apc*^{Min/+} mice and *wt* littermates of 'non-hygienic' status to use as cell recipients. Mice were humanely euthanized according to institutional criteria (i.e., poor body condition score, large tumor

size) or when exhibiting other signs of distress. Experiments were conducted using six mice per treatment group as noted throughout the text.

Experimental Design

A total of 24 mice were used for these experiments. Twenty C57BL/6 *wt* (N=8) or *Apc*^{Min/+} (N=12) littermate mice were included in treatment regimens or as experimental controls. Cell recipient and control mice were subdivided into large cages with ten mice (six *Apc*^{Min/+} and four *wt* mice) per cage to permit optimal co-housing for treatment and microbiome analyses. Fecal specimens were collected individually prior to treatment and then again every three weeks until the end of the study. An additional cohort of restricted flora C57BL/6 *wt* mice (N=4) were housed separately in a different animal facility and used as donors of 'hygienic' CD4⁺ lymphocytes. Recipient mice were injected with CD4⁺ lymphocytes (N = six *Apc*^{Min/+} mice per treatment group), or underwent sham injection with media only, at three months of age.

Adoptive Transfer of Purified CD4⁺ T Cells into Recipient Mice

CD4⁺ lymphocytes isolated using spleens and mesenteric lymph node from Taconic restricted flora *wt* mice using magnetic beads (Dyna) and then sorted by hi-speed flow cytometry (MoFlow2) to obtain purified populations of CD4⁺ lymphocytes and determined to be ~98% pure as previously described elsewhere [16]. Anesthetized recipient mice aged three months were injected intraperitoneally with 3×10^5 T cells as previously described.

Gut Microbiome Analyses

We performed on mouse stool high-throughput sequencing of the V4 region of the 16S gene using an Illumina HiSeq platform. After quality filtering, we recovered an average of 26,879 reads per sample from 58 samples collected from 18 animals, including 12 harboring the *Apc*^{Min/+} mutation and 6 littermates with a *wt* genotype. We clustered these sequences into 1703 operational taxonomic units for further analysis as previously described [42]. The OTU level analysis for min study utilizes four groups of mice, untreated *Apc*^{Min/+}, untreated *wt*, *Apc*^{Min/+} mice after lymphocyte transfer and *wt* mice after lymphocyte transfer. Each of the 1703 OTUs in our dataset is considered as to whether that OTU is enriched in any given treatment group relative to any other treatment group, plotted with

the log-fold differences for each OTU and group relative to the untreated *wt* group. Red color reflects values below *wt* and blue reflects values above *wt*. For the $p = 0.05$ group the range is 4.5 to -4.5 log-fold changes.

Quantitation of Intestinal Tumors

Intestinal tumors were counted using a stereomicroscope at x10 magnification. Location of tumors was determined relative to the distance from the pylorus.

Histopathology and Immunohistochemistry

For histologic evaluation, formalin-fixed tissues were embedded in paraffin, cut at 4 μ m, and stained with hematoxylin and eosin or immunohistochemistry (IHC). Polypoid adenomas were classified according to the worst preneoplastic lesion they contained. Prenaloplastic lesions were classified as low (LGD) and high grade dysplasia (HGD) or Carcinoma *in situ* (CIS) using histomorphological criteria that have been earlier described [21, 44]. MPO-specific immunohistochemistry and quantitative histomorphometry of MPO-positive cells were performed as previously described [44].

Statistical Analyses

Adenomatous polyp counts and tumor-associated MPO⁺ neutrophils were compared between groups using Mann-Whitney U analysis. The staging of polyps according to their most advanced dysplasia/adenoma lesion was compared between groups with the Chi-square test. Statistical significance was set at $P < 0.05$. Analyses were performed with the Graphpad Prism version 5.0 for windows, GraphPad software, San Diego, CA.

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The authors have declared that no competing financial interests exist.

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