Initiation of inflammatory tumorigenesis by CTLA4 insufficiency due to type 2 cytokines

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Genetically predisposed CTLA4 insufficiency in humans is associated with gastric cancer development, which is paradoxical to the prototypical role of CTLA4 in suppressing antitumor immunity. CTLA4 is a critical immune checkpoint against autoimmune disorders. Autoimmunity has been implicated in protumor or antitumor activities. Here, we show that CTLA4 insufficiency initiates de novo tumorigenesis in the mouse stomach through inflammation triggered by host-intrinsic immune dysregulation rather than microbiota, with age-associated progression to malignancy accompanied by epigenetic dysregulation. The inflammatory tumorigenesis required CD4 T cells, but not the T_\text{H}1 or T_{\text{H}}17 subsets. Deficiencies in IL-4 and IL-13 or IL-4 receptor \(\alpha\) broke the link between inflammation and initiation of tumorigenesis. This study establishes the causality of CTLA4 insufficiency in gastric cancer and uncovers a role of type 2 inflammation in initiating gastric epithelial transformation. These findings suggest possible improvement of immune therapies by blocking tumorigenic type 2 inflammation while preserving antitumor type 1 immunity.

INTRODUCTION

Gastric (stomach) cancer (GC) is the second most lethal and the fourth most common cancer, causing more than 700,000 deaths per year worldwide (Lozano et al., 2012). GC is usually diagnosed at age 60 yr or older, with a higher risk in minorities. However, a recent study found an upward trend in incidence of noncardia GC (which refers to GC in all areas except the top part of stomach) in young white Americans (ages 25–39 yr; Anderson et al., 2010). GC represents a prototype of inflammatory carcinogenesis in solid tumors. Indeed, it is the study of GC that has provided some of the early evidence for the role of inflammation in cancer development. GC often develops occultly until a sign of metastatic cancer emerges, such as the “telltale” lymph node metastasis termed Virchow’s node (Siosaki and Souza, 2013), which is named after Virchow, who made the original observation in the 19th century and also proposed the link between inflammation and cancer.

Gastric adenocarcinoma (GA) accounts for most GC cases. Its origin remains unclear. In a classical paradigm known as the Correa cascade, the etiology of GA is described as a histopathological process proceeding from gastritis, intestinal metaplasia (IM), and dysplasia to carcinoma (Correa, 1988). A new type of gastric metaplasia, spasmylocytic poly-

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cribes (30% in mRNA and 18–46% in proteins; Kuehn et al., 2014). Genetic studies of 251 cases of human GA from different ethnic populations have also found a paradoxical association with CTLA4 because the risk alleles of CTLA4 promoter and exon 1 linked to GC (Hadinia et al., 2007; Hou et al., 2010) are known to cause reduced CTLA4 expression (Ligers et al., 2001; Anjos et al., 2002; Wang et al., 2002). Of note, GC was also found in a patient with a deficiency of LRBA (LPS-responsive vesicle trafficking, beach- and anchor-containing) protein (Bratanič et al., 2017), a defect that causes secondary CTLA4 loss (Lo et al., 2015). CTLA4 is an immune checkpoint controlling T cell homeostasis (Tivol et al., 1995; Waterhouse et al., 1995; Chambers et al., 1997). It is a prototypical inhibitor of antitumor immunity (Chambers et al., 2001). Although the genetic evidence of CTLA4 insufficiency in human GC etiology is paradoxical to the prototypical role of CTLA4 in antimùm tumor immunity, the new data are conceptually consistent with the inflammatory etiology of human GC in general and suggest new pathways of inflammatory tumorogenesis in humans.

We have created transgenic CTLA4 shRNA knockdown (CTLA4KD) mice to mimic CTLA4 insufficiency in humans. The transgene encodes a CTLA4-specific shRNA driven by a U6 promoter and reduces CTLA4 expression to ~40% of controls. This model has been used to study how CTLA4 insufficiency impacts immune regulation in various genetic backgrounds (Chen et al., 2006; Miska et al., 2012, 2014; Devarajan et al., 2014). We adapted the CTLA4KD model to study the role of CTLA4 insufficiency in GC development. We found that CTLA4 insufficiency, modeled by CTLA4KD or antibody blockade, caused the initiation of inflammatory tumorogenesis in the stomach of mice with susceptible genetic backgrounds. Furthermore, this study also provides the first evidence, to our knowledge, for a novel role of TGFβ cytokines IL-4/IL-13 in causing premalignant differentiation in gastric epithelia and thus mediating the link between inflammation and initiation of gastric tumorogenesis.

RESULTS

CTLA4 insufficiency modeled by transgenic RNA interference (RNAi) initiated the SPEM premalignancy development in the stomach

To understand the human GC genetic risks linked to CTLA4 insufficiency, we used the CTLA4KD mouse model. We first studied the animals on the BALB/c × C57BL/6 (B6) F1 mixed genetic background (called CB6F1). CB6F1 mice are a robust model with a noninbred yet homogenous genetic makeup (Miller et al., 2005). The CTLA4KD and PL4 vector control transgenic lines on the B6 genetic background (CTLA4KD/B6 or PL4/B6; Miska et al., 2012, 2014) were crossed with BALB/c mice to generate CTLA4KD mice and PL4 controls on the CB6F1 genetic background. Transgene-negative littermates were also used as controls. Supporting the genetic associations of CTLA4 insufficiency with human GC, CTLA4KD mice exhibited a type of gastric premalignant pathology termed SPEM. As shown in Fig. 1, inflammatory infiltration in the stomach mucosa of CTLA4KD mice was detectable at 3 wk of age, and SPEM started to develop at 4 wk of age. At ~20 wk of age, all CTLA4KD mice exhibited extensive SPEM (100% penetrance; Fig. 1, A–C). Gross examination of the stomach identified a “wrinkled” appearance of the external surface (Fig. S1, A and B). At the mucosal surface, enlarged rugae were readily visible (Fig. S1 C); histological examination revealed massive SPEM (Fig. S1, D–G). CTLA4 insufficiency induced SPEM on the B6 genetic background, but it occurred mildly, with a late onset, and only developed in some animals; however, CTLA4 insufficiency induced robust SPEM pathology in mice on the BALB/c inbred genetic background (Fig. S1, H and I).

SPEM spontaneously progressed to adenocarcinoma with age in mice with CTLA4 insufficiency

GC development perhaps represents one of the clearest examples of age-associated carcinogenesis. We examined the impact of age on GC development in CTLA4KD mice. The SPEM lesion in this model persisted until ~52 wk of age, but by ~78 wk of age, all mice had dysplastic lesions in the gastric mucosa exhibiting cytological atypia and architectural complexity. Moreover, the frankly malignant pathology was evident with destructive growth beyond the mucosa that breached through the muscularis mucosae, with growth in the submucosa and even the muscularis externa. The cancerous growth had 100% penetrance and mainly consisted of adenocarcinoma with differentiated glandular structure, although heterogeneous neoplastic tissues could be identified (Fig. 1, A and C; and Fig. S2 A). Of note, human GC may manifest as well-differentiated adenocarcinoma (Kushima et al., 2006; Yao et al., 2006), including the GC associated with CTLA4 insufficiency (Hayakawa et al., 2016), which was unexpectedly more resistant than poorly differentiated GA to chemotherapy drugs (Machara et al., 1987).

Besides pathological characterization, we also assessed the mucosal tumors in the CTLA4KD mice with biological tests. We made a cell line from an aging CTLA4KD mouse, which formed tumors in recipient mice (Fig. S2 B). Furthermore, we tested freshly isolated mucosal cells by implanting them subcutaneously into new recipient mice on the CB6F1 background. Gastric mucosal cells from aging CTLA4KD mice, but not the age-matched controls, formed tumors after injection into new recipients on the CB6F1 background (Fig. 1, D and E), confirming their transformed and immortalized status. Reflecting a feature of the original tumors (Fig. 1 A) in CTLA4KD mice, the new subcutaneous tumors that formed from the implanted cells had secretory glands (Fig. 1 F).

SPEM is characterized by overexpression of Trefoil factor 2 (TFF2; Goldenring et al., 2010), whereas loss of TFF1 causes GC (Soutto et al., 2011). In gastric mucosae of CTLA4KD mice, Tff1 gene expression was substantially lower than controls, whereas Tff2 gene expression was increased as...
much as ~10-fold over controls (Fig. S3 A). We also assessed
the mRNA expression of 84 genes commonly associated
with cancer development and found that many genes exhib-
ited no substantial changes in expression, whereas some had
moderate alteration in the expression in the tumor at ~78
wk of age (not depicted). Furthermore, we assessed the ex-
pression of a few genes from the early to late stages of tumor
development. We detected subtle or no changes in Myc and
p53 expression but found a dynamic profile of Mmp9 as well
as cell cycle control genes Ccna1 and Cdkn1c (which have
been associated with human GC; Sapari et al., 2012; Loh et
al., 2014) along the inflammatory oncogenic cascade, espe-
cially during the transition from SPEM to GA (Fig. S3, B–E).
Therefore, with the histological phenotype, the characteristic
gene expression and the spontaneous progression to malig-
nancy, the SPEM induced by CTLA4 insufficiency indeed
represents a premalignancy.

CTLA4 insufficiency modeled by anti–CTLA4 antibody
blockade induced the SPEM premalignancy in mice
To corroborate the role of CTLA4 insufficiency in gastric
tumorigenesis by methods independent of RNAi, we treated
mice on the CB6F1 background with anti-CTLA4 mAbs
to down-modulate CTLA4. To recapture genetically pre-
disposed CTLA4 insufficiency, mice were treated with an-
ti-CTLA4 antibodies from 3–4 d of age for 4 wk and analyzed
1 wk after the treatment. The treatment induced obvious al-
teration detectable from the external surface (Fig. 2 A) and
the luminal (mucosal) side of the stomach (Fig. 2 B), as well as
inflammatory infiltration and characteristic SPEM pathology
(Fig. 2, C–E) in the stomach in five of seven treated animals.
The data corroborate the observations of gastric tumorigene-
sis in CTLA4KD mice and are consistent with the association
of CTLA4 insufficiency with human GC.

Gastric tumorigenesis caused by CTLA4
insufficiency was caused by host–intrinsic immune
dysregulation rather than microbiota
To investigate whether undetected overgrowth of normal flor-
al Helicobacter contributed to the gastric tumorigenesis in
CTLA4KD mice, we purified DNA from stomach samples
and conducted a group-specific PCR assay for the 16S rRNA
gene of all known Helicobacter species or for all bacteria. As
shown in Fig. S4 (A), Helicobacter 16S rDNA was detectable
in the gastric mucosa of normal mice. Both pan–Helicobacter
16S rDNA and pan–bacterial 16S rDNA assays indicated a
lower bacterial load in the gastric mucosa of CTLA4KD mice
surveyed up to ~80 wk of age, suggesting that the gastric tu-
morigenesis in CTLA4KD mice occurred independently of
pathogenic Helicobacter infections.

We next tested whether microbiota was required for the
gastric tumorigenesis. CTLA4KD mice were rederived and
maintained in germ-free (GF) facilities. The animals were an-
alyzed at 9–10 wk of age. Similar to age-matched CTLA4KD
mice maintained in specific pathogen–free (SPF) facilities,
GF CTLA4KD animals developed extensive SPEM in the
gastric mucosa (Fig. 3, A and B). Therefore, microbiota was
not required for the initiation of tumorigenesis by CTLA4
insufficiency, although we cannot rule out the effect of po-
tential microbial products in the sterilized food because it was
not synthetic. Indeed, microbiota-independent autoimmune
responses were evident in both cellular and humoral immune
responses. There was a high titer of serum autoantibodies
against gastric mucosa in CTLAKD mice even in the ab-
sence of microbiota (Fig. 3, C and D). Likewise, heightened
T$_{H}$1, T$_{H}$2 and T$_{H}$17 responses were detected in CTLA4KD
mice regardless of the GF or SPF conditions (Fig. 3 E and
Fig. S4, B–D). Together, these results indicate that the intrin-
sic immune dysregulation induced by CTLA4 insufficiency
was sufficient to cause de novo tumorigenesis in this model,
although the results do not rule out the contribution of mi-
crobial agents in GC development.

Gastric tumorigenesis induced by CTLA4 insufficiency
was transferrable with immune cells and required CD4 T cells
To examine whether the effect of CTLA4 insufficiency on
gastric tumorigenesis indeed originated from immune cells,
we reconstituted neonatal immunodeficient (Rag1$^{-/-}$) mice
with 15 million spleen cells from CTLA4KD or littermate
control donors. Reconstituted animals were analyzed 6–10
wk later. Indeed, CTLA4KD donor cells, but not the controls,
cauced SPEM in reconstituted Rag1$^{-/-}$ recipients (Fig. 4 A).
Depletion of CD4 T cells, but not CD8 cells, abrogated the
inflammatory tumorigenesis by CTLA4KD donor cells.
We then conducted the adoptive transfer experiment with a mixture of CD25− spleen cells from CTLA4KD donors with the CD25+ fraction from the spleen of normal mice, or vice versa. The former, but not latter, group exhibited inflammation and SPEM in the stomach (Fig. 4, E and F), strongly suggesting that the defect of CTLA4 insufficiency that causes de novo inflammatory tumorigenesis resides in the CD4+CD25− pathogenic effector T (T eff) cell rather than regulatory T (Treg) cell subset. Of note, the role of CD4 Teff cells is unlikely to be caused by their helper function for antibody production because serum transfer from CTLA4KD donors did not cause pathology in the stomachs of recipient mice (not depicted).

TH1 and TH17 cells were not required for tumorigenesis caused by CTLA4 insufficiency To examine the role of T H1 cells in inflammatory tumorigenesis caused by CTLA4 dysregulation, we used IFN-γ−deficient mice on the BALB/c background. Anti-CTLA4 mAb initiated SPEM efficiently in mice on the BALB/c background. IFN-γ deficiencies did not inhibit the extent of inflammatory pathology or SPEM development (Fig. 5 A), indicating that T H1 responses were not required for the initiation of inflammatory tumorigenesis caused by CTLA4 insufficiency.

We then tested the role of T H17 cells. In our models, IL−17A rather than IL−17F was detected at a high levels. Therefore, we used IL−17A knockout mice to study the role of T H17 cells. As shown in Fig. 5 B, deficiencies in IL−17A did not reduce inflammatory infiltration in the gastric mucosa. Importantly, IL−17A knockout did not curtail the initiation of tumorigenesis, as the extent of SPEM between CTLA4KD IL17A+ and CTLA4KD IL17A− mice did not differ significantly (Fig. 5 B).

IL−4 and IL−13 played an essential role in mediating the link between inflammation and initiation of tumorigenesis Because our original hypothesis on the causal role of T H1 and T H17 cells in the inflammatory tumorigenesis turned out to be incorrect (Fig. 5), we examined a potential role for T H2 dysregulation in gastric tumorigenesis. IL−4 and IL−13 are essential for T H2 differentiation and effector function (Ansel et al., 2006). The IL−4 and IL−13 genes are located in adjacent

Figure 2. CTLA4 insufficiency modeled by anti−CTLA4 monoclonal antibody blockade induced SPEM in wild−type mice. Wild-type mice on the mixed CB6F1 background were treated with monoclonal antibodies against CTLA4 or control, from 3–4 d of age for 4 wk. The animals were analyzed at 5 wk of age. (A) Images of external stomach. (B) Images of the luminal side in the stomach. (C) Representative images of H&E−stained sections (low−power images followed with high−power images of the highlighted areas). Bars: (low power) 500 µm; (high power) 100 µm. (D and E) Summary of histopathology scores. Data represent six to seven mice in each group pooled from two experiments. Each data point represents one animal [mean ± SEM; Mann−Whitney test]. *, P < 0.05; **, P < 0.01.
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Figure 3. Gastric tumorigenesis initiated by CTLA4 insufficiency occurred independently of microbiota, with evidence of autoimmunity against gastric mucosal tissue. CTLA4KD mice and controls in SPF versus germ-free (GF) facilities were analyzed between 9–10 wk of age. (A) Representative histopathology images. Bars, 100 µm. (B) Summary of pathology scores (Mann–Whitney test). (C) Serum or plasma samples from CTLA4KD and wild-type littermates maintained in GF or SPF facilities were assayed for gastric mucosal autoantibodies. Autoantibodies that bound to gastric mucosal antigens were revealed by secondary antibodies conjugated with fluorescent probes (red); DAPI counterstained for nuclei (blue). Bars, 100 µm. (D) Titration of the autoantibodies (assay range, 60–60,000; Kruskal–Wallis test). (E) The lymphocytes from CTLA4KD and control mice were analyzed by intracellular cytokine staining. Summary of the total numbers of IFN-γ–producing TH1, IL-17A–producing TH17, and IL-4– or IL-13–producing TH2 effector cells in the draining lymph nodes of CTLA4KD mice versus controls in SPF versus GF environments. Each data point represents one animal (mean ± SEM; Mann-Whitney test). For all panels, data represent four to seven mice pooled from two analysis experiments with mice from one GF derivation. **, P < 0.01; ***, P < 0.001.
Figure 4. CD4 T cells were required for gastric tumorigenesis initiated by CTLA4 insufficiency. Splenocytes, or CD4- or CD8-depleted fractions of splenocytes, were prepared from CTLA4KD or littermate control donors. For some experiments, splenocytes from CTLA4KD and normal mice were separated into CD25+ and CD25− fractions and cross-mixed at 1:25 ratio with CD25+ cells from CTLA4KD donors and CD25− cells from normal controls, respectively,
positions. Their functions overlap but are not completely redundant (Hallett et al., 2012; Wynn, 2015). Although IL-4/IL-13 dysregulation is implicated in cancer cell survival and proliferation (Hallett et al., 2012), whether it has a role in the early stage of tumorigenesis (i.e., the differentiation of premalignant lineages) remains unclear.

To obtain direct evidence for IL-4/IL-13 in the initiation of tumorigenesis, we used genetic ablation. Recently, a knockin line with loxp sites flanking the adjacent IL-4 and IL-13 genes (the IL4/IL13flox line) was generated and enabled conditional knockout of both IL-4 and IL-13 (Liang et al., 2011). In preliminary studies, we obtained IL4/IL13flox mice and crossed them with CMV-Cre mice to generate a line of germline knockout on both IL4 and IL13 genes, IL4/IL13°.

We crossed IL-4Rα knockout mice (Noben-Trauth et al., 1997). IL-4Rα knockin line with loxP sites flanking the adjacent IL-4 and IL-13 receptors (Hallett et al., 2012). They exhibited robust SPEN pathology, littermate IL4/IL13° mice had no SPEN lesions in the stomach even at 8–10 wk of age, which otherwise would have developed at ~4 wk of age (see Fig. 1). However, the extent of inflammatory pathology was comparable between CTLA4KD IL4/IL13° and CTLA4KD IL4/IL13° littermates (Fig. 6 A). Indeed, destruction of parietal cells in the CTLA4KD IL4/IL13° mice was evident in histopathology (Fig. 6 A) and reflected in the elevated pH of gastric luminal fluid (not depicted).

The finding that IL-4/IL-13 deficiencies suppressed SPEN development prompted us to explore a potential therapeutic modality: neutralizing type 2 cytokines to block the initiation of inflammatory tumorigenesis. We tested anti-IL-4 antibody treatment in the CTLA4KD model. As shown in Fig. 6 B, although neutralizing IL-4 in CTLA4KD mice did not reduce the extent of inflammatory infiltration, it significantly inhibited SPEN pathology.

IL-4 and IL-13 may act directly on the epithelial compartment, or signal intrinsically in immune cells and indirectly affect epithelial transformation. As an initial assessment of this possibility, we obtained IL-4 receptor α (IL4Rα) knockout mice (Noben-Trauth et al., 1997). IL-4Rα is a subunit for both IL-4 and IL-13 receptors (Hallett et al., 2012). We crossed IL-4Rα° mice with a Rag1° line and generated IL4Ra°Rag1° double knockout mice and IL4Rα°Rag1° controls. These mice were used as recipients for adoptive transfer of spleen cells from CTLA4KD or control donors. The transferred spleen cells were not only capable of producing IL-4 and IL-13 but also had intact IL4Rα–mediated signaling. The reconstituted animals thus had IL4Rα deficiency or sufficiency in the stromal and epithelial lineages. To make the experiments more rigorous, the IL4Rα°Rag1° mice or IL4Rα°Rag1° controls were reconstituted with spleen cells at either an adult age (Fig. 6 C) or neonatal age (Fig. 6 D). Indeed, IL4Rα deficiency in the recipient abrogated the initiation of tumorigenesis, but not the inflammation (Fig. 6, C and D). This effect was not caused by reduction in recruitment of transferred CTLA4KD T cells to the stomach mucosa. There were also similar frequencies of F4/80° cells (macrophages; Fig. 6 E) or Gr1° granulocytes (not depicted) in inflamed stomach mucosa of IL4Rα° or IL4Rα° recipients. Further studies with definitive lineage tracing tools are needed to pinpoint the IL4Rα signaling to a specific epithelial lineage in the stomach mucosa. However, it is worth noting that the predominant cells recruited to the stomach mucosa at this early stage were T cells, and the SPEN cells were likely differentiated from resident cells in the stomach, in particular, by transdifferentiation from chief cells (Nam et al., 2010). Collectively, these results strongly suggest that the inflammatory tumorigenesis is triggered by proximal “cross talk” between dysregulated T1/2 cells and epithelial cells, with a major role of IL4Rα–mediated signaling in the epithelial compartment, which initiates the early transformative events.

**Inflammatory tumorigenesis caused by CTLA4 insufficiency was associated with epigenetic dysregulation reflected in human GC**

Chronic cytokine stimulation may lead to epigenetic dysregulation. Indeed, IL-4 signaling has long been known to cause modification of DNA methylation and drive lineage specification of immune cells (Ansel et al., 2006). IL-4/IL-13 receptors are expressed by human GC cells (Morisaki et al., 1994) as well as in the SPEN and GA in the CTLA4KD model (Fig. S5 B). Thus, IL-4/IL-13 signaling may, directly or indirectly, contribute to epigenetic dysregulation that promotes GC development. One of the prominent epigenetic changes in a tumorigenic cascade is an altered profile of methylation in genomic DNA at the 5 position of cytosine (5mC; Jones, 2012). The abundance of 5mC in the genomes...
makes 5mC content an insensitive parameter for quantifying changes. Prompted by a study of melanoma-associated loss of 5-hydroxymethylcytosine (5hmC; Lian et al., 2012), the hydroxylated product of 5mC, we quantified 5hmC content in genomic DNA of gastric mucosae from CTLA4KD mice and controls as a global assessment of methylation-based epigenetic changes (Fig. 7, A and B). We detected a lower 5hmC content in GC samples in aging CTLA4KD mice than in littermate controls, but not in the young groups. We examined regulatory genes in the 5hmC pathway, ten-eleven translocation methylcytosine dioxygenase (Tet) and isocitrate dehydrogenase (Idh) genes. We detected a consistent change of gene expression only in Idh2 throughout the progression from SPEM to GA, but there was a significant reduction of Tet3 expression at the early stage of tumorigenesis (Fig. S5 C). Interestingly, introducing IL-4/IL-13 deficiency into the CTLA4KD model restored Tet3 expression (Fig. 7 C) but did not rectify Idh2 dysregulation (Fig. 7 D). Therefore, the epigenetic dysregulation associated with inflammatory tumorigenesis caused by CTLA4 insufficiency may be contributed by IL-4/IL-13–dependent and independent mechanisms.

We examined 5hmC in human GC. We obtained GA samples and healthy stomach mucosal tissues and conducted 5hmC staining as described previously (Lian et al., 2012; Minor et al., 2013). As shown in Fig. 7 (E and F), the proportion of 5hmC+ nuclei was substantially reduced in GA cells compared with healthy controls. These results in human GC, taken together with the evidence from CTLA4KD mice, suggest that 5hmC dysregulation may be involved in gastric epithelial transformation driven by type 2 inflammation.

**DISCUSSION**

Control of autoimmunity requires critical immune checkpoints such as CTLA4, which regulates not only T cell homeostasis but also immune tolerance induction to specific self-antigens (Tivol et al., 1995; Waterhouse et al., 1995; Chambers et al., 1997; Ise et al., 2010). CTLA4 is well known as an archetypical inhibitor of antitumor immunity. As such, the concept that CTLA4 insufficiency can cause cancer is paradoxical to the basic tenet that blocking CTLA4 induces antitumor immunity. This paradox is real and relevant, however, as indicated by the association of CTLA4 insufficiency and GC in humans. The data from this study with animal models establish the causality of CTLA4 insufficiency in de novo cancer development in the stomach. CTLA4 modulation by RNAi silencing or antibody blockade in mice initiated spontaneous tumorigenesis in the stomach, without a requirement for microbiota, but is accompanied by autoimmune responses against gastric mucosal tissue. These results suggest a critical role of host-intrinsic immune dysregulation in the initiation of inflammatory tumorigenesis.

Epidemiological studies have associated autoimmune disorders with a variety of cancers in humans (Volkers, 1999;
A study with a CD4 T cell receptor transgenic model has also demonstrated a causal role of autoimmune immunity in GC development (Nguyen et al., 2013). On the other hand, experimental and clinical evidence indicates that autoimmune immunity may function as an effector mechanism for tumor killing as well. Thus, autoimmune immunity can be a “double agent” in mediating either protumor or antitumor activities. Of note, it is increasingly recognized that autoimmune toxicity might be a critical barrier that limits cancer immunotherapy. Furthermore, inflammatory effectors induced by checkpoint blockade might underlie the clinical observations that in some cases, immunotherapy might exacerbate cancer progression (Toomer and Chen, 2014; June et al., 2017; Sharon, 2017). Which role autoimmune immunity assumes perhaps depends on the susceptible type of target cells in an inflammatory setting as well as the dominant types of immune effectors and inflammatory pathways.

In a broad stroke, immunity and immune-mediated inflammation are categorized into either type 1 or type 2 (Gause et al., 2013; Wynn, 2015; Gandhi et al., 2016). Refuting our original hypothesis, the data from our experiments show that it is not the type 1 immunity by IFN-γ-producing T_{H}1 or IL-17–producing T_{H}17 cells that causes tumorigenesis in this model, although it causes inflammation and tissue damage. Rather, it is the type 2 inflammation driven by IL-4/IL-13 that mediates the link between inflammation and initiation of tumorigenesis in this model of GC caused by immune dysregulation. Of note, suboptimal expression of CTLA4 in the CTLA4KD model does not compromise the in vivo and in vitro suppressive activity of CD4^{+}Foxp3^{+} T_{reg} cells (Devarajan et al., 2014), although the presence of CTLA4 is required for T_{reg} cell function (Wing et al., 2008). Given the requirements for CD4^{+}CD25^{+} T cells and IL-4/IL-13, a plausible conclusion is that IL-4/IL-13–producing T_{H}2 cells drive the initiation of GC development, although definitive evidence with cell-specific targeting is needed to pinpoint the exact source of IL-4/IL-13 that causes the inflammatory tumorigenesis. Furthermore, we should note that our conclusion on the requirement of IL-4/IL-13, but not IFN-γ and IL-17, is limited to the initiation phase in terms of SPEM differentiation. Many other cytokines have been well documented for their role in stomach cancer development; for example, IL-1β (Tu et al., 2008) and TNF-α (Oguma et al., 2008). Although neutralizing TNF-α or blocking IL-1R1 with in vivo antibody treatment did not inhibit inflammation or SPEM in CTLA4KD mice (unpublished data), further studies are needed to examine their role in the progression of inflammatory tumorigenesis.

T_{H}2 cells, through complex interactions with several subsets of innate immune cells, play a key role in driving type 2 immune responses, which are characterized by production of type 2 cytokines, including IL-4, IL-5, IL-9, IL-13, IL-25, and IL-33 (Gause et al., 2013; Wynn, 2015). The role of IL-4/IL-13–producing T_{H}2 cells and innate immune cells is well documented in facilitating tumor cells evading immune attack by antagonizing tumor-killing T_{H}1, T_{H}17, or cytotoxic T lymphocytes and/or promoting immunosuppressive myeloid cells. This has been demonstrated in breast cancer as well as in many other types of cancers as a shared mechanism driving tumor progression (Bronte et al., 2003; DeNardo et al., 2009; Gabrilovich and Nagaraj, 2009; Gocheva et al., 2010). Whether dysregulation of T_{H}2 cells may cause the early stage tumorigenic differentiation or cellular transformation has remained unclear, although observations have been made that in some cases of human GC associated with H. pylori, IL-4– or IL-13–producing T_{H}2 cells were associated with the transition from gastritis to dysplasia (Ren et al., 2001). Our study identifies an essential role for T_{H}2 cells in driving the differentiation of the SPEM premalignant lineage and thus initiating tumorigenesis. In addition, IL-4/IL-13 production by T_{H}2 cells is likely also involved in the progression from SPEM to cancer through the induced IL-4/IL-13 receptors in the epithelium, which remains to be examined with lineage-specific and time-controlled targeting.

It should be emphasized that the particular regimen of anti-CTLA4 treatment used in this study was purposed to corroborate the effect of genetically predisposed CTLA4 insufficiency with a method independent of the RNAi approach. The relevance of the results to clinical anti-CTLA4 therapies cannot be determined. Anti-CTLA4 treatment has conferred remarkable benefits of cancer survival in patients. On the other hand, inflammatory toxicity was a common side effect, particularly in the gastrointestinal tract (Phan et al., 2003; Beck et al., 2006; Hodi et al., 2010). Both the upper and lower gastrointestinal tracts were affected. For example, in some patients who received anti-CTLA4 treatment...
and underwent esophagogastroduodenoscopy with biopsies, inflammatory pathology in the stomach was identified by histological analyses, although in general, enterocolitis was detected more frequently than gastritis (Beck et al., 2006). Whether and how the de novo carcinogenic effect uncovered in our study has any relevance to clinical anti-CTLA4 therapies remains to be assessed in the future. Nevertheless, as cancer immunotherapy becomes a mainstay, the long-term effect of autoimmune-derived inflammatory signals that arise from deactivating immune tolerance mechanisms may need to be studied. It may also be worth noting that clinical trials with anti-CTLA4 treatment did not show benefit in GC patients. Human GC cell lines, similar to the mouse GC in the CTLA4KD model, express functional IL-4 receptor (Morisaki et al., 1994; Essner et al., 2001). Blocking a critical immune checkpoint gene such as CTLA4 unleashes a “mixed bag” of both type 1 and type 2 inflammatory effectors. The data from this study suggest possible improvement of immune therapies by blocking IL-4/IL-13–mediated protumor type 2 inflammation while preserving antitumor type 1 immunity.

Overall, the data from this study are consistent with the premise that type 1 immunity in general is geared toward tissue destruction, whereas type 2 immunity is more attuned to damage repairing (Gause et al., 2013; Wynn, 2015). In that regard, de novo tumorigenesis in a setting of chronic type 2 inflammation perhaps represents an aberrant process of tissue repairing driven by IL-4/IL-13, at least in some solid organs. CTLA4KD mice not only model GC caused by CTLA4 insufficiency but also capture a converging point of histopathological progression from premalignancy (SPEM) to cancer (GA), regardless of whether the original initiator of inflammatory tumorigenesis is extrinsic microbes such as Helicobacter species or intrinsic immune dysregulation. Collectively, the results from this study suggest de novo tumorigenesis driven by immune dysregulation. In brief, CTLA4 insufficiency causes chronic inflammation, with Th2 responses and heightened production of IL-4/IL-13–mediated protumor type 2 inflammation while preserving antitumor type 1 immunity.

Figure 7. Inflammatory carcinogenesis in the stomach of mice with CTLA4 insufficiency was associated with epigenetic dysregulation in the 5hmC pathway. (A) Relative quantification of 5hmC content in DNA from stomach mucosal tissue of CTLA4KD mice and littermate controls (experiment 1, n = 2–3 mice in each group; experiment 2, each bar represents one animal; experiment 3, n = 3 mice in each group, mean ± SEM). (B) Representative dot-blot of 5hmC staining of genomic DNA (top) compared with methylene blue staining of DNA (loading control). (C and D) mRNA expression of epigenetic regulatory genes Tet3 and Idh2 in gastric mucosal tissue samples of 8- to 10-wk-old CTLA4KD IL4/IL13flox, CMV-Cre, Rag1° transgenic controls. RU, relative units. Data represent three to six mice per group pooled from two experiments. Each data point represents one animal (mean ± SEM; ANOVA). (E and F) 5hmC staining in human GC (adenocarcinoma). Cryosections of frozen GC samples and controls were stained for 5hmC (Red). Results are representative of five normal mucosal samples and six GC samples. Representative staining is shown in C (top panels, no 1° Ab staining control of normal tissues; middle and bottom panels, 5hmC staining of healthy and tumor tissue sections, respectively). DAPI counterstained cell nuclei (blue). Bars, 100 µm. Percentages of 5hmC+ nuclei in the normal versus GC group, with a total of 7,230 and 7,434 nuclei randomly sampled, respectively (n = 5–6 tissue samples; mean ± SEM; Student’s t test), are summarized in D. Data represent two experiments. Each data point represents one sample. * P < 0.05; ** P < 0.01; *** P < 0.001.
mice were from The Jackson Laboratory. The IL4/IL13flox mice were crossed with CMV-Cre mice to generate germline deletion of both IL-4 and IL-13 genes. GF CTLA4KD mice were rederived by embryo transfer at Taconic. Other animals were maintained in an SPF barrier facility. The studies were approved by the Institutional Animal Care and Use Committee at the University of Miami.

Antibody treatment
Anti-CTLA4 antibodies (clone UC10–4F10–11) or controls (hamster Ig or PBS vehicle) were injected intraperitoneally into BALB/c or CB6F1 mice, at a dose of 30 µg per gram body weight, biweekly for 4 wk. The same schedule was used for treatments with anti–IL-4 mAbs (clone 11B11) at a dose of 40 µg per gram body weight. The animals were analyzed at 5–6 wk of age.

Adoptive transfer of immune cells
Sterile cellular preparations were made with the spleen of CTLA4KD/CB6F1 and littermate control donors. 15–20 × 10⁶ splenocytes, or equivalent numbers of cells depleted of CD4+ or CD8+ cells, were used to reconstitute neonate Rag1− mice on the CB6F1, BALB/c, or B6 genetic background. In some experiments, the total splenocytes were separated into CD25+ and CD25− fractions. Based on the typical yield at 1:25 ratio from the separation procedure using antibody and magnetic beads, a mixture of the CD25+ and CD25− fractions was made at 1:25 ratio with splenocytes from CTLA4KD donors and littermate controls, respectively, or vice versa. Each Rag1− recipient was reconstituted with the mixture with a total 12 × 10⁶ cells at 2–3 d of age. Mice were analyzed 6–10 wk later.

Histopathology
Stomach samples were fixed in 10% formalin solution. Paraffin-embedded sections and H&E staining were done by the Pathology Research Resource Core at the University of Miami. Images were taken with a Vistavision microscope (VWR) or with an Olympus BH-2 microscope. The inflammatory infiltration in the CTLA4KD model is mainly limited to the submucosa and basal area of the mucosa. It is scored according to the following criteria: scores 1, 2, 3, and 4 indicate that <10%, 10–49%, 50–90%, and >90%, respectively, of the length of the tissue sections was affected by inflammatory infiltration along the stomach sections with mucosal tissues. SPEM or neoplasia is scored as follows: scores 1, 2, 3, and 4 indicate that <10%, 10–49%, 50–90%, and >90%, respectively, of the stomach mucosal area was affected.

Mucosa cell isolation and subcutaneous implantation to test transformation status
Stomachs from CTLA4KD mice and controls were dissected. The mucosa was excised and cut into small pieces. Tissues were treated with EDTA dissociation and collagenase digestion according to a procedure described before (Lui et al., 2015). The mucosal pieces were incubated in a solution consisting of 10% FBS, 1× HBSS, 5 mM EDTA, and 15 mM Hepes and incubated at 37°C for 15 min on a horizontal shaker set to 250 RPM. Two rounds of dissociations were performed with the tissue. For each round, the dissociated cells were collected and filtered through an 80-µm filter. The filtrate was washed twice with 2% FBS and PBS and set on ice. The nondissociated tissue was thoroughly washed free of EDTA using 2% FBS and PBS. For collagenase digestion, the nondissociated tissue was resuspended in a digestion buffer consisting of type IV collagenase (100 U/ml; Sigma), and incubated at 37°C for 1 h on a horizontal shaker set at 250 RPM. The digested tissue was washed with 2% FBS and PBS and passed through an 80-µm filter. The filtrate was collected and pooled with the earlier isolations. This procedure was used for isolation of immune cells as well as mucosal cells.

Quantification of gastric normal flora bacteria by quantitative PCR for Helicobacter or total bacterial loads.
The SPEM pathology caused by CTLA4 modulation is similar to that in B6 mice infected with Helicobacter felis, which prompted us to examine potential overgrowth of normal flora Helicobacter. As in many other facilities (Bohr et al., 2006), our SPF facility does not exclude Helicobacter species that are not considered pathogens. To investigate whether the metaplasia in CTLA4KD mice was caused by undetected normal flora Helicobacter overgrowth or undetected infection of pathogenic Helicobacter, we purified DNA from stomach samples and conducted a group-specific PCR assay for the 16S rRNA gene of all known Helicobacter species (Bohr et al., 2006), or for all bacteria. SYBR green quantitative PCR was conducted with primers specific to the 16S rRNA gene of all Helicobacter species, all bacterial species, or the eukaryotic gene β-casein. The relative levels of 16S rRNA gene were normalized with the level of β-casein DNA in each sample.

Quantitative RT-PCR
The stomach sample was cut along the greater curvature and was flattened. The exposed luminal side was washed with sterile PBS. The clean stomach samples were transferred...
Quantification of 5hmC in DNA and immunofluorescence staining for 5hmC in cell nuclei of tissue sections

DNA was purified from gastric mucosal tissue samples and analyzed via dot-blot as described in previous studies (Lian et al., 2012; Minor et al., 2013), with specific antibodies for 5hmC, and counterstained with methylene blue to normalize for total quantity.

Human GA samples were obtained from the Tissue Bank Core of the Sylvester Cancer Center at the University of Miami. For three of the samples, normal stomach mucosal tissue from the same patient was also obtained as matched controls. The tissues were embedded in OCT compound, and cryosections of the tumors and normal tissues at 7 μm thickness were prepared. The tissue sections were fixed with paraformaldehyde and stained with specific antibodies against 5hmC, as described in previous studies (Lian et al., 2012; Minor et al., 2013). In brief, cryosections were fixed in 4% paraformaldehyde for 15 min. After three washes with PBS, slides were treated with prewarmed 1 N HCl for 30 min at 37°C. Sections were then blocked with 3% normal donkey serum/0.4% Triton X-100 in PBS for 1 h at room temperature. Rabbit anti-5hmC antibody (Active Motif) was then added, incubated for overnight at 4°C, and followed with Cy3-conjugated donkey anti–rabbit antibody (Jackson ImmunoResearch Laboratories) in 2% normal donkey serum/0.1% Triton X-100 in PBS for 45 min at room temperature. After three washes in PBS, the slides were counterstained with DAPI mounting medium and imaged with a Leica inverted TCS SP-5 broadband confocal microscope (Leica 40×/1.25-0.75NA HCX PL APO oil-immersion lens). Images were then linearly contrasted and noise was removed using ImageJ software.

Statistics

Student’s t tests or Mann–Whitney tests were used for single comparisons, and ANOVA was performed for multiple group analyses. For nonparametric multiple group analyses, Kruskal–Wallis analyses were performed with false discovery rate adjustment. The χ² test was used to assess incidence of inflammation and tumorigenesis (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, not statistically significant).

Online supplemental material

Fig. S1 shows the characteristic SPEM pathology in the CTLA4KD models, which varies in extent on different genetic backgrounds. Fig. S2 presents evidence for malignant transformation of the gastric mucosal cells of aging CTLA4KD mice. Fig. S3 shows mRNA expression profiles in gastric mucosae of CTLA4KD mice for some genes associated with stomach cancer as well as cancer development in general. Fig. S4 shows the dysregulation of both type 1 and type 2 cytokine responses in CTLA4KD mice in the absence of detectable infection by pathogenic Helicobacter. Fig. S5 presents evidence of type 2 cytokine–driven signaling and epigenetic alteration in the gastric mucosal cells.

Flow cytometry

Flow cytometry analyses were conducted with a standard procedure. The cells were stained with fluorescent antibody conjugates to determine cell phenotype. Intracellular cytokine staining was done with a standard procedure. The following antibody conjugates were used: PETR anti–CD8; eFluor450–conjugated anti–CD44; APC-eFluor780–conjugated anti–CD62L; PE-Cy7 and APC-eFluor780–conjugated anti–CD25 (Thermo Fisher Scientific). Intracellular cytokine staining was done with procedures described before (Devarajan et al., 2014; Pua et al., 2016). Cells were stimulated for 6 h in phorbol dibutyrate and ionomycin (Sigma), with brefeldin A (Thermo Fisher Scientific) added 4 h after the beginning of stimulation. Cells were stained for surface makers, and then fixed for 8 min in 4% paraformaldehyde. Cells were permeabilized in 0.5% saponin and blocked for 5 min at room temperature, followed by addition of both PE–Cy7 or eFluor450–conjugated anti–IL-17A and PE-Cy7–conjugated anti–IFN-γ (BioLegend), APC-conjugated anti–IL-4, and PE-conjugated anti–IL-13 (Thermo Fisher Scientific) for 20 min at 4°C. Staining of c-Myc was done by fixation and permeabilization of the gastric tumor cells or normal gastric mucosal cells with fixation and permeabilization buffers (Thermo Fisher Scientific) followed by intracellular staining with Alexa Fluor 647–labeled anti–human c-MYC mAbs (clone 9E10; Thermo Fisher Scientific), which cross-reacts with mouse c-Myc, or Alexa Fluor 647–labeled IgG1 isotype control (BioLegend). Cells were analyzed with flow cytometers (LSR–II and Fortessa; Becton Dickinson) or CytoFLEX (Beckman Coulter).
REFERENCES


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Author contributions: J. Miska, J.B. Lui, and Z. Chen designed the experiments. J. Miska and J.B. Lui performed most of the experiments. K.H. Toomer, P. Devarajan, and Z. Chen performed some experiments. X. Cai, J. Houghton, D.M. Lopez, M.T. Abreu, and G. Wang helped design and interpret some experiments and edit the manuscript. J. Miska, J.B. Lui, and Z. Chen wrote the manuscript. All authors reviewed and approved the manuscript.

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