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Unique invariant natural killer T cells promote intestinal polyps by suppressing TH1 immunity and promoting regulatory T cells

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Abstract

CD1d-restricted invariant natural killer T (iNKT) cells are known as potent early regulatory cells of immune responses. Besides the established roles in the regulation of inflammation and autoimmune disease, studies have shown that iNKT cells have important roles in tumor surveillance and the control of tumor metastasis. Here we found that absence of iNKT cells dramatically decreased the total number of intestinal polyps in $APC^{Min/+}$ mice, a model for colorectal cancer. Polyp iNKT cells were enriched for IL-10 and IL-17 producing cells, showed a distinct phenotype being $CD4^+$, $NK1.1^-$ $CD44^{int}$ and $PD\text{-}1^{lo}$, and they were negative for the NKT cell transcription factor PLZF. Absence of iNKT cells was associated with a reduced frequency of Treg cells and lower expression levels of FoxP3 protein and transcript uniquely in the polyps, and a switch to an inflammatory macrophage phenotype. Moreover, in iNKT cell deficient $APC^{Min/+}$ mice, expression of T helper (TH) 1-associated genes, such as *IFN-γ* and *Nos2*, was increased in polyps, concomitantly with elevated frequencies of conventional $CD4^+$ and $CD8^+$ T cells in this tissue. The results suggest that a population of regulatory iNKT cells locally promote intestinal polyp formation by enhancing Treg cells and immunosuppression of anti-tumor TH1-immunity.

Keywords

NKT cells; Immunoregulation; Tumor; Intestine; Treg

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AUTHOR CONTRIBUTIONS

YW planned and performed experiments, analyzed data and prepared manuscript figures and text. SS planned and performed experiments and reviewed the manuscript. LL performed experiments. SP provided reagents, designed experiments, and reviewed the paper. SC initiated and designed the study, analyzed data and wrote the paper.

DISCLOSURE

The authors declare no conflict of interest.

INTRODUCTION

Lipid reactive CD1d-restricted natural killer T (NKT) cells are potent early regulatory cells of diverse immune responses. Besides the established roles in the regulation of immunity to infection, and in inflammation and autoimmune disease, many studies have attributed NKT cells an important role in tumor surveillance and the control of tumor metastasis [1]. NKT cells can be divided into two functionally distinct categories according to their expression of T cell receptors (TCR) [2, 3]. An evolutionarily conserved category of NKT cells (termed invariant, iNKT cells, or type 1 NKT cells) expresses a semi-invariant TCR containing a specific TCRα-chain (in humans Vα24-JαQ, in mice Vα14-Jα18) [4]. CD1d presents glycolipids to NKT cells, and essentially all iNKT cells are activated by the artificial lipid ligand α-galactosylceramide (αGalCer), first described for its strong anti-tumor effect [5]. The remaining CD1d-reactive TCRαβ cells have diverse TCR (termed diverse, dNKT cells, or type 2 NKT cells) [2]. Studies employing αGalCer treatment have established that iNKT cells have anti-tumor functions in several murine experimental models. Moreover, in some models iNKT cells demonstrate “natural” tumor surveillance, i e in the absence of activation with administered iNKT cell ligands [6–8]. Interferon (IFN)- γ production by iNKT cells has been identified as an essential component of their anti-tumor activity. Further, several reports found that cancer patients have reduced levels of NKT cells that show a functional deficiency in IFN-γ production, and that higher levels of NKT cells correlated with improved prognosis [9]. On the other hand, dNKT cells have been found to suppress $CD8^+$ T cell mediated tumor immunity in an IL-13 and TGF-β dependent manner [9]. Findings in peripheral blood from myeloma patients seem to support this dichotomy. An expanded population of dNKT cells was demonstrated, and interestingly, these cells produced IL-13 and were reactive to an inflammation-associated lysophospholipid presented on CD1d [10]. Thus, different roles have often been associated with the two NKT subsets, and a model has been put forward proposing that iNKT cells promote tumor immunity, while dNKT cells suppress tumor immunity [9].

Human inflammatory bowel disease (IBD) increases the risk of developing colorectal cancer; consistent with a role for chronic inflammation in promoting carcinogenesis [11]. In this context, it is significant that NKT cells were demonstrated to promote intestinal inflammation in Crohn's disease and mouse models [12–14]. The demonstrated role of NKT cells in chronic intestinal inflammation, raises the question of whether these enhance inflammation-driven intestinal tumor formation by their capacity to promote inflammation, or prevent tumor formation at this site through their ability to provide tumor surveillance and tumor prevention. $Apc^{Min/+}$ mice are a model for human colorectal cancer (CRC) [15], which is the leading cause of cancer-related mortality worldwide. The $Apc^{Min/+}$ mice have a truncated adenomatous polyposis coli (*Apc*) gene, a mutation that leads to the spontaneous formation of benign intestinal adenomatous polyps in heterozygote mice, recapitulating early events in human colorectal carcinogenesis. Several studies have demonstrated the critical role of inflammation driving the formation of polyps in human CRC and mouse models of the disease [16], and blocking of both innate and adaptive immune signals prevents tumor formation in $Apc^{Min/+}$ mice[17, 18]. Here, we have used the $Apc^{Min/+}$ mouse model to

determine the role of CD1d-restricted NKT cells in the regulation of polyp formation in the intestine.

RESULTS

iNKT cells naturally promoted intestinal tumor development

We first determined whether the heterozygous *Apc* mutation in $Apc^{Min/+}$ mice affected the numbers and the functions of iNKT cells. In our animal facility, 10–12 week old $Apc^{Min/+}$ mice had no macroscopically visible intestinal polyps, but early polyp formation could be seen on sections using a microscope (Fig. 1A). iNKT cell frequencies were similar in the spleen, mesenteric lymph nodes (MLN) (Fig. 1B) and liver (data not shown) of $Apc^{Min/+}$ and $Apc^{+/+}$ mice at this age. Further, $Apc^{Min/+}$ and $Apc^{+/+}$ mice responded to αGalCer stimulation with vigorous production of cytokines at comparable levels detected in the serum at 2 to 24 h (Fig. 1C). Thus, iNKT cells in 10–12 week old $Apc^{Min/+}$ mice were present in equal frequencies and demonstrated a normal *in vivo* responsiveness to αGalCer when compared to their $Apc^{+/+}$ littermate control mice.

At 15 weeks of age, intestinal polyps were visible in all $Apc^{Min/+}$ mice with a median of around 20 polyps over the entire length of the intestine, with no significant difference between male and female $Apc^{Min/+}$ mice (data not shown). To investigate the influence of iNKT cells on the natural course of polyp development in $Apc^{Min/+}$ mice, we crossed the mice with $Ja18^{-/-}$ mice that specifically lack iNKT cells. The splenomegaly[19] was reduced in $Apc^{Min/+}Ja18^{-/-}$ mice compared to the $Apc^{Min/+}Ja18^{+/-}$ controls (Fig. 1D), and $Apc^{Min/+}Ja18^{-/-}$ had a ~75% reduction in the median number of polyps in the small intestine (SI) compared to the heterozygote ($Apc^{Min/+}Ja^{18+/-}$) littermate controls (Fig. 1E). We also crossed $Apc^{Min/+}$ mice with CD1d-deficient mice lacking all NKT cells. Compared to $Apc^{Min/+}CD1d^{+/-}$ littermate controls, $Apc^{Min/+}CD1d^{-/-}$ mice demonstrated a 56% decrease in SI polyp counts (Fig. 1F). Neither of the two NKT deficient mice harboring the $Apc^{Min/+}$ mutation had significantly reduced numbers of polyps in the colon. Thus, two different mutations resulting in iNKT cell deficiency had reduced polyp numbers, demonstrating that iNKT cells naturally promote tumor development in this model. Moreover, the similar reduction in polyp numbers in mice lacking iNKT cells and all NKT cells suggests that dNKT cells do not have significant effects on tumor development in this model.

We next investigated the effect of activation of iNKT cells during polyp development. Mice were treated from 5–15 weeks of age with αGalCer that induces a mixed T helper (TH)1/TH2 cytokine profile, or with the modified ligand C20:2 that induces preferential TH2 cytokine production by iNKT cells [20]. αGalCer treatment reduced polyp numbers in both SI and colon, while SI polyp numbers in mice treated with C20:2 were significantly higher (Fig. 1G). This demonstrated that ligand activated iNKT cells have the capacity to control polyp development in both SI and colon, and indicated that activation of TH1 cytokine secretion by iNKT cells led to suppressed polyp development, while induction of iNKT cell derived TH2 cytokines rather enhanced polyp development.

Unique phenotype and functions of iNKT cells in polyps of $Apc^{Min/+}$ mice

To address the underlying mechanisms for the promotion of polyps in $Apc^{Min/+}$ mice by iNKT cells, we first performed a broad analysis of iNKT cells in polyps and different lymphoid organs. Polyp infiltrating lymphocytes contained around 0.6% iNKT cells, which is similar to iNKT cell percentages in LP lymphocytes from $Apc^{Min/+}$ and $Apc^{+/+}$ mice (Fig. 2B). In MLN and spleen the frequencies of iNKT cells were comparable in both mice (Fig. 2B). Due to the splenomegaly of $Apc^{Min/+}$ mice, the absolute number of splenic iNKT cells was slightly but significantly increased, while in contrast, the total numbers of CD4 and CD8 T and B cells remained the same (data not shown). *In vitro* stimulation of splenocytes induced similar frequencies in both mice of IL-4 and IFN-γ producing iNKT cells (data not shown)

In an extended phenotypic analysis we did not detect differences between iNKT cells when comparing the same tissues from $Apc^{Min/+}$ and $Apc^{+/+}$ mice, but we confirmed previous publications showing that iNKT cells at different locations have distinct phenotypes[21] (Fig. 2C). In polyps of $Apc^{Min/+}$ mice, the fraction of iNKT cells expressing CD4 and NK1.1 was lower than in other organs. In contrast, CD69 (not shown) and CD44 was expressed by almost all polyp iNKT cells, but CD44 levels were strongly decreased compared to splenic iNKT cells, and only around 10% of iNKT cells in polyps displayed the marker of activation/exhaustion, PD-1 (CD279) (Fig. 2C). Similarly, LP iNKT cells demonstrated low proportions of cells displaying NK1.1 and high CD69, however, the majority of LP iNKT cells expressed PD-1. The iNKT population in MLN of both mice had similarly low frequencies of $NK1.1^+$, $CD69^+$ cells and $PD\text{-}1^+$ cells (around 20%). Therefore, polyp iNKT cells displayed a cell surface phenotype that was different from iNKT cells in the other organs analyzed.

Expression of the transcription factor promyelocytic leukemia zinc finger (PLZF) is a known feature of both developing and mature iNKT cells[22]. In addition, iNKT cells can be divided into distinct functional subsets that express characteristic sets of transcription factors determining the pattern of cytokines secreted upon activation[23], analogous to T helper (Th)1, Th2 and Th17 cells. Three major functional subsets have been defined, iNKT1, iNKT2 and iNKT17, that are distributed in different ratios in various lymphoid organs. We stained polyp iNKT cells for intracellular transcription factors and compared them to iNKT cells in LP, MLN and spleen (Fig. 2D–F). Surprisingly, polyp iNKT cells were essentially negative for PLZF, with a similar median fluorescence intensity (MFI) level to splenic $CD4^+$ T cells. These PLZF-negative iNKT cells fall outside the established gating for iNKT1/2/17 cell subsets (see gating in Fig. 2D). Here, we have termed the PLZF-negative iNKT cells "iNKT-P^{neg}". Strikingly, over 90% of iNKT cells in polyps were iNKT-P^{neg}. iNKT cells in LP of both mice expressed PLZF, but at lower levels than iNKT cells from spleen and MLN. Among iNKT cells in control $Apc^{+/+}$ LP, around 30% were iNKT-P^{neg} by our definition, and around 55% were iNKT1, while LP of $Apc^{Min/+}$ mice had slightly but significantly lower frequency of iNKT1 cells and a somewhat increased frequency of iNKT-P^{neg} cells. Splenic iNKT cells consisted almost exclusively of iNKT1 cells, while MLN iNKT cells displayed equal frequencies of iNKT1 and iNKT2 cells and a distinct iNKT17 population of around 10%, and there was no difference between iNKT cells from $Apc^{Min/+}$ and $Apc^{+/+}$ mice in

these organs. Around 10% of all iNKT cells in MLN, LP and polyp expressed RORγt (Fig. 2F), while the levels of RORγt were somewhat lower in polyp iNKT cells compared to other organs. Some of the RORγt$^+$ iNKT cells in LP, and the majority in polyps, were PLZF negative (thereby falling within the iNKT-P^{neg} definition in Fig. 2D). Thus, polyp iNKT cells displayed a transcription factor pattern that was different from the established iNKT functional subsets, most notably by lacking the NKT cell transcription factor PLZF. This suggests that polyp iNKT cells possess a distinct functional capacity that underpins tumor promotion occurring at this site.

Considering that iNKT cells in polyps did not express the expected transcription factors that determine iNKT cell functional status, we determined intracellular cytokines in iNKT cells activated *in vivo* with αGalCer (Fig. 2G, H). As shown before[24], we found that MLN iNKT cells responded poorly to systemicαGalCer stimulation (data not shown). Unstimulated iNKT cells in polyps contained detectable frequencies of cells positive for IL-2, IL-4, IL-13, TNF-α and IL-17 but not IFN- γ, something that was also seen in LP, but was absent in splenic iNKT cells. After *in vivo* stimulation, iNKT cells in polyps harbored significant frequencies of cells positive for IFN-γ'γ (around 20%), IL-2, IL-4, IL-13 and TNF-α (all around 10%), however the frequencies were lower than found in splenic iNKT cells. Strikingly, however, polyp iNKT cells were strongly enriched for cells containing IL-10 or IL-17 compared to both spleen and LP iNKT cells, particularly evident at 4 hours (Fig. 2H). Taken together, this demonstrates that αGalCer activated polyp iNKT cells secreted a different pattern of cytokines compared to spleen and LP iNKT cells, notably being enriched for IL-10 and IL-17 producing cells.

iNKT cells controlled accumulation, activation and functional status of polyp infiltrating T cells

We next defined differences in T cells infiltrating the polyp in iNKT cell deficient *Apc*$^{Min/+}$*Jα18*$^{-/-}$ and *Apc*$^{Min/+}$*Jα18*$^{+/-}$ mice. Lack of iNKT cells resulted in elevated frequencies of CD8$^+$ T cells in polyps as well as of conventional CD4$^+$ T cells (CD4$^+$ FoxP3$^-$, convCD4 cells) in both polyps and MLN of *Apc*$^{Min/+}$*Jα18*$^{-/-}$ mice (Fig. 3A). Further, in the absence of iNKT cells the expression of PD-1 was reduced on CD8 T cells in both polyps and LP and on convCD4 T cells in LP, consistent with a lower degree of exhaustion (Fig. 3B, C). In polyps, where PD-1 expression on convCD4 cells was very low, the frequency of PD-1 positive cells was slightly increased. Moreover, in polyp, LP and MLN there was a significant reduction in the expression of the IL-33 receptor ST2, associated with TH2 cells, by convCD4 cells (Fig. 3C), suggesting that iNKT cells provide an environment that is more supportive of this TH subset. Both CD8 and convCD4 T populations displayed strongly reduced frequencies of CD69$^+$ cells in polyps of *Apc*$^{Min/+}$*Jα18*$^{-/-}$ mice compared to *Apc*$^{Min/+}$*Jα18*$^{+/-}$ mice. CD69 is known as a marker of early activation of T cells in lymphoid organs and tissue resident memory T cells, however, studies also suggest that CD69 negatively regulates proinflammatory functions of T cells in tissues (see[25] for review). In the absence of iNKT cells, a lower frequency of convCD4 and CD8 T cells within polyp tissue was engaged in proliferation, as indicated by reduced Ki67 expression. Taken together, this demonstrates that, directly or indirectly, iNKT cells control

accumulation and activation/functional status of convCD4 and CD8 T cells in the polyp tissue.

iNKT cells promoted a regulatory, and suppressed a Th1/proinflammatory polyp microenvironment

To determine the immune and tumor related molecular signatures that distinguish the iNKT cell mediated promotion of tumor burden in $Apc^{Min/+}$ mice, a quantitative PCR array screen was performed for the expression of a set of genes relevant for immune responses, tumor growth and apoptosis (Figure 3A, B). We considered that iNKT cells may promote tumor development by proinflammatory actions increasing the production of tumor growth factors, but also the possibility that tumor enhancement was due to increased immunoregulation resulting in decreased tumor immunity. Gene expression in polyp and LP from $Apc^{Min/+}J\alpha 18^{-/-}$ and $Apc^{Min/+}J\alpha 18^{+/-}$ mice were compared, resulting in the identification of several genes that were altered in expression in the absence of iNKT cells (Fig. 3A, B). Lack of iNKT cells resulted in upregulated expression in polyps of genes associated with a Th1/proinflammatory immune response, such as those encoding IFN-γ, iNOS (*Nos2*), IL12p40, T-bet (*Tbx21*), granzyme B (*Gzmb*) and Stat-1. In contrast, transcript levels of *Foxp3*, the master transcription factor for regulatory T cells (Treg), in polyps were decreased. Interestingly, iNKT cell deficiency resulted in enhanced Th1/inflammation associated gene expression also in LP (increased expression of genes encoding iNOS, IFN-γ, CD8β, CXCL9, CXCL10, granzyme B). We performed quantitative PCR on tissue samples from individual mice for a set of genes to verify the array results (Fig. 3C). The results confirmed significantly higher expression levels of *Ifng* and *Nos2* in both polyp and LP in the absence of iNKT cells. In iNKT sufficient control mice, the levels of both *Il17a* and *Foxp3* transcripts were higher in polyp tissue compared to LP. Notably, absence of iNKT cells was associated with a decrease in *Foxp3* expression in the polyps but not LP. This strongly indicates that iNKT cells promoted polyp development by enhancing immune regulation and suppressing Th1 associated tumor immunity.

iNKT cells supported the expression of FoxP3 and an activated T regulatory (Treg) cell phenotype in polyps

The decreased *FoxP3* expression in polyps from $Apc^{Min/+}$ $J\alpha 18^{-/-}$ mice compared to $Apc^{Min/+}J\alpha 18^{+/-}$ mice prompted a comprehensive analysis of Treg cells by flow cytometry (Fig. 5). In iNKT cell deficient polyps, but not LP or other lymphoid organs, the frequency of Treg cells (both when gated as $CD25^+$ $FoxP3^+$ cells or as $FoxP3^+$ cells) among $CD4^+$ cells was significantly decreased (Fig. 5A, B). Notably, also the MFI level of FoxP3 in $CD25^+$ $CD4^+$ T cells was reduced in $Apc^{Min/+}J\alpha 18^{-/-}$ mice. The same was true when FoxP3 levels were compared within the $CD25^+$ $FoxP3^+$ gate, suggesting that iNKT cells promote Treg in a manner that maintains higher levels of FoxP3 protein. Further, iNKT cells reinforced expression of the IL-33 receptor ST2 on both polyp and LP Treg cells. Extended phenotypic analysis revealed that presence of iNKT cells was associated with an increased fraction of polyp Treg cells expressing CD69 (Fig. 5C), and of LP Treg cells expressing PD-1, while KLRG1 expression was not influenced by iNKT cells (not shown). Taken together, this demonstrates that iNKT cells drive Treg cell maintenance and/or activation both in polyps and LP of $Apc^{Min/+}$ mice.

Absence of iNKT cells led to a systemic shift in macrophage phenotype from M2 to M1

As we found that *Nos2*, expressed by macrophage subtype 1 (M1) cells, was the most strongly upregulated gene in $Apc^{Min/+}Ja^{18-/-}$ polyp and LP compared to $Apc^{Min/+}Ja^{18+/-}$ tissues, we investigated innate immune cells in polyps, LP and spleen (Fig. 6). MLN contained very low frequencies of these cells (data not shown). In the presence of iNKT cells there was an elevated frequency of $CD11c^{hi}$ dendritic cells in the polyps compared to iNKT cell deficient mice (Fig. 6A, B). There was also a several-fold higher proportion of cells with a phenotype of myeloid derived suppressor cells (MDSC) commonly found in tumors[26]. Further, there was a significant increase in the frequency of $F4/80^+$ macrophages in the spleen (Fig. 6A, B), consistent with the splenomegaly in $Apc^{Min/+}Ja^{18+/-}$ mice (Fig. 1D). We next determined the macrophage expression of iNOS and CD206, characteristic of M1 and M2 macrophages, respectively. Strikingly, iNKT cell deficiency resulted in a highly significant shift in the macrophage phenotype in all tissues, from a mixed/M2 dominated population (high frequency of $CD206^+$/low frequency of $iNOS^+$ cells) in $Apc^{Min/+}Ja^{18+/-}$ mice, to a macrophage population strongly skewed towards the M1 phenotype in $Apc^{Min/+}Ja^{18-/-}$ mice (high frequency of $iNOS^+$ and strongly diminished proportion of $CD206^+$ macrophages) (Fig. 6C, D). In the spleen, the proportion of macrophages expressing CD11c, also described as an M1 macrophage marker, was increased in the absence of iNKT cells, while, surprisingly, in polyps of the same mice the frequency of macrophages expressing CD11c was reduced (Fig. 6D). Interestingly, in polyps and other tissues analyzed we found that CD11c and iNOS were expressed by distinct subsets of $F4/80^+$ cells (data not shown), indicating heterogeneity of macrophages beyond a simple M1/M2 division. Further analysis of the MDSC population revealed that the majority of MDSC in iNKT sufficient $Apc^{Min/+}$ mice were polymorphonuclear MDSC (PMN-MDSC; $Ly6C^{int}/Ly6G^+$). This population was strongly reduced in $Apc^{Min/+}Ja^{18-/-}$ mice lacking iNKT cells and similar in frequencies to mononuclear MDSC (M-MDSC; $Ly6C^{hi}/Ly6G^-$) in these mice (Fig. 6E, F). Thus, in the absence of iNKT cells, there was a strong shift from $CD206^+$ M2 towards $iNOS^+$ M1 macrophages in all tissues analyzed, and a reduction of PMN-MDSC most significantly in polyps.

To reinforce the notion that iNKT cells regulate the inflammatory microenvironment in polyps, we transferred sorted iNKT cells to $Apc^{Min/+}Ja^{18-/-}$ mice and three weeks later investigated the effects of the transfer on macrophage phenotypes. Transfer of iNKT cells resulted in a striking reduction of iNOS expression and increase in CD206 expression among macrophages in the polyps (Fig. 6G, H). In contrast, there was no significant change in macrophage expression of these markers in LP or spleen after iNKT cell transfer. Transfer of iNKT cell depleted T cells did not change the macrophage phenotype in any of the organs compared to non-transferred mice. This is consistent with a model in which iNKT cells promote an anti-inflammatory environment specifically in the polyps resulting in the enhancement of M2 macrophages.

DISCUSSION

iNKT cells are well known for their potent tumor immunosurveillance and the suppression of tumors after αGalCer therapy. In such models, activated iNKT cells combat tumors by the

production of IFN-γ and downstream activation of cells including NK cells, dendritic cells and cytotoxic CD8 T cells, and by being directly cytotoxic against CD1d-expressing tumor cells (see review[27]). In contrast, in the present study, using two different mutant mouse strains that lack iNKT cells, we demonstrate that iNKT cells promoted the development of spontaneous intestinal polyps in $Apc^{Min/+}$ mice, a model for early stages of human colorectal cancer. Surprisingly, we find that iNKT cell promotion of polyps was associated with an immunoregulatory polyp microenvironment, characterized by increased Treg and PMN-MDSC populations and an enhanced M2 macrophage phenotype, but suppressed TH1-immunity and reduced frequencies of tumor infiltrating conventional CD4 and CD8 T cells. The results are summarized in the simplified model in Fig. 7.

The absence of iNKT cells in $Apc^{Min/+}$ mice was associated with increased expression of IFN-γ and iNOS, and a systemic switch in macrophage phenotype from M2 to M1. These changes are likely to be pivotal for the reduced tumor burden in $Apc^{Min/+}J\alpha 18^{-/-}$ mice. Firstly, $Apc^{Min/+}$ mice lacking iNOS have significantly more adenomas than iNOS positive littermate controls[28]. This is in line with human studies showing that high infiltration of cells positive for iNOS correlated with significantly improved prognosis in CRC [29]. Moreover, M2 or M2-like tumor associated macrophages are known to support tumor progression and suppress tumor immunity, while M1 macrophages are involved in anti-tumor immunity[30]. Secondly, IFN-γ is shown to be tumor suppressive in $Apc^{Min/+}$ mice[31.] The source of IFN-γ was not investigated in our studies, but $CD8^+$ T cells are likely candidates. Consistently, we find that the frequency of infiltrating CD8 T cells, as well as CD4 T cells, was increased in the absence of iNKT cells. This may be an indirect effect and result from the reduction of Treg frequency and activation status in $Apc^{Min/+}J\alpha 18^{-/-}$ mice, as it has been shown that depletion of Treg in $Apc^{Min/+}$ mice results in the influx of T cells into the polyps[32]. Also in some other murine tumor models iNKT cells have been shown to promote tumor growth, associated with suppressed CD8 CTL, decreased IFN- γ and elevated IL-13 production[33, 34]. The underlying mechanism of suppression of tumor immunity by iNKT cells in these models may be similar to what has been described for dNKT cells in a series of studies by Terabe and Berzofsky and co-workers [35, 36]. Here, IL-13 production by dNKT cells induced TGF-β production by $CD11b^+$ $Gr\text{-}1^+$ myeloid cells, which in turn inhibited the activation of tumor specific $CD8^+$ effector cells resulting in enhanced tumor growth. Such myeloid cells may be involved in the suppression of immunity in $Apc^{Min/+}$ polyps as we found that the presence of iNKT cells also increased the polyp frequency of cells with a phenotype of MDSC that express Ly6G, a marker of PMN-MDSC that are strongly associated with tumor promoting activity[26]. Tumor infiltration by CD8 T cells has been associated with improved prognosis in many cancers including CRC[37], and more recently, extended studies of infiltrating immune cells in human CRC have established their important prognostic value, more broadly demonstrating that TH1 immunity and CD8 T cells in tumor infiltrates correlate with increased disease free survival of patients [38]. Thus, the suppression of TH1 immunity in $Apc^{Min/+}$ polyps by the presence of iNKT cells, is likely to be a major cause for the increased polyp numbers. In contrast, expression of IL-17A is associated with tumor promotion in human CRC and $Apc^{Min/+}$ mice [18, 39]. We found that IL-17A transcript levels were highly variable in polyps in the presence of iNKT cells, but not significantly altered in polyps in iNKT cell deficient $Apc^{Min/+}$ mice.

iNKT cell regulation of the immune environment in the polyps may partly be due to the promotion by iNKT cells of infiltrating Treg cells that had a phenotype associated with a higher activation state and elevated effector function. We show that in the presence of iNKT cells, the amount of FoxP3 mRNA in polyps was increased, the frequency of positive cells and the fluorescence intensity of FoxP3 staining in Treg cells were augmented. Such differences were not detected in other tissues. Moreover, in the presence of iNKT cells, Treg cells in polyps showed a marked upregulation of ST2. ST2 was recently demonstrated to be expressed on high proportions of Treg cells in colon as well as in adipose tissue[40–42]. IL-33 promoted their immunoregulatory function and maintenance of the Treg phenotype, including maintenance of FoxP3 expression, and increased expression of its own receptor ST2 on Treg. Importantly, ST2 deficiency protects from tumor development in an induced mouse model of CRC[43]. Taken together, this suggests that iNKT cells promote the accumulation, activation and/or homeostasis of highly active immunoregulatory Treg cells in the polyp tissue. It is interesting to note that the induction of Treg cells by iNKT cells has also been shown to play a role for tolerance in autoimmune settings[27].

Although other tissues were also influenced, the most striking differences resulting from the absence of iNKT cells in $Apc^{Min/+}$ mice were revealed in the polyps, especially with regards to Treg frequency and phenotype, suggesting that iNKT cells regulate immune cells in this tissue. This was also suggested by the polyp specific effect on MDSC by depletion of iNKT cells in $Apc^{Min/+}$ mice, and by the promotion of an M2 macrophage phenotype specifically in the polyps after transfer of iNKT cells to $Apc^{Min/+}Ja^{18-/-}$ mice. We found that iNKT cells infiltrated the $Apc^{Min/+}$ polyps, but they were present in a relatively low frequency at this site. Polyp iNKT cells were predominantly NK1.1^{-}, CD44^{int}, PD-1^{-} and around half of them were negative for CD4, which differed from iNKT cells in LP and lymphoid organs, and strikingly, they were negative for PLZF. Moreover, while polyp iNKT cells had a generally lower cytokine production *in vivo* in response to αGC, they demonstrated a highly increased capacity to make IL-10 and IL-17. These polyp iNKT cells share some functional features and cell surface phenotype with so called iNKT10 cells that arise after strong αGC stimulation *in vivo*[44], and with iNKT cells that are present in white adipose tissue[44, 45]. Both adipose tissue iNKT and iNKT10 cells produced IL-10 upon activation, but had a decreased production of other tested cytokines compared to splenic iNKT cells in naive mice, and were ascribed regulatory functions. Similar to iNKT cells in $Apc^{Min/+}$ mice, the induced iNKT10 cells suppressed tumor immunity and this effect was dependent on IL-10[44]. Adipose tissue iNKT cells were shown to favor a non-inflammatory adipose environment by promoting the homeostasis and regulatory function of adipose tissue Treg through the production of IL-2, and moreover, IL-10-dependently induced an anti-inflammatory state in local macrophages[45]. Here, we demonstrate that polyp iNKT cells had very similar effects on polyp Treg cells and macrophages, despite the 7-fold lower frequency of iNKT cells in polyps (around 0.6%) compared to adipose tissue (around 4%[45]). The production of IL-10 and IL-2 by polyp iNKT cells may contribute to these effects. It was proposed that the adipose tissue regulatory iNKT phenotype was induced by chronic local stimulation. This may be true also for polyp iNKT cells, and is consistent with their low but constitutive production of some cytokines. A local instruction of the iNKT cell phenotype in polyps was also suggested by the fact that transfer of liver iNKT cells, that were predominantly iNKT1

(data not shown), to $Apc^{Min/+}Ja18^{-/-}$ mice reconstituted the polyp macrophage skewing towards M2 similar to what was found in $Apc^{Min/+}Ja18^{+/-}$ mice. A different scenario was proposed in a recent publication demonstrating that unique features of the TCR could determine the development of iNKT cells with a phenotype of adipose iNKT cells in the thymus, followed by subsequent accumulation of these iNKT cells in adipose tissue[46]. It remains to be investigated to what extent the tissue microenvironment induces the polyp iNKT-P^{neg} or adipose tissue phenotype of iNKT cells, and which signals may be required for the development of these unusual iNKT cell phenotypes.

The fact that iNKT cells have been shown to be protective in several tumor models have led to early clinical trials that have shown some promising outcomes in cancer patients that have received iNKT cell directed therapy[47]. Our results reveal novel aspects on iNKT cell regulation of tumor immunity, and encourage more detailed studies of the relative contributions of iNKT cells to anti-tumor immunity and immunoregulation in tumor immunity to fully understand their role and underlying mechanisms at different stages, and to evaluate the prospect of iNKT cell directed therapy in these diseases.

METHODS

Mice

The $Apc^{Min/+}$ mutation occurred and is maintained on the C57BL/6 genetic background [15]. Sex- and age matched mice were used in all experiments. $Apc^{Min/+}$ mice were crossed with $Ja18^{-/-}$ mice to obtain $Apc^{Min/+}Ja18^{-/-}$ and littermate control $Apc^{Min/+}Ja18^{+/-}$ mice. $Apc^{Min/+}$ mice were also crossed with $CD1d^{-/-}$ mice to obtain $Apc^{Min/+}CD1d^{-/-}$ and littermate control $Apc^{Min/+}CD1d^{+/-}$ mice. Both male and female mice from these crosses were used for experiments. All mice were bred and maintained at the department of Experimental Biomedicine, University of Gothenburg. Animal experiments in this study were approved by the animal ethics committee in Gothenburg.

Quantification of polyps

Mice were sacrificed at 15 or 20 weeks of age. The intestines were flushed with phosphate buffered saline (PBS) from both ends using blunt end gavage needles to remove fecal material, and cut into three equal length segments: duodenum, jejunum and ileum. Each segment was then cut open longitudinally and polyps were counted under stereomicroscope.

In vivo treatment with glycolipid

Mice were injected intraperitoneally (i. p.) with 4μg of glycolipids (α-GalCer C26:0, α-GalCer C20:2) in 200μl of PBS with a final concentration 0.1% DMSO and 0.05% Tween-20. Vehicle control was prepared and injected in an identical manner. 5-week old female $Apc^{Min/+}$ mice were treated with glycolipids or vehicle control on day 1, 2, 7, 14, 21, 28 and 60, and the mice were sacrificed at 15 weeks of age.

Histology

Tissue rolls of 12-week old mouse intestine were fixed with formalin at room temperature for 24 hours. After processing, the formalin fixed, paraffin-embedded tissues were vertically

sectioned lengthwise, and sections stained with hematoxylin and eosin (performed by Histo-Center AB, Västra Frölunda, Sweden).

Lymphocyte preparation

Spleen, MLN and SI were collected from 15-week old $Apc^{Min/+}$ or $Apc^{Min/+}$ $J\alpha 18^{-/-}$ mice and their littermate control mice. Single cell suspensions from spleen and MLN were prepared. LP lymphocytes and tumor infiltrating lymphocytes were isolated from the small intestine after removal of Peyer's patches. The tissue was dissected into tumor and unaffected tissue to be processed separately. Unaffected tissue was cut into small pieces. Tumor and unaffected tissues were dissociated with Lamina Propria dissociation kit (Miltenyi Biotech). Undigested tissue was removed by filtration and lymphocytes counted using trypan blue.

Flow cytometry

See Supplemental Material.

In vivo stimulation with αGalCer and serum cytokine determination

αGalCer were first dissolved in 100% DMSO, followed by a dilution with 0.5% Tween-PBS to achieve a stock concentration of 172.4 μg glycolipid/ml in 1% DMSO and 0.5% tween in PBS. This stock was stored at −20°C. Upon use, the glycolipid stock was thawed and sonicated for 5 min and immediately heated at 80°C for 2 min in glass vials. After vortexing for 1 min, the required volume of glycolipid stock was dissolved in pre-warmed (37°C) PBS and kept in an 80°C bath until shortly before injection. Each mouse was injected i. p. with 4μg of αGalCer in 200μl of PBS with a final concentration 0.1% DMSO 0.05% Tween-20. Mice were bled before αGC injection, and at different times (2h, 8h, 24h respectively) after injection. Serum was prepared by centrifugation and stored at −20° C. Cytokine content was measured using a CBA Mouse Th1/Th2/Th17 Cytokine Kit (BD Bioscience) and the samples were analyzed with a LSRII Flow Cytometer (BD Bioscience).

RNA isolation, cDNA synthesis and quantitative PCR

See Supplemental Material.

Quantitative RT^2 profiler™ PCR arrays

The 384 (4×96) wells custom-made PCR array (SA Bioscience) layout contained 4 replicate primer assays for each of 86 target genes and 7 housekeeping genes. In addition, 4 wells contained mouse genomic DNA controls, and 12 wells contained positive PCR controls. Prepared cDNA from pools of 5 mice were added (for polyps in duplicates) to RT2 SYBR Green Mastermix and the mix was aliquoted into the PCR array plates. PCR reactions were performed by the Genomics Core Facility, The Sahlgrenska Academy, and relative expression levels were determined using data from the real-time cycler and the ^{CT} ΔΔmethod.

iNKT cell isolation and adoptive transfer

Hepatic mononuclear cells (for hepatic mononuclear cell isolation see Supplemental Material) were stained with TCRβ and PBS57 loaded CD1d tetramer-PE and sorted with FACSAria II (BD). CD1d-tetramer^{+} iNKT cells (5×10^5) or CD1d-tetramer-negative TCRβ^{+} cells were injected i.v. into 13 and 14.5 week old $Apc^{Min/+}Ja18^{-/-}$ mice. Non-transferred mice were used as control. Mice were sacrificed 3 weeks after the first injection. Lymphocytes from spleen, MLN, polyps and unaffected lamina propria were isolated and analyzed with flow cytometry.

Statistical analysis

Calculation of statistical significance was performed using nonparametric Mann-Whitney test or two-way ANOVA comparison with Bonferroni post-tests. P values of <0.05 were considered significant. Statistical analyses were performed on Prism GraphPad 6. Results are presented as mean ± SD in the figures.

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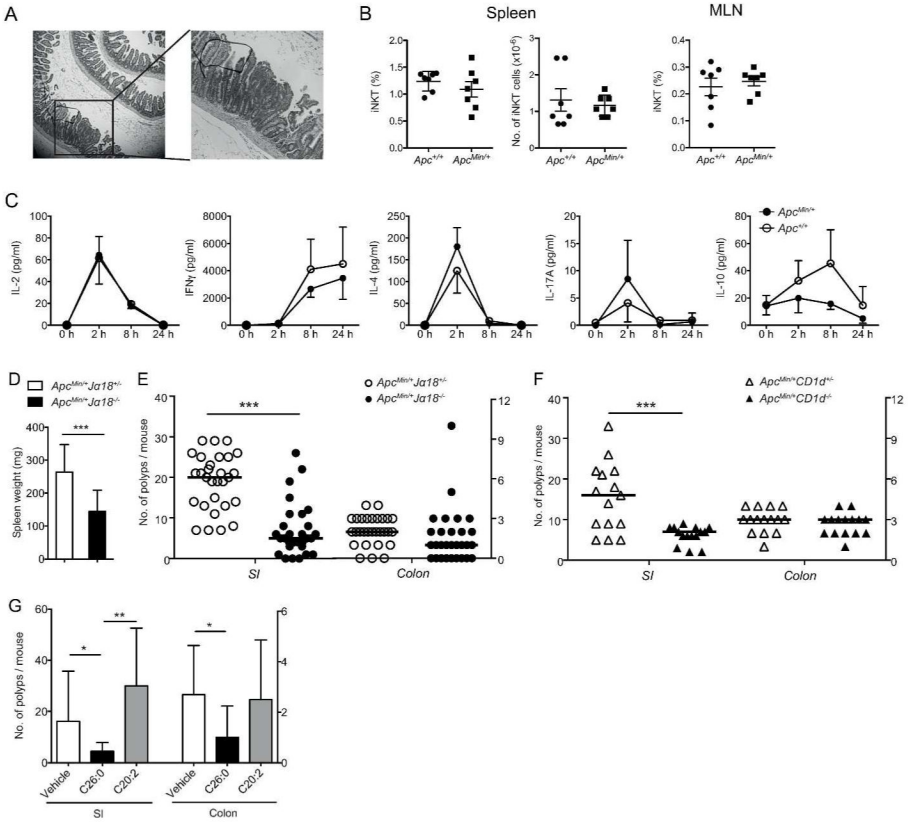

Figure 1. *APC*$^{MIN/+}$ mice lacking iNKT cells had a decreased number of intestinal tumors

(A) Small intestinal lesion in 12-week old *Apc*$^{Min/+}$ mouse. Tissue sections were stained with hematoxylin/eosin, right panel shows the close up of boxed part from left panel. (B) iNKT cells (TCR^{+}αGalCer (PBS57) loaded CD1d tetramer^{+} cells) in *Apc*$^{+/+}$ and *Apc*$^{Min/+}$ littermate mice were analyzed by flow cytometry. Symbols represent values from individual mice, mean ± SD is indicated (n=7). (C) αGalCer was injected into *Apc*$^{Min/+}$ and *Apc*$^{+/+}$ mice, and serum cytokines were analyzed before (0 h) and at 2, 8 and 24 hours after injection. Data (mean ± SD, n=3–5) are from one of two similar independent experiments. Spleen weight (D), and polyp numbers in small intestine (SI) and colon (E) in 15-week old *Apc*$^{Min/+}$*Ja18*$^{-/-}$ mice lacking iNKT cells compared to heterozygote control littermate *Apc*$^{Min/+}$ *Ja18*$^{+/-}$ mice. (F) Development of intestinal polyps in 20-week old *Apc*$^{Min/+}$ *CD1d*$^{-/-}$ mice lacking CD1d compared to heterozygote control littermate *Apc*$^{Min/+}$ *CD1d*$^{+/-}$ mice. Symbols represent individual mice, and median ± SD is indicated of 15 (D and F) and 25 (E) mice. (G) Polyp numbers in *Apc*$^{Min/+}$ mice treated from 5 weeks of age with αGalCer (C26:0) or the TH2-cytokine skewing analogue C20:2 and sacrificed at 15 weeks of age. Data are presented as median ± SD (n=10). Mann-Whitney test (B–F) and two-way ANOVA comparison with Bonferroni post-tests (G) were used for statistical analyses. * $p<0.05$, ** $p < 0.01$, *** $p < 0.001$.

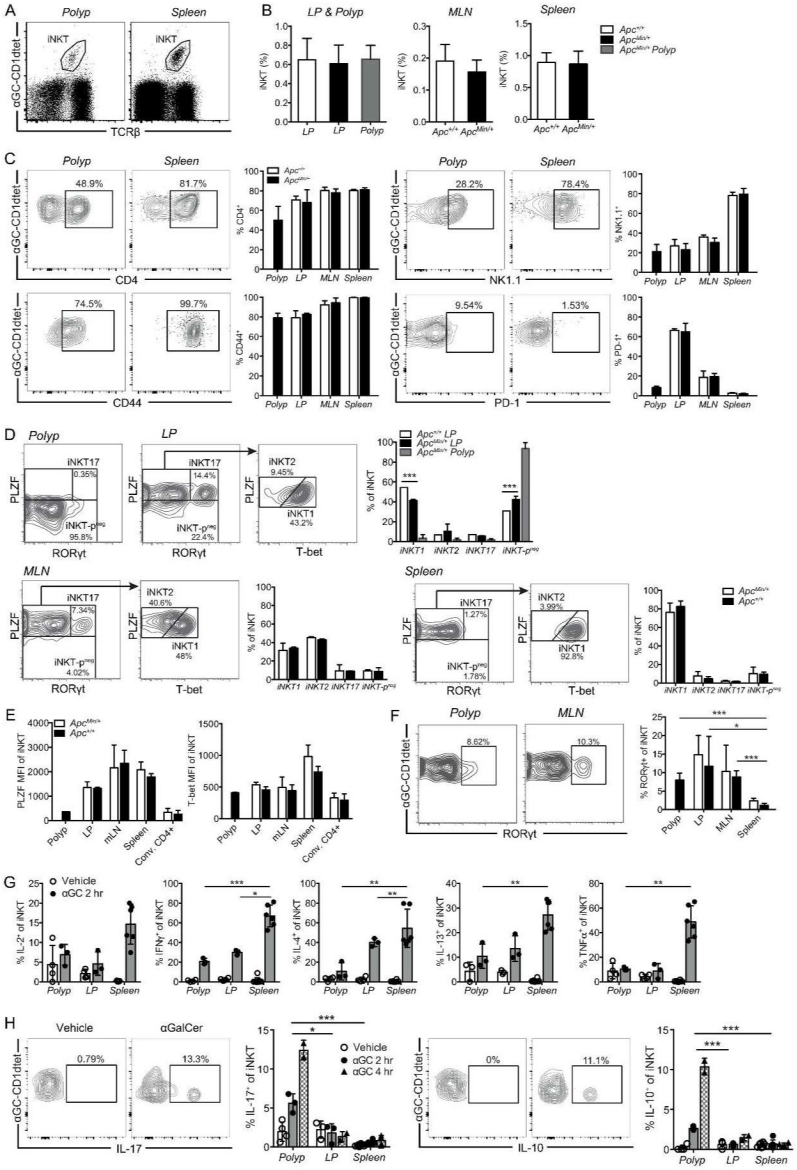

Figure 2. Polyp iNKT cells in *APC*$^{MIN/+}$ mice displayed unique phenotype and functions, and lacked PLZF expression

iNKT cells were identified in 15 week old mice using αGalCer (PBS57) loaded CD1d-tetramers and anti-TCRβ and gated as shown (A). (B) Frequencies of iNKT cells in 15-week old *Apc*$^{Min/+}$ and *Apc*$^{+/+}$ mice. (C) Representative stainings of CD4, NK1.1, CD44 and PD-1 expression on iNKT cells from polyp and spleen of *Apc*$^{Min/+}$ mice, and summary plots of marker expression by iNKT cells in different tissues of *Apc*$^{Min/+}$ and *Apc*$^{+/+}$ mice. Data are presented as mean ± SD of 10 mice. (D) iNKT cells were defined as iNKT1, iNKT2 and iNKT17 cells according to the expression of PLZF, T-bet and RORγt as presented by the gates in the FACS plots. Representative stainings of iNKT cells from *Apc*$^{Min/+}$ mice are shown. Bar graphs in (D) show summary plots of the proportions of iNKT1, iNKT2, iNKT17 and iNKT-P^{neg} (PLZF-negative iNKT) cells in LP and polyp, MLN and spleen of *Apc*$^{+/+}$ and *Apc*$^{Min/+}$ mice. (E) Summary plot of PLZF and T-bet staining median

fluorescence intensity (MFI) in different organs. The levels of PLZF and T-bet in splenic conventional CD4 (Conv. $CD4^+$) T cells are shown for comparison. (F) Representative staining of RORγt in iNKT cells and summary plot of the frequencies of $ROR\gamma t^+$ iNKT cells in different organs. Data from at least three independent experiments have been pooled and are presented as mean ± SD of 3 to 5 mice (D–F). (G, H) Cytokine production by iNKT cells after 2 and 4 hours of *in vivo* αGalCer stimulation. Data derive from two independent experiments, and 6 to 8 mice per group. Each data point represents an individual mouse (spleen) or pooled organs from 2 mice (LP and polyp). Flow cytometry plots in (H) show representative staining of IL-10 and IL-17 in iNKT cells 4 hours after αGalCer injection. Data represent mean ± SD, and Mann-Whitney test was used for statistical analyses. * $p<0.05$, ** $p < 0.01$, ***$p<0.001$.

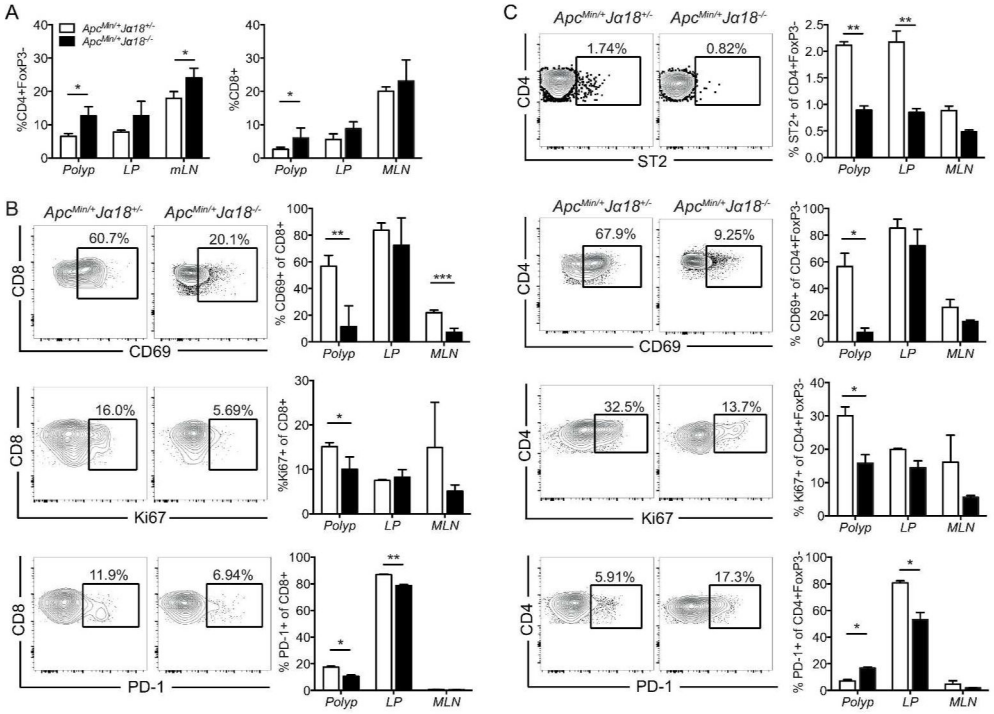

Figure 3. Increased frequency of conventional T cells in polyps in the absence of iNKT cells

(A) Frequency of conventional $CD4^+$ ($CD4^+FoxP3^-$) cells and $CD8^+$ T cells among lymphocytes in $Apc^{Min/+}$ $Ja18^{-/-}$ mice and their $Apc^{Min/+}$ $Ja18^{+/-}$ littermate controls. (B) Representative staining and summary data of CD69, Ki67 and PD-1 expression on $CD8^+$ T cells in $Apc^{Min/+}Ja18^{-/-}$ and $Apc^{Min/+}$ $Ja18^{+/-}$ mice. (C) Representative staining and summary data of ST2, CD69, Ki67 and PD-1 expression on conventional $CD4^+$ T cells in $Apc^{Min/+}Ja18^{-/-}$ and $Apc^{Min/+}$ $Ja18^{+/-}$ mice. Data from at least three independent experiments are presented as mean ± SD of 7 to 10 mice (CD69) and 3 to 6 mice (Ki67, PD-1, ST2) per genotype. Mann-Whitney test was used for statistical analyses. * $p < 0.05$, ** $p < 0.01$, ***$p<0.001$.

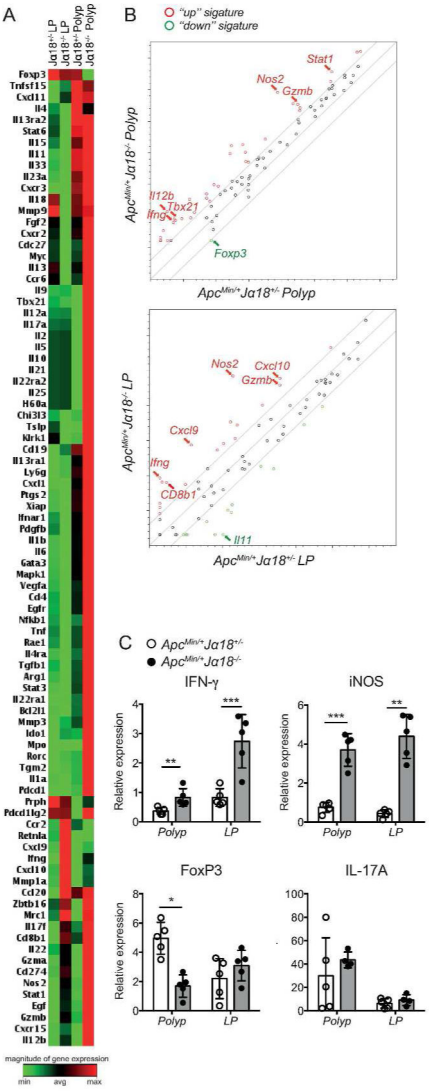

Figure 4. Absence of iNKT cells promoted expression of inflammatory genes and reduced immunoregulatory genes in polyp tissue

The expression of mRNA from unaffected LP and polyp tissue of 15 week old *Apc*$^{Min/+}$ *Jα18*$^{-/-}$ and *Apc*$^{Min/+}$ *Jα18*$^{+/-}$ mice was examined by RT2 PCR profiler array with a selection of genes of relevance for immunity and tumor progression (A, B, each sample was a pool of mRNA from 5 mice) and real time PCR (C). (A) Heat map of gene expression. (B) Normalized expression values for polyp (upper) and LP (lower) tissues. *Apc*$^{Min/+}$ *Jα18*$^{-/-}$ ‘‘up’’ (red) and ‘‘down’’ (green) signature transcripts are highlighted. The central line indicates unchanged gene expression, and the boundary lines indicate 4-fold regulation. (C) The expression of selected genes were examined by real time PCR and normalized against β-actin. Symbols represent individual mice, and data are presented as mean ± SD of 5 mice. Mann-Whitney test was used for statistical analyses. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

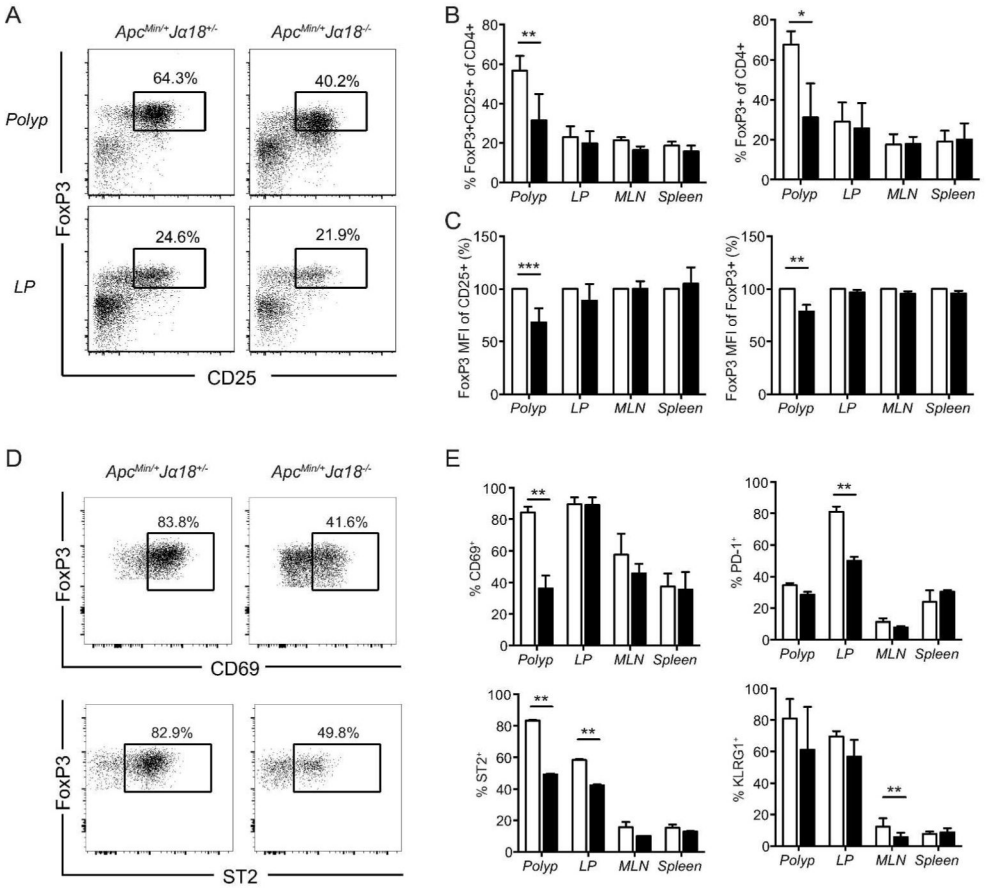

Figure 5. iNKT cells supported the expression of FoxP3 and an activated polyp Treg cell phenotype

(A) Representative stainings show a decreased level of FoxP3 protein in Treg cells from $Apc^{Min/+}Ja18^{-/-}$ and $Apc^{Min/+}$ $Ja18^{+/-}$ mice. (B) Frequencies of $CD25^+FoxP3^+$ T cells (left) and total $FoxP3^+$ T cells (right) among $CD4^+$ T cells in $Apc^{Min/+}Ja18^{-/-}$ and $Apc^{Min/+}$ $Ja18^{+/-}$ mice. (C) MFI of FoxP3 staining in $CD25^+$ cells (left) or in $FoxP3^+$ cells (right) in different tissue in the presence and absence of iNKT cells. (D) Representative staining of CD69 and ST2 on Treg cells in polyps from $Apc^{Min/+}Ja18^{-/-}$ mice and $Apc^{Min/+}$ $Ja18^{+/-}$ mice. (E) Surface expression of CD69, PD-1, KLRG1 and ST2 on Treg cells in $Apc^{Min/+}Ja18^{-/-}$ mice and their $Apc^{Min/+}$ $Ja18^{+/-}$ littermates. Data are presented as mean ± SD of 7 mice from three independent experiments (A–B) and 3 mice from a representative experiment of three performed (C), and 3 mice from two independent experiments (E). Mann-Whitney test was used for statistical analyses. *p<0.05, ** p < 0.01, *** p < 0.001.

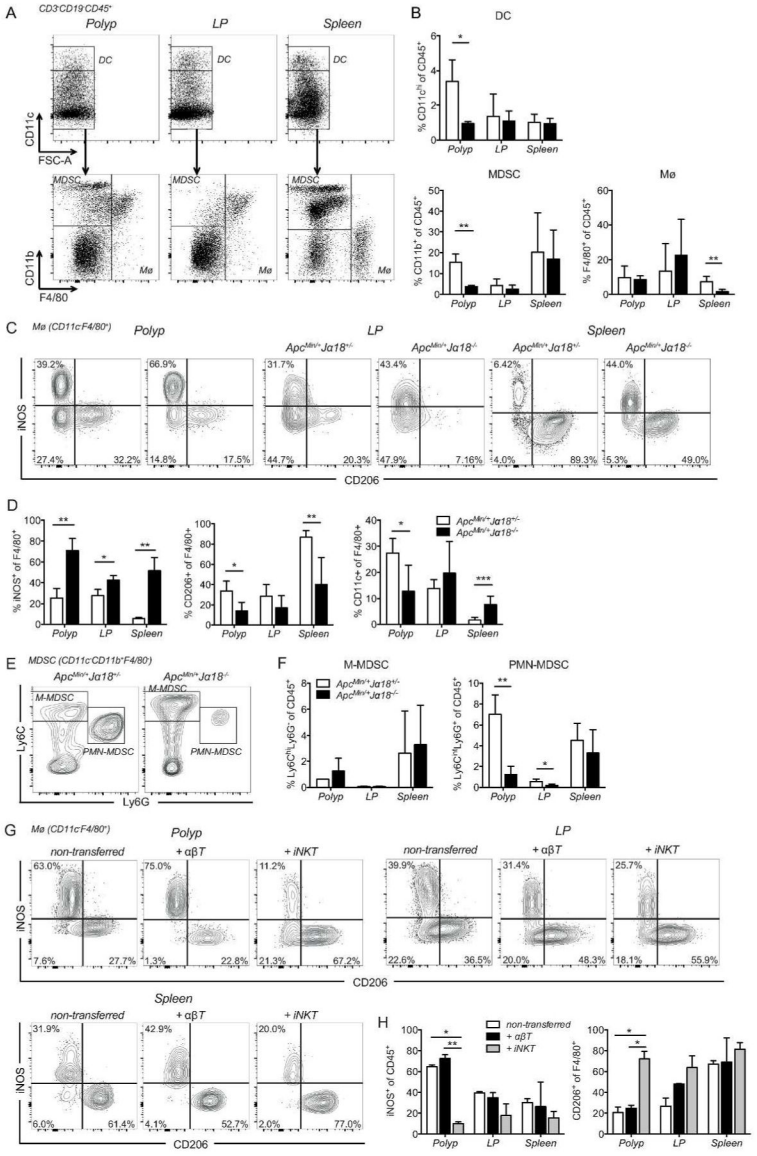

Figure 6. Lack of iNKT cells resulted in a skewed macrophage phenotype from M2 to M1

Innate immune cells were investigated by flow cytometry in polyps, LP and spleen of $Apc^{Min/+}$ $Ja18^{-/-}$ and $Apc^{Min/+}$ $Ja18^{+/-}$ mice. (A) Gating strategies for dendritic cells (DC, $CD3^{-}CD19^{-}CD45^{+}CD11c^{hi}$), MDSC ($CD3^{-}CD19^{-}CD45^{+}CD11c^{lo/neg}CD11b^{+}$) and macrophages (Mø, $CD3^{-}CD19^{-}CD45^{+}CD11c^{lo/neg}F4/80^{+}$) are shown for $Apc^{Min/+}$ $Ja18^{+/-}$ polyp, LP and spleen. (B) Frequency of DC, MDSC and Mø among $CD45^{+}$ cells in indicated tissues. (C) Representative staining of iNOS and CD206 expression on macrophages. (D) Frequency of macrophages expressing iNOS, CD206 and CD11c in the same tissues. Data from three independent experiments are presented as mean ± SD of 6 mice. (E) Gating strategy for M^{-}MDSC ($CD3^{-}CD19^{-}CD45^{+}CD11c^{lo/neg}CD11b^{+}Ly6C^{hi}Ly6G^{-}$) and PMN-MDSC ($CD3^{-}CD19^{-}CD45^{+}CD11c^{lo/neg}CD11b^{+}Ly6C^{int}Ly6G^{+}$). (F) Frequency of M-MDSC and PMN-MDSC in $Apc^{Min/+}$ $Ja18^{-/-}$ and $Apc^{Min/+}$ $Ja18^{+/-}$ mice. (G) $Apc^{Min/+}$ $Ja18^{-/-}$ mice

were adoptively transferred with 5×10^5 hepatic iNKT cells or CD1d-tetramer-negative αβ T cells (αβT) twice, at 13 and 14.5 weeks of age and sacrificed at 16 weeks of age. Non-transferred mice were also used as control. Representative stainings of iNOS and CD206 expression on macrophages are shown for each group. (H) Frequency of macrophages expressing iNOS and CD206 in the same tissues. Data from are presented as mean ± SD of 4 mice. Mann-Whitney test was used for statistical analyses. * $p<0.05$, ** $p < 0.01$, *** $p < 0.001$.

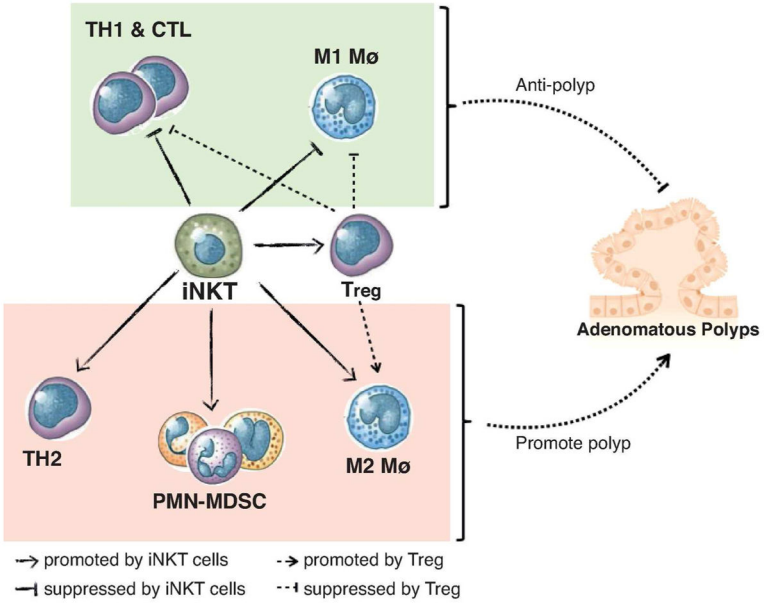

Figure 7. iNKT cells suppressed TH1 immunity and promoted an immunoregulatory microenvironment in polyps

The findings are summarized in this proposed model for iNKT cells promotion of intestinal polyps.