Title: Granzyme B skews CD4+ T cell differentiation resulting in increased intestinal pathogenicity

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Abstract: CD4⁺ T cell activation and differentiation are important events that set the stage for proper immune responses. Many factors are involved in the activation and differentiation of T cells, and these events are tightly controlled to prevent unwanted and/or exacerbated immune responses that may harm the host. It has been well documented that granzyme B, a potent serine protease involved in cell-mediated cytotoxicity, is readily expressed by certain CD4⁺ T cells, such as regulatory T cells and CD4⁺CD8αα⁺ intestinal intraepithelial lymphocytes, both of which display cytotoxicity associated with granzyme B. However, because not all CD4⁺ T cells expressing granzyme B are cytotoxic, additional roles for this protease in CD4⁺ T cell biology remain unknown. Here, using a combination of in vivo and in vitro approaches, we report that granzyme B-deficient CD4⁺ T cells display increased IL-17 production. In the adoptive transfer model of intestinal inflammation, granzyme B-deficient CD4⁺ T cells triggered a more rapid disease onset than their WT counterparts, and presented a differential transcription profile. Similar results were also observed in granzyme B-deficient mice infected with Citrobacter rodentium. Our results suggest that granzyme B modulates CD4⁺ T cell differentiation, providing a new perspective into the biology of this enzyme.

Introduction
Granzymes are a family of serine proteases expressed by many immune cell populations. There are five different granzymes in humans (A, B, H, K and M) and 11 in mice (A, B, C, D, E, F, G, K, L, M and N), which differ in function and substrate specificity.¹ Most research has focused on granzymes A and B due to their hallmark role in CD8⁺ T and NK cell-mediated cytotoxicity against tumor cells or virus-infected cells.² However, mounting evidence indicates that granzymes are also involved in other processes, including inflammatory responses,³⁻⁵ remodeling of the extracellular matrix,⁶ and cardiovascular disorders.⁷

Activated CD4⁺ T cells express granzyme B, and some of these cells exhibit lytic activity similar to CD8⁺ T cells.⁸⁻⁹ Antigen-specific CD4⁺ T cell lytic activity mediated by granzyme B can be triggered by viral and bacterial infections.¹⁰⁻¹² Similarly, some tumor-infiltrating CD4⁺ T cells also present anti-tumor cytotoxicity mediated by granzyme B.¹³ In addition, one of the mechanisms by which regulatory CD4⁺ T cells in humans and mice control immune responses is through granzyme B-mediated cell death.¹⁴⁻¹⁸

After activation, CD4⁺ T cells can differentiate into a diverse set of T helper (Th) cells depending on environmental cues and transcription factors, which ultimately leads Th cells to produce a specific cytokine profile.¹⁹ In certain populations of differentiated CD4⁺ T cells, granzyme B controls CD4⁺ T cell immune responses by initiating activation-induced cell death.²⁰ Downregulation of granzyme B, for example when Th2 cells are cultured in the presence of vasoactive intestinal peptide, reduces Th2 cell death.²⁰ Moreover, granzyme B expression in Th2 cells is enhanced by IL-10R signaling, which results in cell death.²¹ These studies clearly indicate that granzyme B is involved in controlling some immune CD4⁺ T cells responses. However, it is unclear whether this enzyme is actively involved during the process of Th differentiation.
In this report, we present data indicating that granzyme B is expressed during CD4+ T cell differentiation. Cells lacking granzyme B show a different cytokine profile than granzyme B-competent cells, primarily presenting increased IL-17 production. Moreover, using the adoptive transfer model of intestinal inflammation, we show that granzyme B-deficient CD4+ T cells activated in vivo have a different gene expression profile and display increased pathogenicity relative to WT cells. Similarly, we present evidence indicating that granzyme B deficiency predisposes mice to higher susceptibility to Citrobacter rodentium infection. These results indicate that granzyme B is required for proper CD4+ T cell differentiation, and in its absence, CD4+ T cells may acquire an aberrant phenotype characterized by increased IL-17 production and pathogenicity.

Results

Th0 and Th1, but not Th17 CD4+ T cells express granzyme B during differentiation

To determine the role of granzyme B in T cell activation and differentiation, we cultured naïve CD4+ T cells in Th0, Th1 and Th17 differentiation conditions. One and three days after culture, RNA was extracted to determine granzyme B expression relative to naïve CD4+ T cells. Under Th0 conditions, expression of granzyme B at day 1 did not increase above the levels observed for naïve CD4+ T cells (Figure 1a, top), but presented an almost two-fold increase at day 3 (Figure 1a, bottom). Th1 cells expressed a 6-fold increase in granzyme B mRNA over naïve T cells at day 1, decreasing to 2-fold at day 3. On the other hand, CD4+ T cells under Th17 differentiation conditions did not induce granzyme B mRNA expression at either time point (Figure 1a).

Around 15% of Th0 and Th1 cells expressed granzyme B intracellularly at day 5 (Figure 1b), whereas this enzyme was not detected in Th17 cells, confirming the results for granzyme B
mRNA expression. Granzyme B in Th1 cells was not detected at 1 day after differentiation but it was conspicuously expressed at day 3 and day 5 (Figure 1c). On the other hand, Th17 cells did not express granzyme B at any of the time points analyzed (Figure 1c). Because Th1 and Th17 cultures contain different cytokine/antibody cocktails, and granzyme B is expressed in the former but not in the latter helper cells, we investigated what factors present in these culture conditions induce or repress granzyme B expression. For this purpose, we activated CD4$^+$ T cells under Th0 conditions (anti-CD3/CD28, IL-2, and irradiated spleen cells), in the presence of individual factors present in Th1 and Th17 differentiation cocktails (Figure 1d). IL-2 alone induced ~7.5% of CD4$^+$ T cells to express granzyme B, which was not significantly different from cells cultured without IL-2 or in the presence of IL-12, IL-1β, and IL-23, these last two cytokines involved in Th17 differentiation. However, Th17-inducing cytokines IL-6 and TGFβ, as well as the blocking anti-IFNγ antibody significantly ablated granzyme B expression. These results indicate that CD4$^+$ T cell activation induces granzyme B activation, whereas some cytokines and anti-IFNγ inhibit its expression.

Because most of our differentiation cultures included spleen cells as a source of APC, we determined whether these cells influence granzyme B expression during CD4$^+$ T cell activation. As shown in Figure 1e, CD4$^+$ T cell activation in the absence of APC (but including IL-2), resulted in less than 2% of CD4$^+$ T cells expressing granzyme B, whereas irradiated spleen APC significantly increased granzyme B expression.

Overall, these results indicate that granzyme B is expressed during Th0 and Th1 differentiation, with irradiated APC being an important expression-enhancing factor. On the other hand, Th17 differentiation conditions ablate granzyme B expression. This effect can be attributed to IL-6, TGFβ, and anti-IFNγ.
As shown in Figure 1b, during Th0 and Th1 differentiation, two populations of CD4<sup>+</sup> T cells can be distinguished: WT-gzmB<sup>pos</sup> and WT-gzmB<sup>neg</sup>. To determine whether these populations present different cytokine profiles, we determined the frequencies of IFN<sub>γ</sub> and IL-17-producing cells in each of these subpopulations. Around half of Th0 WT-gzmB<sup>pos</sup> expressed IFN<sub>γ</sub>, whereas only around 5% of the WT-gzmB<sup>neg</sup> CD4<sup>+</sup> T cells were positive for IFN<sub>γ</sub> (Figure 1f). Th0 cells derived from granzyme B-deficient (Gzmb<sup>−/−</sup>) mice presented decreased levels of IFN<sub>γ</sub>-producing cells in comparison to WT-gzmB<sup>pos</sup> cells, but significantly more IFN<sub>γ</sub><sup>+</sup> cells than WT-gzmB<sup>neg</sup> cells (Figure 1f). On the other hand, IL-17 production was not significantly different among Th0 cells from any of the CD4<sup>+</sup> T cell populations.

Th1 differentiation induced high levels of IFN<sub>γ</sub><sup>+</sup> cells in all CD4<sup>+</sup> T cell populations analyzed; however, WT-gzmB<sup>pos</sup> cells presented the highest levels of IFN<sub>γ</sub><sup>+</sup> cells in comparison to WT-gzmB<sup>neg</sup> or cells derived from Gzmb<sup>−/−</sup> mice (Figure 1g). Interestingly, under Th1 conditions, cells from Gzmb<sup>−/−</sup> mice presented significantly greater levels of IL-17<sup>+</sup> cells when compared to WT-gzmB<sup>pos</sup> and WT-gzmB<sup>neg</sup> cells (Figure 1g). These results suggest that granzyme B may be involved in preventing IL-17 production when CD4<sup>+</sup> T cells are cultured under Th1 conditions.

**Mesenteric lymph node APC enhance granzyme B and IL-17 expression**

As shown in the previous section, addition of irradiated spleen APC to the cultures enhanced granzyme B expression by activated CD4<sup>+</sup> T cells under Th0 conditions (Figure 1e). Because APC activity varies between lymphoid organs, we investigated whether APC from spleen and mesenteric lymph nodes (MLN) induced differential granzyme B expression, and if so, whether IFN<sub>γ</sub> and IL-17 expression is affected. Spleen APC induced greater granzyme B
expression than their MLN counterparts in WT CD4+ T cells (Supp. Figure 1a). IFNγ production between WT-gzmB<sup>pos</sup> CD4+ T cells was similar regardless of the source of irradiated APC, which was also observed between WT-gzmB<sup>neg</sup> CD4+ T cells (Supp. Figure 1b). However, irradiated APC from MLN induced higher IL-17 production than spleen APC in the WT-gzmB<sup>pos</sup> CD4+ T cell population (Supp. Figure 1c). Granzyme B-deficient CD4+IFNγ<sup>+</sup> T cells were similar between cultures containing spleen or MLN APC (Supplemental Fig. 1d). However, increased IL-17 production was also observed in granzyme B-deficient CD4+ T cells differentiated in the presence of MLN APC (Supp. Figure 1e).

Overall, these results indicate that APC present in distinct immunological compartments stimulate differential granzyme B expression. Moreover, APC that induce CD4+ T cells to express less granzyme B also seem to promote increased IL-17 production (particularly in the WT-gzmB<sup>pos</sup> and granzyme B-deficient CD4+ T cell populations), suggesting an inverse relationship between granzyme B expression and IL-17 production.

**In vivo activation of granzyme B-deficient CD4+ T cells results in increased pathogenicity**

To further understand the role of granzyme B in CD4+ T cell differentiation, we adoptively transferred naïve CD4+CD45RB<sup>hi</sup> T cells from WT or Gzmb<sup>-/-</sup> mice into Rag2<sup>-/-</sup> recipient mice. Because it is well-established that transfer of naïve CD4+ T cells into immunocompromised recipients results in chronic intestinal inflammation, 22, 23 this set up allows us to: 1) determine whether granzyme B-deficiency alters CD4+ T cell pathogenicity, and 2) whether absence of granzyme B influences the cytokine profile of CD4+ T cells activated in vivo (discussed in the next section).
In our animal colony, transfer of naïve CD4+ T cells from WT mice typically results in disease symptoms by 6 weeks after transfer. However, when naïve CD4+ T cells from Gzm\(b^{-/-}\) mice were transferred into Rag2\(^{-/-}\) mice, weight loss was evident starting at 14 days after transfer, and by day 21 these mice had lost around 20% of their original weight (Figure 2a). At this point, the experiments were terminated to comply with our institution’s animal welfare regulations. In contrast, at day 21, Rag2\(^{-/-}\) mice that received WT cells were either gaining or maintaining their weight (Figure 2a). These results suggest that absence of granzyme B resulted in more pathogenic T cells. To determine whether this was a CD4+ T cell intrinsic effect, we adoptively transferred naïve CD4+ T cells from WT or Gzmb\(^{-/-}\) donor mice into Rag2\(^{-/-}\) Gzmb\(^{-/-}\) mice. Donor CD4+ T cells from WT mice did not cause early weight loss in the double knock-out recipient mice, while CD4+ T cells deficient in granzyme B caused similar weight loss as in Rag2\(^{-/-}\) recipient mice (Figure 2a). These results indicate a granzyme B-intrinsic effect in CD4+ T cell pathogenesis. Weight loss in mice receiving granzyme B-deficient cells was accompanied by other signs of disease, such as diarrhea, pilo-erection, and hunching (Figure 2b). As expected, colon pathology based on immune cell infiltration, loss of goblet cells, epithelial damage and hyperplasia was higher in mice receiving CD4+ T cells from Gzm\(b^{-/-}\) donors (Figure 2c). Finally, granzyme B-deficient CD4+ T cells reconstituted the recipient mouse colons better than WT cells, regardless of the recipient mice (Figure 2d).

In summary, our data indicate that absence of granzyme B in CD4+ T cells activated \textit{in vivo} results in accelerated pathogenicity and increased cell reconstitution.

\textbf{Granzyme B-deficient CD4+ T cells present increased IL-17 production \textit{in vivo}.}
To determine whether granzyme B deficiency results in differential CD4+ T cell cytokine profiles \textit{in vivo}, we adoptively transferred naïve CD4+CD45RB\textsuperscript{hi} T cells from WT or \textit{Gzmb}\textsuperscript{-/-} mice into \textit{Rag2}\textsuperscript{-/-} recipient mice. Three weeks after transfer, donor-derived cells were recovered from the MLN and lamina propria and analyzed for IFN\textgamma and IL-17 expression. As shown in Figure 3, IFN\textgamma was the predominant cytokine produced by donor CD4+ T cells derived from WT and \textit{Gzmb}\textsuperscript{-/-} mice. In cells recovered from the MLN, there were similar levels of IFN\textgamma production between WT and \textit{Gzmb}\textsuperscript{-/-} CD4+ T cells (Figure 3a). However, CD4+ T cells from \textit{Gzmb}\textsuperscript{-/-} donor mice presented significantly increased IL-17 production in comparison to WT CD4+ T cells (Figure 3a). A similar pattern was observed in donor-derived cells recovered from the lamina propria (Figure 3b).

In summary, \textit{in vivo} activated CD4+ T cells deficient in granzyme B presented increased IL-17 production. These results suggest that absence of granzyme B results in skewed \textit{in vivo} differentiation.

\textbf{Granzyme B deficient CD4+ T cells present a distinct gene expression profile}

Because granzyme B-deficient CD4+ T cells had increased IL-17 production when activated \textit{in vivo}, we investigated whether these cells presented a distinct gene expression profile in comparison to \textit{in vivo} activated CD4+ T cells from WT mice. For this purpose, CD4+CD45RB\textsuperscript{hi} T cells from WT and \textit{Gzmb}\textsuperscript{-/-} donor mice were adoptively transferred into \textit{Rag2}\textsuperscript{-/-} mice. Three weeks after transfer, cells were recovered from the MLN, their RNA extracted and gene expression profile determined by RNAseq. As shown in Figure 4a, WT and granzyme B-deficient CD4+ T cells presented significantly different gene expression profiles. We also performed gene set enrichment analysis to determine whether WT and granzyme B-deficient CD4+ T cells recovered
from recipient mice segregated in specific groups. As indicated in Figure 4b, GSEA indicated that these two CD4+ T cell populations presented distinct differential gene expression within several GSEA groups (Figure 4b). These results indicate that granzyme B-deficient CD4+ T cells, in the adoptive transfer system, follow a distinct differentiation pathway compared to WT CD4+ T cells.

We validated the results generated from the RNAseq analysis by comparing the expression of selected genes in naïve CD4+ T cells (Figure 4c, top), and donor-derived cells recovered 21d post-adoptive transfer (Figure 4c, bottom). Hallmark genes involved in Th17 responses, such as Rorc and Il17a were similarly expressed in naïve CD4+ T cells, regardless of the granzyme B status, but their expression had significantly increased in recovered granzyme B-deficient CD4+ T cells. Ddit4, a gene important for optimal T cell proliferation,24 is highly expressed in T cells from multiple sclerosis patients, and is important in Th17 differentiation.25 Ddit4 expression was 3-fold higher in activated CD4+ T cells derived from Gzmb−/− mice in comparison to WT CD4+ T cells (Figure 4c). Granzyme B-deficient CD4+ T cells presented a 2-fold increase in Il7r expression (Figure 4c), which mediates IL-7 signaling, a well-known lymphopoietic cytokine also expressed in T cells undergoing homeostatic expansion.26 Plexin D1, encoded by the Plxnd1 gene, binds semaphorins27 and is critical for directing thymocyte migration.28 Although the role of Plxnd1 is not well known in mature T cell biology, granzyme B-deficient CD4+ T cells presented approximately a 4-fold increase in expression. Interestingly, donor CD4+ T cells derived from Gzmb−/− mice presented decreased Il10ra expression in comparison to WT counterparts (Figure 4c).

Overall, these results show that in the adoptive transfer model of CD4+ T cell activation, granzyme B deficiency results in a distinct gene expression profile, characterized by genes relevant to Th17 differentiation and T cell proliferation.
Granzyme B-deficient CD4+ T cells possess better reconstitution capabilities

As indicated in Figure 2d, granzyme B-deficient CD4+ T cells showed greater reconstitution in the colon than WT CD4+ T cells when transferred into Rag-2−/− recipient mice. However, this observation could be due to inflammation-driven proliferation, which was more prevalent in Rag2−/− mice receiving CD4+ T cells from Gzmb+/− mice. To further determine the reconstitution potential of CD4+ T cells derived from WT and Gzmb−/− mice, we performed competitive adoptive T cell transfer experiments. For this purpose, naïve CD4+ T cells from WT CD45.1 and Gzmb−/− CD45.2 mice (1x10^5 each) were adoptively co-transferred into the same recipient mice. Of the total donor-derived cells obtained from the MLN, granzyme B-deficient CD4+ T cells presented ~60% reconstitution, whereas WT-derived cells showed ~40% reconstitution (Figure 5a). Although the IFNγ profile of co-transferred cells was similar to cells transferred alone, co-transferred WT cells presented increased IL-17 production in comparison to WT CD4+ T cells transferred alone (Figure 5b). These results raise the possibility that granzyme B-deficient CD4+ T cells influenced the behavior of granzyme-competent CD4+ T cells. Rag2−/− mice co-transferred with WT and GzB−/− CD4+ T cells displayed similar weight loss (Figure 5c) and colon pathology (Figure 5d) to mice transferred only with CD4+ T cells from Gzmb−/− mice, suggesting that the increased pathogenicity of the latter cells was maintained even in the presence of granzyme-competent CD4+ T cells.

Granzyme B-deficient mice present normal cellularity and IFNγ /IL-17 production

Since in vivo activated granzyme B-deficient CD4+ T cells possess differential gene expression and cytokine profiles, it is possible that these differences are also observed in naïve
cells. To investigate this possibility, we enumerated the CD4$^+$ T cell cellularity of spleen, MLN, and IEL compartments from WT and Gzmb$^{-/-}$ mice. As indicated in Figure 6a, frequencies and numbers of TCR$\beta^+$CD4$^+$ T cells were similar among the two groups of mice. Ex vivo IFN$\gamma$ and IL-17 production by non-stimulated (ns) or stimulated (s) naïve CD4$^+$ T cells derived from the MLN and IEL showed no distinguishable difference between cells from WT and Gzmb$^{-/-}$ mice (Figure 6b).

In summary, naïve WT and Gzmb$^{-/-}$ animals appear to have similar CD4$^+$ T cell cellularity and basal IFN$\gamma$ and IL-17 production in peripheral lymphoid organs and the intestines.

Granzyme B deficiency results in increased disease severity during Citrobacter rodentium infection

As demonstrated in the previous section, naïve Gzmb$^{-/-}$ mice have a normal CD4$^+$ T cell compartment compared to WT mice. However, because we observed that activated T cells from Gzmb$^{-/-}$ mice became more pathogenic and had a distinct gene expression profile in the adoptive transfer model of colitis, we investigated whether Gzmb$^{-/-}$ mice respond properly to antigenic stimulus. For this purpose, we infected WT and Gzmb$^{-/-}$ mice with the extracellular bacterium Citrobacter rodentium, which preferentially colonizes the colon of mice and induces an IL-17/IL-22 immune response. After infection, WT mice maintained similar weight throughout the course of the experiment; however, starting at 9 days post-infection, Gzmb$^{-/-}$ mice lost weight, reaching approximately ~20% loss of the starting weight by day 12 (Figure 7a). Gzmb$^{-/-}$ mice also presented other signs of disease, such as diarrhea, hunched posture, and pilo-erection, which were mostly absent in WT mice (Figure 7b). Despite similar colon bacterial burden (Figure 7c) Gzmb$^{-/-}$ mice developed higher pathology characterized by increased infiltration and epithelial injury than WT
mice (Figure 7d). Analysis of cytokine production showed that in the spleen, MLN and LP of infected Gzm\(b^{-/-}\) mice, there was a significant increase in CD4\(^+\) T cells expressing IL-17, while IFN\(\gamma\) production was increased only in the MLN (Figure 7e). Because innate immune cells are important for the response against \(C.\) \(rodentium\), we investigated whether the absence of granzyme B in innate immune cells was responsible for the observed increased pathology in infected Gzm\(b^{-/-}\) mice. For this purpose, Rag-2\(^{-/-}\) and Rag-2\(^{-/-}\)Gzm\(b^{-/-}\) mice were infected with \(C.\) \(rodentium\) and monitored for 14 days. Both groups of mice maintained similar weights throughout the course of the experiment (Supplemental Figure 2a), indicating that the effect caused by granzyme B-deficiency is most likely associated with adaptive immune cells. To further confirm these results, we treated Rag-2\(^{-/-}\) and Rag-2\(^{-/-}\)Gzm\(b^{-/-}\) mice with anti-CD40 antibodies, which induce acute intestinal inflammation in the absence of T or B cells.\(^{29}\) As shown in Supplemental Figure 2b, Rag-2\(^{-/-}\) and Rag-2\(^{-/-}\)Gzm\(b^{-/-}\) mice presented similar weight loss throughout the course of the experiment. Overall, these results recapitulate what was observed in the adoptive transfer model of colitis, and indicate that the absence of granzyme B increased pathogenicity, which is associated with adaptive immune responses.

**Discussion**

Granzyme B expression in CD4\(^+\) T cells is well-documented, and has been primarily studied in the context of granzyme B/perforin-dependent regulatory T cell suppressor activity.\(^{30}\) However, as our data indicate, granzyme B expression appears to be a common feature among a significant fraction of activated CD4\(^+\) T cells. Interestingly, not all differentiation conditions induced granzyme B expression. For example, Th0 and Th1 cells rapidly express granzyme B starting at 1-day post activation, whereas Th17 cells do not express this enzyme at any time after
activation. In the former conditions, expression of granzyme B is primarily driven by the presence of irradiated splenocytes, indicating the possibility that cell-to-cell contact is important for granzyme B expression.

Although none of the cytokines involved in Th1 differentiation induced granzyme B expression above the levels observed with the addition of irradiated splenocytes, anti-IFNγ and some cytokines present in Th17 cocktails, such as IL-6 and TGFβ prevented the expression of the enzyme, which suggests that restriction of granzyme B expression is necessary for Th17 polarization. Studies in cytotoxic CD8+ T cells have shown that activation of these cells with anti-CD3 in the presence of IL-6 induces granzyme B expression, but not in the presence of TGF-β.31 Here we show that in CD4+ T cell differentiation, IL-6 does not enhance the expression of granzyme B, indicating that CD8+ and CD4+ T cells possess different mechanisms for modulating granzyme B production.

Granzyme B expression may not only be limited during the initial CD4+ T cell priming and differentiation. For example, TGFβ is known for its role in regulatory T cell differentiation, and some of these cells require granzyme B for their suppressor functions. However, as our results indicate, TGFβ ablates expression of this enzyme, suggesting that regulatory T cells must acquire granzyme B expression during post-priming events. Similarly, a fraction of effector CD4+ T cells that migrate into the intestinal intraepithelial lymphocyte compartment acquire granzyme B expression after transcriptional reprogramming.32

One of the most intriguing questions is: Why do CD4+ T cells express granzyme B during Th0/Th1 activation and differentiation? During in vitro Th0/Th1 differentiation WT-gzmBpos and WT-gzmBneg cells have a distinct IFNγ profile, suggesting that these cells may represent different lineages, and the activity of granzyme B influences their outcome. This is better exemplified when
analyzing granzyme B-deficient CD4+ T cells, where in vivo and in vitro activation skew them towards an IL-17-producing phenotype, with a distinct gene expression signature. We postulate that granzyme B-deficient IL-17+ cells represent a lineage of cells that underwent aberrant differentiation. Therefore, expression of granzyme B during Th0/Th1 activation serves as a checkpoint for proper CD4+ T cell differentiation, preventing IL-17 production and increased pathology. These observations have important significance because it has been reported that allelic variants of granzyme B correlate with autoimmune disorders,33-35 raising the possibility that in certain CD4+ T cell-mediated disorders, lack of proper granzyme B function increases the probability of improper differentiation and increased pathogenesis potential. If this idea is correct, then WT-gzmBpos CD4+ T cells may represent cells in which the differentiation signals have the potential to skew cells into an unwanted phenotype, and granzyme B expression in these cells ensures the correct differentiation process.

Granzyme B activity in lymphocyte development and/or differentiation has been previously reported.36 These authors showed that granzyme A and B are important for the development of a subset of TCRneg intestinal intraepithelial lymphocytes known for the expression of intracellular CD3γ. Development of these cells requires granzyme B to cleave and inactivate the intracellular domain of Notch1. This raises the interesting possibility that during CD4+ T cell differentiation, granzyme B prevents unwanted phenotypes by disrupting signals coming from the environment, such those provided by Notch and its ligands, which are known to influence CD4+ T cell differentiation.37 Our observation that granzyme B expression was increased in the presence of irradiated splenocytes, supports this hypothesis and we are currently investigating what signals derived from APC enhance granzyme B expression.
It has been reported that granzyme B-deficient CD4+CD25- T cells used in murine models of graft versus host disease (GVHD), induced faster disease onset with increased lethality, in comparison to their WT counterparts. Although this group did not provide a mechanistic explanation for the increased pathogenicity observed, they suggested that increased proliferation and decreased cell death may be involved. According to our data, we believe that in the GVHD model, differential proliferation and survival of granzyme B-deficient CD4+ T cells are the result of aberrant CD4+ T cell differentiation, which allows escape of highly pathogenic clones. Further investigations in the GVDH model are needed to test this hypothesis.

In summary, granzyme B has been known for its role in cell mediated-cytotoxicity, and while many groups have reported potential extracellular roles for this enzyme, here we present *in vitro* and *in vivo* evidence supporting a novel intrinsic function for granzyme B during CD4+ T cell differentiation. Although many questions remain to be answered regarding how granzyme B activity is involved in this process, our results provide a strong foundation for a better understanding of the function of this enzyme in CD4+ T cell biology.

**Methods**

*Mice.* C57BL/6J and C57BL/6J.CD45.1 mice were originally purchased from The Jackson Laboratory (000664, and 002014 respectively) and have been maintained and acclimated in our colony for several years. Granzyme B (Gzmb)-/- mice were kindly provided by Dr. Xuefang Cao. CD57BL/6J Rag2-/- mice were kindly provided by Dr. Luc Van Kaer. Rag2-/-Gzmb-/- were generated in our colony. All mice were between 6 to 10 weeks of age at the time of experimentation. Male and female mice were used for all experiments, except for *Citrobacter*
rodentium infection where 7-week-old females were used. Mice were maintained in accordance with the Institutional Animal Care and Use Committee at Vanderbilt University.

Reagents and flow cytometry. Fluorochrome-coupled anti-mouse CD4 (GK1.5), CD8α (53-6.7), CD45RB (C363.16A), granzyme B (NGZB), IFNγ (XMG1.2), IL-17a (TC11-18H10), TCRβ (H57-597) and ghost viability dyes were purchased from ThermoFisher, BD Bioscences or Tonbo. Biotinylated anti-CD8α (53-1.7) and CD19 (1D3) antibodies were purchased from Tonbo. Anti-CD40 antibody was purchased from BioXcell. Surface cell staining was performed following conventional techniques. For intracellular cytokine staining, cells were stimulated with PMA and ionomycin in the presence of Golgi Stop (BD Biosciences) for 4hr prior to staining. Extracellular markers were stained, cells were fixed briefly with 2% paraformaldehyde, followed by permeabilization and intracellular staining using the BD Cytofix/Cytoperm kit, according to manufacturer’s instructions. All stained samples were acquired using BD FACS Canto II, 3- or 4-Laser Fortessa, or 5-Laser LSR II flow cytometers (BD Bioscences). Data were analyzed using FlowJo software (Tree Star).

Lymphocyte isolation. Spleen and MLN lymphocytes were isolated by conventional means. IEL and LP cells were isolated by mechanical disruption as previously reported. Briefly, after flushing the intestinal contents with cold HBSS and removing excess mucus, the intestines were cut into small pieces (~1 cm long) and shaken for 45 minutes at 37°C in HBSS supplemented with 5% fetal bovine serum and 2 mM EDTA. Supernatants were recovered and cells isolated using a discontinuous 40/70% Percoll (General Electric) gradient. To obtain lamina propria lymphocytes,
intestinal tissue was recovered and digested with collagenase (187.5 U/ml, Sigma) and DNase I (0.6 U/ml, Sigma). Cells were isolated using a discontinuous 40/70% Percoll gradient.

In vitro CD4 T cell activation/differentiation. Naïve CD4 T cells were isolated from the spleens of the indicated mice by magnetic sorting using the Miltenyi or StemCell naïve CD4+ T cell isolation kits according to the manufacturer’s instructions. Splenocytes or MLN cells used as APC were incubated for 1hr at 37°C, non-adherent cells were removed, adherent cells were washed and resuspended in complete RPMI, and irradiated with 7 Gy (700 rads). $5 \times 10^5$ CD4+ T cells with or without $5 \times 10^5$ irradiated APC were incubated in the presence of plate bound anti-CD3 (5µg/ml) and soluble anti-CD28 (2.5µg/ml), for the indicated times, under the following conditions: Th0 (10ng/mL IL-2), Th1 (10ng/mL IL-2, 20ng/mL IL-12, and 10µg/mL anti-IL-4), and Th17 (5ng/mL TGFβ, 20ng/mL IL-6, 10ng/mL IL-1β, 10ng/mL IL-23, 10µg/mL anti-IL-4, and 10µg/mL anti-IFNγ).

Adoptive transfer of naïve CD4 T cells. Splenocytes from WT or Gzmb-/- mice were depleted of CD19+ and CD8α+ cells using magnetic bead sorting (Miltenyi). Enriched cells were stained with antibodies directed against TCRβ, CD4 and CD45RB, and naïve CD4+ T cells were sorted as CD4+CD45RBhi using a FACSAria III at the Flow Cytometry Shared Resource at VUMC. $1 \times 10^5$ cells were adoptively transferred into the indicated recipient mice. Starting weight was determined prior to injection. Mice were monitored and weighed weekly. At the end time point, mice were sacrificed and donor-derived cells were isolated from the indicated organs or tissues. Colon histopathology was performed in a blind fashion by a GI pathologist (MBP), following established parameters. For competitive transfer experiments, $1 \times 10^5$ CD4+CD45RBhi cells from both WT-
CD45.1 and Gzmb−/− CD45.2 mice were adoptively co-transferred into Rag2−/− mice and analyzed as indicated above.

Transcription profile analysis. For gene expression analysis, TCRβ+CD4+ T cells were purified by flow cytometry from a pool of MLN cells comprised of 1 male and 1 female animal with similar disease progression 21 days after adoptive transfer. RNA was isolated using the RNeasy micro kit (Qiagen). cDNA library preparation and RNAseq was performed by the VANTAGE Core at VUMC on an Illumina NovaSeq 6000 (2 x 150 base pair, paired-end reads). The tool Salmon was used for quantifying the expression of RNA transcripts. The R project software along with the edgeR method was used for differential expression analysis. For gene set enrichment analysis (GSEA), RNAseq data was ranked according to the t-test statistic. The gene sets curated (C2), GO (C5), immunological signature collection (C7) and hallmarks of cancer (H) of the Molecular Signatures Database (MSigDB) were used for enrichment analysis. GSEA enrichment plots were generated using the GSEA software from the Broad Institute with 1000 permutations.

Real time PCR. For in vitro analysis, RNA was extracted from MACS-enriched, differentiated CD4+ T cells at day 1 and 3 of culture. For in vivo analysis, RNA was isolated from flow cytometry purified donor-derived TCRβ+CD4+ T cells as described above. cDNA was synthesized using the RT2 First Strand kit (Qiagen). Real time PCR was performed using RT2 SYBR Green Mastermix (Qiagen) on an Applied BioSystems Proflex PCR cycler. All primers were purchased from Qiagen.

Citrobacter rodentium infection. Seven-week old female WT and Gzmb−/− animals were infected with C. rodentium as previously described. Briefly, mice were infected with 5x10⁸ CFU
exponentially grown bacteria by oral gavage. Starting weight was determined prior to gavage.

Mice were monitored and weighed daily for 14d. At the end time point, cells were isolated from the indicated organs or tissues. Cellularity was determined, intracellular cytokine staining was performed, and colon histopathology was performed in a blind fashion by a GI pathologist (MBP) as previously described.46

Anti-CD40 disease induction. Rag-2⁻/⁻ and Rag-2⁻/⁻Gzmb⁻/⁻ mice were injected i.p. with 150µg/mouse of anti-CD40 (BioXcell). Mice were monitored and weighed daily for 7 days.

Acknowledgments

This work was supported by NIH grant R01DK111671 (D.O.-V.); National Library of Medicine T15 LM00745 (M.J.G.); Vanderbilt Training in Cellular, Biochemical and Molecular Sciences Training Program, T32 GM008554-25 (K.G.M.); Vanderbilt Training in Gastroenterology T32 grant NIH T32 DK007673 (A.N.); and the Digestive Disease Research Center at Vanderbilt University Medical Center, NIH grant P30DK058404 (M.B.P., K.T.W., and D.O.-V.). We thank the Flow Cytometry Shared Resource and the Vanderbilt Technologies for Advanced Genomics (VANTAGE) for their invaluable support.

Competing interests: The authors declare no competing interests.

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**Figure legends**

**Figure 1.** Granzyme B expression during T cell differentiation. (a) Naïve CD4\(^+\) T cells from WT mice were cultured in the presence of plate-bound anti-CD3 and soluble CD28 under the indicated differentiation conditions, and in the absence of irradiated splenocytes. At day 1 (top) and 3 (bottom), cells were recovered, RNA extracted and granzyme B mRNA expression determined. (b) Intracellular granzyme B in cells cultured as in (a), but in the presence of 5x10\(^5\) irradiated splenocytes. Left, representative dot plot; live, TCR\(\beta^+\)CD4\(^+\) cells are displayed. Right, data summary. Each dot represents an individual sample. (c) Granzyme B expression of cells cultured in Th1 and Th17 conditions in the presence of irradiated splenocytes at different time points. (d) Cells cultured in Th0 conditions in the presence of the indicated individual cytokines, harvested 5 days after culture. (e) Cells cultured as indicated in (d) in the presence or absence of irradiated APC from spleen. Intracellular IFN\(\gamma\) and IL-17 staining of representative samples cultured in (f) Th0 or (g) Th1 conditions in the presence of irradiated splenocytes. Top, intracellular IFN\(\gamma\) and IL-17 staining representative samples; bottom, data summary. Each dot represents an individual sample. Data are representative of at least 3 independent experiments, performed in triplicate. n=6. For (e), ***P<0.001, Student’s t test; for (g) and (h), *P<0.05; **P<0.01; ***P<0.001; ****P<0.001, One-way ANOVA.

**Supplemental Figure 1.** Granzyme B and cytokine expression in the presence of 5x10\(^5\) irradiated spleen or MLN APC. Naïve CD4\(^+\) T cells from WT mice were cultured in the presence of plate-bound anti-CD3 and soluble anti-CD28 under Th1 differentiation conditions. (a) Granzyme B expression 5 days after culture. (b) IFN\(\gamma\) and (c) IL-17 production from the same cultures. (d) IFN\(\gamma\) and (e) IL-17 expression from granzyme B-deficient CD4\(^+\) T cells under similar cultures as in (a). Each dot represents an individual sample. Data are representative of at least 2 independent experiments, performed in triplicate. n=3-6. *P<0.05; **P<0.01; ***P<0.001. For (a) and (e), Student’s t-test; for (b) and (c), One-way ANOVA.

**Figure 2.** *In vivo* activated granzyme B-deficient CD4\(^+\) T cells present increased pathogenicity. 1x10\(^5\) CD4\(^+\)CD45RB\(^{hi}\) T cells from WT or Gzm\(b^{-}\) donor mice were adoptively transferred into Rag2\(^{-}\) or Rag2\(^{-}\)Gzm\(b^{-}\) recipient mice. (a) Mice were weighed weekly, and (b) monitored for...
signs of disease. Twenty-one days after transfer, (c) colon pathology was scored (left, representative micrographs, 100X magnification; right, data summary), and (d) donor-cell reconstitution of the IEL compartment in the colon was determined. For (a), dotted lines represent untreated \( \text{Rag}2^{-/-} \) and \( \text{Rag}-2^{-/-}\text{Gzmb}^{-/-} \) mice. Each dot represents an individual mouse. Data are representative of at least 3 independent experiments. \( n=8-10 \). For (a), **P<0.01; ****P<0.0001; Student’s t-test for each time point. For (b), # compares WT > \( \text{Rag}2^{-/-} \) to \( \text{Gzmb}^{-/-} \) > \( \text{Rag}2^{-/-} \) and WT > \( \text{Gzmb}^{-/-}\text{Rag}2^{-/-} \) at 14 days, and ## compares WT > \( \text{Rag}2^{-/-} \) to \( \text{Gzmb}^{-/-} \) > \( \text{Rag}2^{-/-} \) and WT > \( \text{Gzmb}^{-/-}\text{Rag}2^{-/-} \) at 21 days; Two-way ANOVA. For (c) and (d), *P<0.05; ***P<0.001; One-way ANOVA.

**Figure 3.** Granzyme B-deficient CD4\(^+\) T cells present skewed IL-17 differentiation in vivo.

1x10\(^5\) CD4\(^+\)CD45RB\(^hi\) T cells from WT or \( \text{Gzmb}^{-/-} \) donor mice were adoptively transferred into \( \text{Rag}2^{-/-} \) recipient mice. Three weeks after transfer, donor-derived cells were recovered from (a) MLN, and (b) lamina propria, and their IFN\(\gamma\)/IL-17 profile was determined by intracellular staining. Dot plots indicate a representative sample. Live, TCR\(\beta\)\(^+\)CD4\(^+\) cells are displayed. Graphs represent the summary. Each dot indicates an individual mouse. For MLN, \( n=11-13 \); for lamina propria, \( n=7-11 \). Data are representative of at least 3 independent experiments. **P<0.01; ****P<0.001. Student’s t-Test.

**Figure 4.** Granzyme B-deficient CD4\(^+\) T cells activated in vivo present a distinct gene expression profile. 1x10\(^5\) CD4\(^+\)CD45RB\(^hi\) T cells from WT or \( \text{Gzmb}^{-/-} \) donor mice were adoptively transferred into \( \text{Rag}2^{-/-} \) recipient mice. Three weeks after transfer, donor-derived cells were recovered from the MLN, sorted for live TCR\(\beta\)\(^+\)CD4\(^+\), their RNA isolated and sequenced. (a) Representative heat map. (b) Representative gene set enrichment analysis. (c) Real-time PCR validation of selected genes. \( n=6-7 \). *P<0.05; **P<0.01. Student’s t-Test.

**Figure 5.** Granzyme B-deficient CD4\(^+\) T cells present greater in vivo reconstitution.

1x10\(^5\) CD4\(^+\)CD45RB\(^hi\) T cells from WT (CD45.1) and \( \text{Gzmb}^{-/-} \) (CD45.2) donor mice were co-transferred into the same \( \text{Rag}2^{-/-} \) recipient mice. As controls, cells from the same donors were independently transferred into \( \text{Rag}2^{-/-} \) recipient mice. Three weeks after transfer, (a) MLN reconstitution (right, representative dot plot displaying live, TCR\(\beta\)\(^+\)CD4\(^+\) cells; left, summary),
and (b) IFNγ/IL-17 production was determined. (c) Rag2−/− mice that received CD4+ T cells from both WT/Gzmβ−/− or only Gzmβ−/− mice were weighed weekly. (d) Colon pathology was determined 3 weeks post transfer. Each dot represents an individual mouse. For (a) to (c), n=8-10; for (e) to (g), n=5-11. Data are representative of at least 2 independent experiments. **P<0.01; Student’s t-Test.

Figure 6. Naïve granzyme B-deficient mice present a normal CD4+ T cell compartment. (a) CD4+ T cell frequencies and cellularity from spleen, MLN, and IEL compartment were determined by flow cytometry. (b) Ex vivo IFNγ and IL-17 production from CD4+ T cells derived from naïve WT and Gzmβ−/− mice. Cells were non-stimulated (ns) or stimulated (s) for 4 h with PMA/ionomycin. Each dot represents an individual mouse. For (a), spleen and MLN, n=6-10; for IEL, n=12-13. For (b), n=6.

Figure 7. Granzyme B-deficient mice present severe disease after *C. rodentium* infection. Indicated mice were orogastrically infected with *C. rodentium* (5x10^8 CFU/mouse) and monitored for (a) weight change, and (b) signs of disease. At 14 days post infection, colons were dissected, (c) colonization/g of colon was determined, and (d) pathology scored as indicated in the Methods section; left, representative micrographs (200X magnification); right, data summary. (e) CD4+ T cell IFNγ and IL-17 expression in the indicated organs was determined. Cells were non-stimulated (ns) or stimulated (s) for 4 h with PMA/ionomycin. Each dot represents an individual mouse. For (a-d), n=7-9; for (e), 3-5. Data are representative of at least 3 independent experiments. For (a): **P<0.01; ***P<0.01; ****P<0.001; Student’s t-Test for each time point. For (b): ****P<0.001; Student’s t-Test for each time point. For (d) and (e): *P<0.05; **P<0.01; One-way ANOVA.

Supplemental Figure 2. (a) Absence of adaptive immune cells rescues granzyme B-deficient mice from severe disease. The indicated mice were orogastrically infected with *C. rodentium* (5x10^8 CFU/mouse) and monitored for weight change for 14 days. Data are representative of at least 2 independent experiments. n=3-4 for sham groups. n=5-7 for infected mice. (b) Granzyme B deficiency does not alter disease progression in the anti-CD40 model of colitis. The indicated
mice were treated i.p. with 150 μg/mouse and weighed daily. Data are representative of at least 3 independent experiments. n=2 for sham groups. n=14 for treated group.
Figure 2

(a) Weight change (%)

(b) Disease severity (max 12)

(c) Pathology

(d) Donor cell (%)
Figure 3

(a) MLN

WT > Rag2<sup>−/−</sup> Gzmb<sup>−/−</sup> > Rag2<sup>−/−</sup>

IL-17

IFN<sub>γ</sub>

CD4<sup>+</sup>IFN<sub>γ</sub><sup>+</sup> cells (%)

CD4<sup>+</sup>IL-17<sup>+</sup> cells (%)

(b) LP

WT > Rag2<sup>−/−</sup> Gzmb<sup>−/−</sup> > Rag2<sup>−/−</sup>

IL-17

IFN<sub>γ</sub>

CD4<sup>+</sup>IFN<sub>γ</sub><sup>+</sup> cells (%)

CD4<sup>+</sup>IL-17<sup>+</sup> cells (%)

** Figure 3 **
Figure 5

(a) Flow cytometry analysis showing donor cell (CD45.2) and WT Gzmb+ frequencies.

(b) Comparison of CD4+ IFN-γ+ (Δ) and CD4+ IL-17+ (○) cell percentages among WT, Gzmb−/−, WT only, and Gzmb−/− only conditions.

(c) Weight change over time after cell transfer.

(d) Pathology assessment.

Figure 5
Figure 6
Figure 7

- **a**
  - Weight change (%)
  - Days after infection
  - WT sham
  - WT C. rod
  - Gzmb-/- sham
  - Gzmb-/- C. rod

- **b**
  - Disease severity (max 12)
  - Days after infection
  - WT C. rod
  - Gzmb-/- C. rod

- **c**
  - CFU/g of tissue
  - WT sham
  - WT C. rod
  - Gzmb-/- sham
  - Gzmb-/- C. rod

- **d**
  - Sham
  - C. rodentium
  - WT
  - Gzmb-/-

- **e**
  - Spleen
  - MLN
  - LP
  - CD4+IFN-γ+ cells (%)
  - CD4+IL-17+ cells (%)
  - WT s
  - WT us
  - Gzmb-/- s
  - Gzmb-/- un