

Oxazolone Colitis, a Th2 Colitis Model Resembling Ulcerative Colitis, Is Mediated by IL-13-Producing NK-T Cells

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Summary

Oxazolone colitis (OC) is an experimental colitis that has a histologic resemblance to human ulcerative colitis. Here we show that IL-13 production is a significant pathologic factor in OC since its neutralization by IL-13R α 2-Fc administration prevents colitis. We further show that OC is mediated by NK-T cells since it can be induced neither in mice depleted of NK-T cells nor in mice that cannot present antigen to NK-T cells and mice lacking an NK-T cell-associated TCR. Finally, we show that NK-T cells are the source of the IL-13, since they produce IL-13 upon stimulation by α -galactosylceramide, an NK-T cell-specific antigen. These data thus describe a cellular mechanism underlying an experimental colitis that may explain the pathogenesis of ulcerative colitis.

Introduction

The human inflammatory bowel diseases (IBD), Crohn's disease (CD), and ulcerative colitis (UC) are believed to be due to an abnormal mucosal T cell responsive to bacterial antigens contained within the gut lumen (Sartor, 1995). In CD, the responding T cells exhibit a Th1 phenotype and thus produce large amounts of interferon- γ (IFN- γ) and tumor necrosis factor (TNF- α); in addition, IL-12 secretion, the driving force of Th1 differentiation, is increased (Parronchi et al., 1997). In UC, the responding T cell is less well defined. In this case, whereas Th1 cytokine production is normal or decreased and some Th2 cytokine production (IL-5 and IL-10) is increased, the production of the signature cytokine associated with the Th2 response, IL-4, is not increased (Fuss et al., 1996). Despite this discrepancy, there is a reason to believe that UC is a Th2-mediated disease; several murine models of mucosal inflammation that are clearly due to excess IL-4 secretion resemble UC on a histopathologic level, whereas the many models due to

Th1 cytokines do not (Boirivant et al., 1998; Mizoguchi et al., 1999).

One such Th2 model is oxazolone colitis, a recently described colitis induced by the intrarectal administration of the haptening agent oxazolone in an ethanol vehicle. In this model, the initial toxic effects of the inducing agent leads to a flooding of the lamina propria with oxazolone and oxazolone-modified bacterial antigens and thus the induction of an immune response that leads to further inflammation. The latter is driven by the production of Th2 cytokines since the inflammation is characterized by increased IL-4 and IL-5 secretion, and the inflammation can be ameliorated by the administration of anti-IL-4 monoclonal antibodies (Boirivant et al., 1998).

The mechanisms by which luminal antigens stimulate an inflammatory immune response in the mucosa in oxazolone colitis and, by extension, in human ulcerative colitis, has until now been unclear. One possibility is that the inflammation is mediated by typical MHC class II-reactive CD4⁺ T cells that have been shown to play a major role in Th1 colitides. Another and, at first glance, more unlikely possibility is that the inflammation is mediated by NK-T cells, i.e., a normally minor T cell population that bears both T cell and NK cell markers and expresses an invariant T cell receptor- α chain (V α 14J α 281 in mice; V α 24J α 18 in humans) and a restricted repertoire of V β chains (Lantz and Bendelac, 1994). Additional characteristics of NK-T cells are that they are either CD4⁺ or CD4⁻ or CD8⁻ cells and that they recognize antigens that are presented by CD1 (CD1d in humans), which is a highly conserved MHC class I-like molecule (Balk et al., 1989) expressed on dendritic cells, macrophages, B cells (Blumberg et al., 1991; Roark et al., 1998), thymic epithelium (Bendelac, 1995), and gastrointestinal epithelial cells (Bleicher et al., 1990; Blumberg et al., 1991). While the natural antigens that are presented by CD1 in vivo are not known, NK-T cells can be stimulated in vitro and in vivo by glycolipids that bind to CD1, such as the prototype or model NK-T cell glycolipid antigen α -galactosylceramide (α GalCer) (Gumpertz et al., 2000; Spada et al., 1998; Saubermann et al., 2000). Finally, activation of NK-T cells via CD1-presented antigen induces rapid NK-T cell release of Th1 and Th2 cytokines, and then apoptosis followed by expansion with continuous antigen exposure (Chen and Paul, 1997; Eberl and MacDonald, 1998; Hayakawa et al., 1992; Yoshimoto and Paul, 1994).

We report here that oxazolone colitis is in fact mediated by NK-T cells and that these T cells are capable of producing large amounts of Th2 cytokines when stimulated by anti-CD3/anti-CD28 or in a CD1-restricted fashion by α GalCer. Initially, this consists of IL-4 secretion, but the latter is rapidly superceded by IL-13 secretion. The IL-13 response appears to be a key pathogenic component of the colitis as IL-13 neutralization by IL-13R α 2-Fc administration prevents its development. Given the resemblance of oxazolone colitis in mice to UC in humans, these data have important implications for the understanding and treatment of human IBD.

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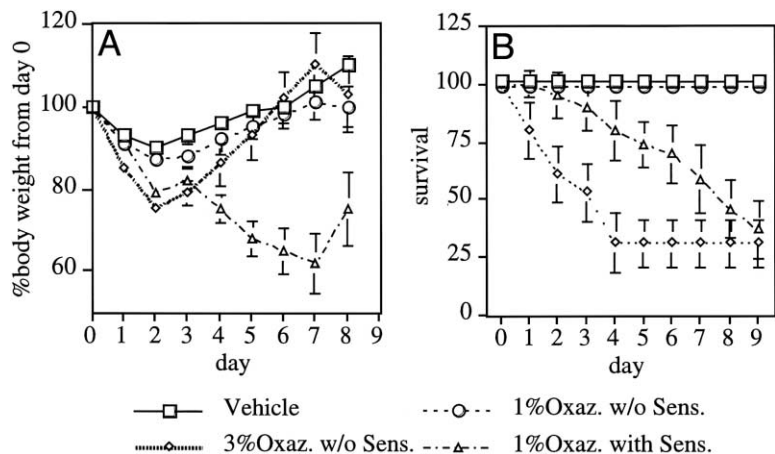
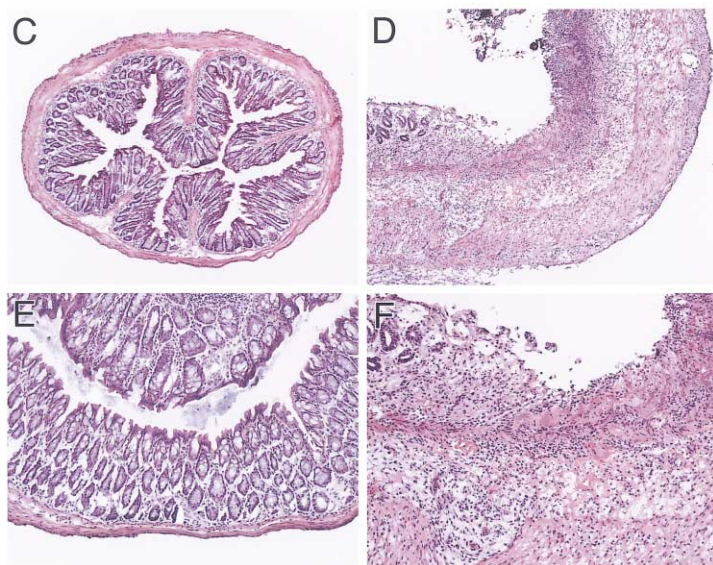


Figure 1. Presensitization before Intrarectal Challenge with Oxazolone Leads to a Chronic Progressive Colitis

Weight loss (A) and mortality (B) of mice after presensitization with vehicle (ethanol) or oxazolone and intrarectal challenge with vehicle or different doses of oxazolone. Each study group analyzed contained \geq ten mice. Data shown represent mean values obtained from three independent experiments. Photomicrograph of an H/E-stained section of colon from a control mouse presensitized and challenged intrarectally with ethanol alone at 7 days after intrarectal challenge, 5 \times (C) and 10 \times (E). Photomicrograph of an H/E-stained section of colon from a mouse presensitized with 3% oxazolone and challenged intrarectally with 1% oxazolone at 7 days after intrarectal challenge, 50 \times (D) and 100 \times (F).



Results

Epicutaneous Presensitization and Intrarectal Rechallenge with Oxazolone Leads to Chronic Progressive Oxazolone Colitis Associated with Th2 Cytokine Production

We have shown previously that intrarectal challenge of naive mice with 3% oxazolone elicits a Th2 response in the lamina propria of the colon, known as oxazolone colitis (Boirivant et al., 1998). However, this dose is highly toxic and produces an accelerated inflammation lasting 3–4 days that leads either to rapid recovery or, alternatively, to death of the mice. To obtain a more long-lived, chronic inflammatory response, we presensitized C57Bl/10 mice with 3% oxazolone by skin painting 5 days before rectal challenge and then administered 1% oxazolone intrarectally to induce colitis. As shown in Figures 1A and 1B, presensitized mice administered 1% oxazolone developed colitis marked by sustained weight loss sometimes culminating in death; nonpresensitized mice given this intrarectal dose had a weight loss

similar to mice administered vehicle (i.e., ethanol) alone. In addition, while nonpresensitized mice administered 3% oxazolone appeared to have only a transient weight loss, this was in reality due to early death of most of the mice and selective survival of a minority that had only mild transient disease. As shown in Figures 1C–1F, histological examination of mouse colons at days 7–10 showed that presensitized mice developed massive bowel wall edema and dense infiltration of the superficial layers of the mucosa with small polynuclear granulocytes; in addition, the epithelial cell layer was disrupted by large ulcerations. This histopathological picture is similar to that seen in human UC and suggests that a similar pathological mechanism contributes to tissue damage in both types of inflammation. Finally, as shown in Figure 2A, mononuclear cells isolated from the lamina propria (LPMC), mesenteric lymph nodes (MLNC), or spleen (SPC) of mice with oxazolone colitis produced large amounts of Th2 cytokines (IL-4, IL-5, IL-13) but only low levels of IFN- γ upon stimulation with anti-CD3 and anti-CD28 antibodies in vitro. In contrast, LPMC

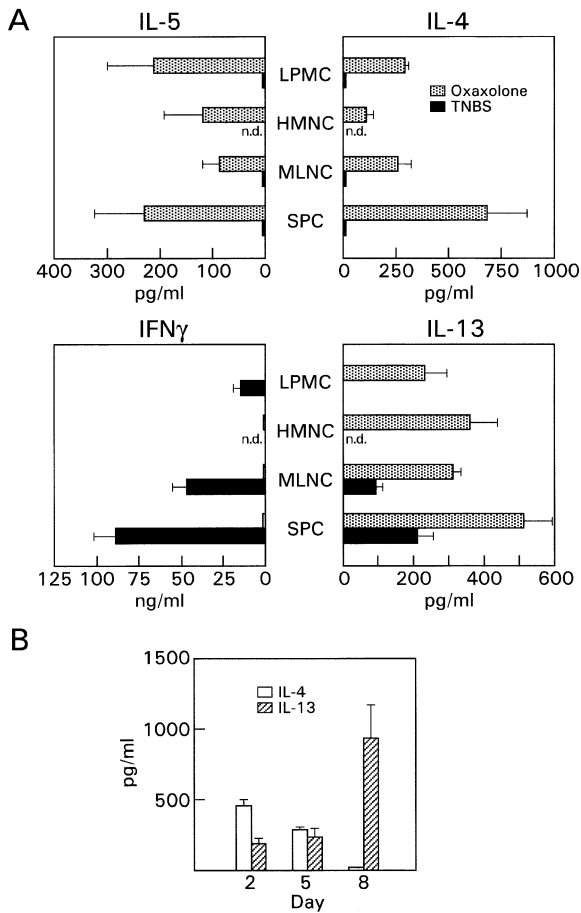


Figure 2. Cytokine Production from Lymphocytes from Mice with Oxazolone Colitis

(A) Lamina propria mononuclear cells (LPMC), hepatic mononuclear cells (HMNC), mesenteric lymph node cells (MLNC), and splenocytes (SPC) were isolated on day 5 after induction of oxazolone colitis (stippled) or TNBS colitis (black) and stimulated *in vitro* for 48 hr with plate-bound anti-CD3 and soluble anti-CD28. Baseline values of LPMC for IL-4 and IL-13 of ethanol-treated mice were nondetectable. The data shown represent the mean values obtained from three independent experiments. All cytokine values obtained with cells from mice with oxazolone colitis were significantly different from cells from mice with TNBS colitis ($p \leq 0.01$).

(B) LPMC were isolated on days 2, 5, or 8 after induction of oxazolone colitis. LPMC were stimulated as above, and the concentrations of IL-4 (open bars) and IL-13 (striped bars) were measured in the supernatants. The data show IL-4 and IL-13 values on day 8 as compared to day 2 in LPMC; $p < 0.01$. The data shown represent the mean values obtained from three independent experiments.

isolated from C57BI/10 mice with TNBS colitis produced undetectable levels of IL-4 and IL-5 and only low levels of IL-13 upon stimulation under the same conditions.

IL-13 Production in Oxazolone Colitis Increases during the Course of Inflammation and Is Essential for the Induction of the Colitis

In further studies of cytokine secretion in oxazolone colitis shown in Figure 2B, we found that IL-4 production by LPMC isolated at different time points during the course of the colitis gradually decreased. In contrast,

IL-13 production by LPMC (as well as MLNC or SPC) during the same time frame increased. This phenomenon has been observed in other animal models of inflammation mediated by Th2 cells (Minty et al., 1997; Urban et al., 1998). To establish a pathogenic role for IL-13 in oxazolone colitis, we neutralized IL-13 by *in vivo* administration of IL-13R α 2 fused to the Fc portion of human IgG1 (IL-13R α 2-Fc) at the time of intrarectal oxazolone administration. This fusion protein binds to IL-13 with high affinity and neutralizes IL-13 bioactivity *in vitro* and *in vivo* (Donaldson et al., 1998; Urban et al., 1998). As shown in Figure 3, mice treated with IL-13R α 2-Fc were protected from the induction of oxazolone colitis; after an initial transient weight loss similar to that observed with administration of ethanol alone, IL-13R α 2-Fc-treated mice rapidly regained their initial body weight, and by day 5 colonic histology was indistinguishable from that of mice that were given ethanol alone (data not shown).

NK1.1-Positive Cells Are Essential for the Induction of Oxazolone Colitis

As described above, mononuclear cells isolated from mice with oxazolone colitis produce increased amounts of IL-13 *in vitro* when stimulated with anti-CD3 and anti-CD28. However, when we purified these mononuclear cells by a negative selection column to enrich for CD3-positive cells (see Experimental Procedures), our ability to stimulate IL-13 production was greatly diminished (data not shown). Since the selection column contains glass beads coated with anti-mouse IgG, it has the potential to retain cells that express Fc γ -receptors (CD16 or CD32) that are coated with immunoglobulins; thus, it seemed possible that IL-13 production by anti-CD3-stimulated LPMC in oxazolone colitis requires a Fc γ -receptor-positive cell. In addition to mast cells and B cells, NK and NK-T cells express CD16 (Koyasu, 1994) and are capable of producing IL-13 (Terabe et al., 2000). To investigate whether either of the latter two cell types are involved in oxazolone colitis we depleted mice of NK1.1 cells by repeated injection of a monoclonal anti-NK1.1 antibody (PK136) before challenge with oxazolone (see Experimental Procedures). Such treatment depleted all NK and NK-T cells, as determined by DX5 and NK1.1 staining of splenocytes (data not shown). As shown in Figures 4A and 4B, we found that mice depleted of these cell types did not develop weight loss or macroscopic/microscopic evidence of colonic inflammation and did not manifest increased Th2 cytokine production after intrarectal administration of oxazolone (data not shown).

In further studies to determine if this pathogenic role for NK1.1-positive cells is specific for oxazolone colitis, we compared NK1.1-depleted C57BI/10 mice with untreated C57BI/10 mice in their susceptibility to TNBS colitis, a Th1 model of colitis resembling human Crohn's disease. As shown in Figures 4C and 4D, depletion of NK1.1-positive cells did not significantly influence weight loss or mortality of mice with TNBS colitis. On the contrary, there was a trend toward greater weight loss in depleted mice. These results suggest that cells bearing NK1.1 in the mucosa play, if anything, an inhibitory role

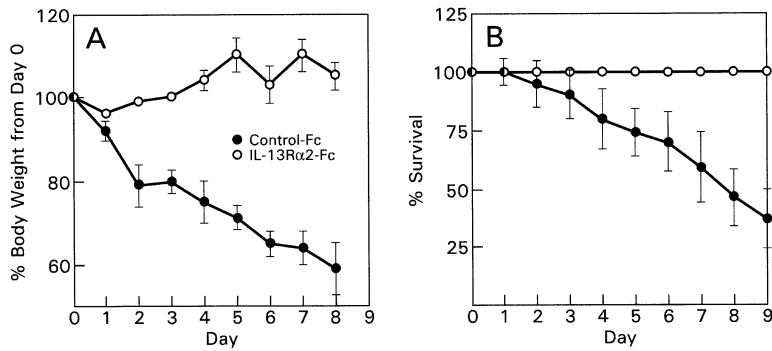


Figure 3. Neutralization of IL-13 Prevents Induction of Oxazolone Colitis

(A) Weight loss and (B) survival in mice pre-sensitized with 1% oxazolone, intrarectally challenged with 1% oxazolone, and then treated with IL13R α 2-Fc or control protein at the time of intrarectal challenge. Each study group analyzed contained \geq ten mice. The data shown represent the mean values obtained from three independent experiments.

for the induction of a Th1-associated inflammation such as that occurring in TNBS colitis, an effect previously noted in other models of Th1-mediated intestinal inflammation (Saubermann et al., 2000).

NK-T Cells Are Required for the Induction of Oxazolone Colitis

Whereas the above studies show that oxazolone colitis is mediated by NK1.1-positive cells, they do not provide information on whether the latter cells are NK or NK-T cells, as the NK1.1 antigen is present on both of these cell types. In an initial set of studies addressing this question, we determined if we could induce oxazolone colitis in β 2m-deficient mice, recognizing that NK-T cells (but not NK cells) require antigen presentation by an atypical class I MHC molecule (Zhang et al., 1996). As shown in Figures 5A–5D, despite the fact that the background strain of the β 2m mice used in these studies is relatively resistant to the development of this form of colitis, the β 2m-deficient mice were clearly free of colitis following administration of oxazolone per rectum as measured by weight loss, survival, and microscopic ex-

amination of lamina propria, whereas littermate controls developed moderately severe disease. These results thus provide strong evidence that oxazolone colitis is mediated by T cells responding to antigens presented by class I MHC molecules, including NK-T cells.

In further studies we focused on whether oxazolone colitis depends on interactions specific for NK-T cells rather than all class I-restricted cells. In one approach to this question, we determined whether oxazolone colitis was affected by blockade of antigen presentation by the atypical MHC class I molecule, CD1, i.e., the presenting molecule directly involved in NK-T cell activation. This was accomplished by administration of a monoclonal anti-CD1 antibody that has been shown to block CD1 in vivo without depleting NK-T cells and without affecting antigen presentation by MHC class II (Park et al., 1998). As shown in Figure 6, administration of this antibody at the time of intrarectal oxazolone administration prevented the development of oxazolone colitis.

In another complementary approach to the same question, we conducted studies of CD1-deficient mice that both lack the ability to present antigens to NK-T

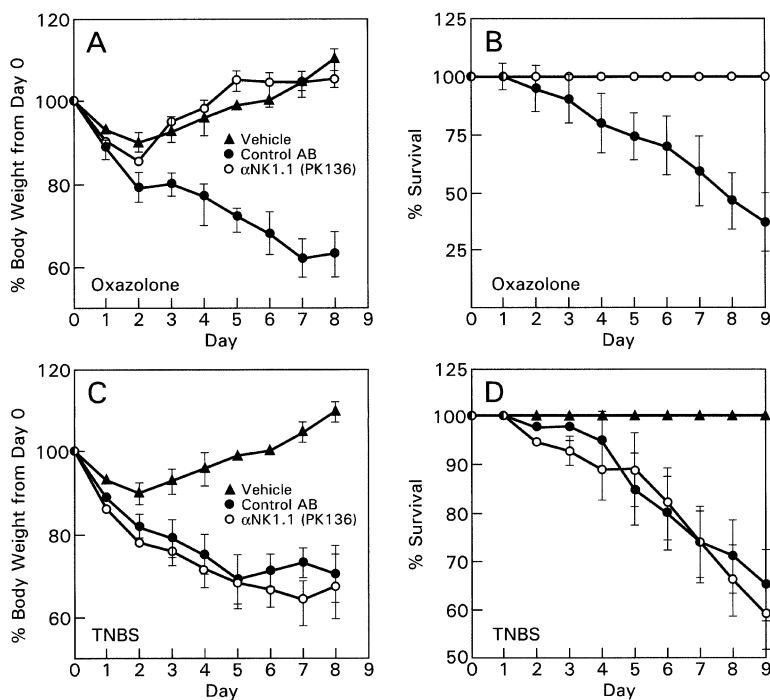


Figure 4. Depletion of NK1.1-Positive Cells Protects Mice from Oxazolone Colitis but Not TNBS Colitis

Weight loss (A and C) and mortality (B and D) after induction of oxazolone colitis (A and B) or TNBS colitis (C and D) or injection of vehicle (ethanol). Mice were injected with control antibody or depleted of NK1.1 cells with anti-NK1.1 (PK136). Each study group analyzed contained \geq ten mice. The data shown represent the mean values obtained from three independent experiments.

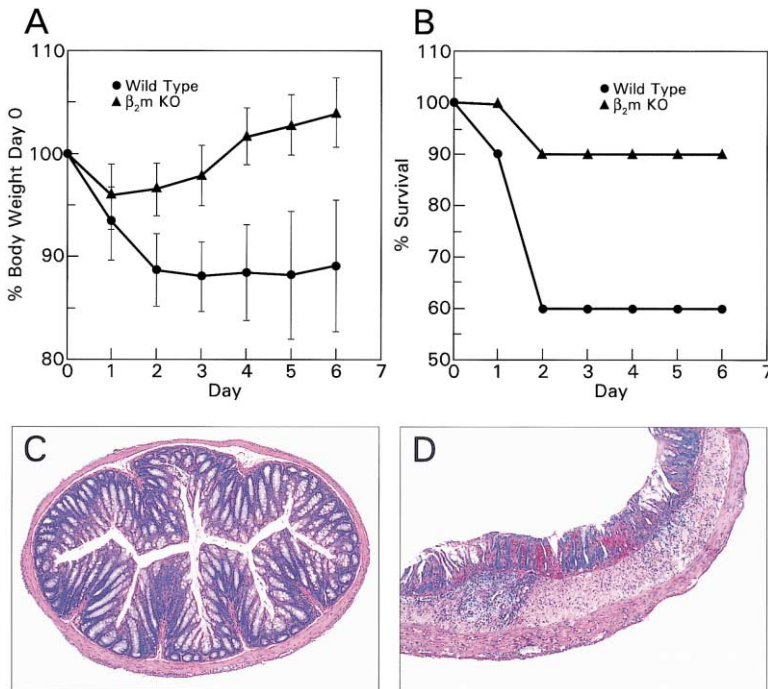


Figure 5. β_2m -Deficient Mice Are Resistant to the Development of Oxazolone Colitis

β_2m -deficient mice (B6/SJL background) and their wild-type littermates (B6/SJL) after induction of oxazolone-colitis. Wild-type mice but not β_2m -deficient mice lost weight and had poor survival (A and B). In addition, whereas β_2m -deficient mice did not manifest microscopic evidence of inflammation (C), wild-type mice manifested inflammation characterized by epithelial ulceration, inflammatory cell infiltration of the mucosal superficial layers, and edematous bowel wall (D). It should be noted that the level of inflammation developing in these wild-type mice was milder than that observed in B10 wild-type mice because the B6/SJL strain is relatively resistant to oxazolone-colitis. Each study group analyzed contained ten mice per group.

cells and lack NK-T cells. As shown in Figure 6, we showed that intrarectal administration of oxazolone to presensitized CD1-deficient mice does not result in the development of colitis as manifested by weight loss or (in data not shown) by histologic changes in colonic tissue. Despite the fact that CD1-deficient mice lack NK-T cells, they have been shown to be fully capable of mounting certain types of Th2 responses (Smiley et al., 1997); thus, this result cannot be attributed to an intrinsic failure of the CD1-deficient mice to mount a Th2 response. In parallel studies also shown in Figure 6, we showed that $J\alpha 281$ -deficient mice that lack NK-T cells expressing the invariant TCR associated with these cells are also resistant to the induction of oxazolone colitis as manifested by weight loss or (in data not shown) by histologic changes in colonic tissue. While most CD1-restricted NK-T cells utilize this invariant TCR, there are some data that suggest the existence of NK-T cells with other TCRs. However, even if these atypical NK-T cells are present in the $J\alpha 281$ -deficient mouse, they are nevertheless insufficient to induce the inflammatory response mediating oxazolone colitis. Thus, it appears likely that this inflammation is in fact mediated by NK-T cells expressing the invariant TCR and that the NK-T cells are $NK1.1^+J\alpha 281^+CD16^+CD4^+$ cells.

NK-T Cells Undergo Expansion during Oxazolone Colitis

In further studies we sought to determine the extent to which the lamina propria of mice with oxazolone colitis is infiltrated with NK-T cells. However, this goal was complicated by the fact that whereas NK1.1 is a frequent marker of NK-T cells, T cells with NK-T cell function have also been identified in the NK1.1-negative population (Hameg et al., 1999). In addition, most NK-T cell markers are dependent on the level of cellular activation. Thus,

while NK-T cells lose their expression of NK1.1 upon activation (Chen and Paul, 1997) another NK/NK-T cell marker, Ly49C, is upregulated on activated NK-T cells. With these limitations in mind, we observed that during the course of oxazolone colitis total $CD3^+$ lymphocyte numbers isolated from mice on day 5 after induction of colitis undergo substantial increases in the lamina propria (10-fold increase) and spleen (2-fold increase). Moreover, in the lamina propria and in the spleen the relative number of NK-T cells increases to a greater extent than other cell populations. Thus, while in the lamina propria of untreated mice, 7% (NK1.1) or 0.4% (Ly49C) of the $CD3^+$ T cells coexpress an NK-T cell marker; in the lamina propria of mice with oxazolone colitis, 21% of the infiltrating T cells express NK1.1 and 34% express Ly49C. Similarly, in the liver, where the highest percentage of NK-T cells can be found, NK1.1 expression on $CD3^+$ T cells increases from 9.9% to 48% of cells. Finally, while in the spleen of untreated mice 3.1% of $CD3^+$ cells express NK1.1⁺, in the spleen of mice with oxazolone colitis 5.1% express NK1.1, and the number of Ly49C-expressing cells increases from 0.6% to 28%. To summarize these findings, T cells with surrogate markers of NK-T cell function undergo a substantial expansion in the lamina propria, the liver, and the spleen during the course of oxazolone colitis.

$CD4^+$ NK-T Cells Produce IL-13 in Response to CD1-Restricted Antigen Presentation

Finally, to investigate the cytokine production of the LPMC and SPC in response to stimulation by antigen presented by CD1, we stimulated these cells with $\alpha GalCer$, a synthetic glycolipid that has been found to activate most invariant NK-T cells in a CD1-dependent fashion (Kawano et al., 1997). MHC-class II-restricted T cells and NK cells are not activated by $\alpha GalCer$; thus,

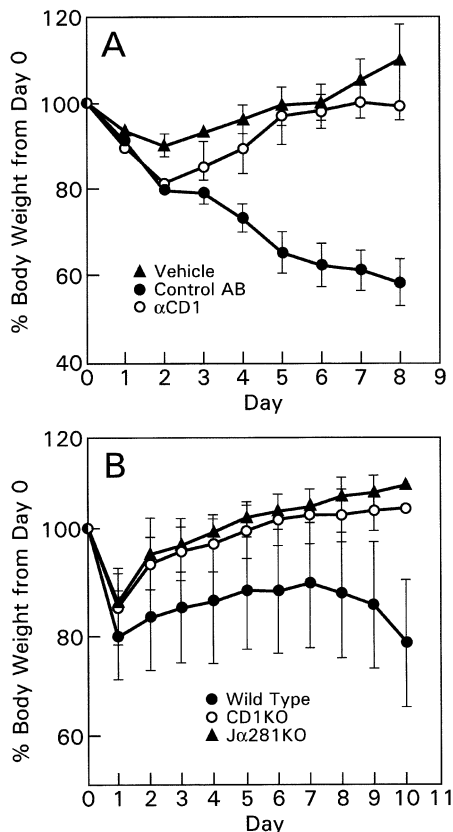


Figure 6. CD1 Antigen Presentation and J α 281 NK-T Cells Are Essential for Induction of Oxazolone Colitis

(A) Weight loss of mice after intrarectal injection of ethanol or oxazolone after i.v. injection of blocking CD1 antibodies (20H2) or control antibody. (B) Weight loss after induction of oxazolone colitis of CD1^{-/-} mice, J α 281^{-/-} mice, and wild-type mice. Each study group analyzed contained \geq ten mice. Weight loss and/or graded histologic changes were statistically significant for both groups of knock-out mice versus wild-type mice (at least a p value \leq 0.03). Both the CD1^{-/-} and J α 281^{-/-} mice were backcrossed onto the BL/6 background (CD1^{-/-} [N10] and J α 281^{-/-} [N6]).

stimulation with α GalCer represents a way to assess NK-T cell activation in unseparated cell mixtures. As shown in Figure 7, when LPMC or SPC from mice with oxazolone colitis were stimulated with α GalCer they produced large amounts of Th2 cytokines, including very high quantities of IL-13. In addition, CD4-positive cells isolated by MACS purified from LPMC or SPC also responded to α GalCer with very high IL-13 production, indicating that many of these CD4⁺ cells in cell populations from mice with oxazolone colitis are CD1-restricted NK-T cells.

Discussion

In the present study we describe an animal model of colitis mediated by Th2 NK-T cells producing IL-13. The colitis was induced by intrarectal administration of a relatively low dose of oxazolone subsequent to skin sensitization with oxazolone. A highly reproducible and chronic colonic inflammation was obtained that,

upon thorough histologic assessment, proved to be similar in several respects to human UC. Skin exposure to oxazolone has previously been shown to induce CD4⁺ T cell-mediated delayed hypersensitivity upon secondary skin challenge; here we show that such exposure also sensitizes mice to a secondary response in the gastrointestinal mucosa. It should be noted, however, that whereas the reaction in the skin is mild and self-limited, it is severe and progressive in the mucosa. This is probably due to the fact that the antigenic milieu of the colon is far different from that in the skin and leads to the stimulation of different effector cells and/or regulatory cells. While the inflammation developing in the colon in oxazolone colitis is similar to a previously reported hapten-induced colitis, TNBS colitis (Neurath et al., 1995), in that it is a CD4⁺ T cell-driven response, it differs from TNBS colitis by the fact that the CD4⁺ T cells produce Th2 rather than Th1 cytokines. Moreover, invariant NK-T cells responding to CD1-restricted antigens rather than conventional T cells responding to MHC class II-restricted antigens appear to be the major cell type responsible for this Th2 cytokine secretion.

The pathogenic pathway leading to tissue injury in TNBS colitis and by extension, in Crohn's disease, can with some confidence be attributed to production of Th1 cytokines such as IFN- γ and TNF- α . These effector molecules induce tissue injury via their activation of downstream effector cells and the resulting secretion of proteases and proapoptotic factors. On the other hand, the pathogenic pathway leading to tissue injury in oxazolone colitis is less clear in that Th2 cytokines (e.g., IL-4, IL-13, IL-10) have been shown to have a generally downmodulatory effect on MHC class II expression by antigen-presenting cells and would thus tend to inhibit activation of T cells (Brown and Hural, 1997; Fiorentino et al., 1991; Moore et al., 1993; Zurawski and de Vries, 1994); in addition, these cytokines would also inhibit downstream effector cells. However, the finding that NK-T cells within lamina propria expand and are essential for disease induction in oxazolone colitis can help explain how a severe progressive inflammation can evolve in spite of these inhibitory effects of Th2 cytokines. One factor that comes into play as a possible basis of NK-T cell-mediated tissue injury is that NK-T cells are activated by antigen in the context of CD1 (CD1d in humans), a molecule widely expressed in the colonic epithelium and dendritic cells (Blumberg et al., 1991; Spada et al., 2000). It is probable that such activation initially involves recognition of antigens associated with CD1 on dendritic cells; however, as the inflammation proceeds there is likely to be disruption of the normal barrier between epithelial cells and lamina propria T cells which leads to activation of NK-T cells by antigen/CD1 on intestinal epithelial cells (R.S.B., unpublished data). More importantly, at this stage, epithelial cells can become cytolytic targets of NK-T cells that have been previously activated by dendritic cells. The end result is loss of epithelial cells, the formation of epithelial ulcers, and the intensification of inflammation due to entry of organisms that can now breach the epithelial barrier. This situation is similar to that described in recent studies of OVA-specific CD8⁺ T cells transferred to mice expressing ovalbumin in the intestinal epithelium. In this study, the transferred cells initially expand

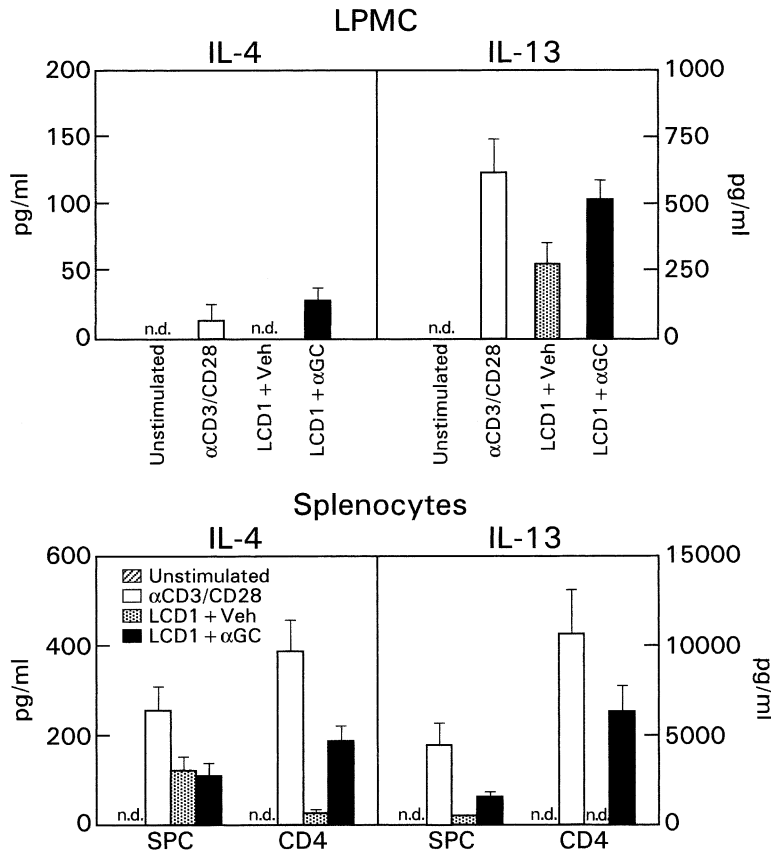


Figure 7. Cytokine Production in Response to α GalCer

LPMC (upper panel), splenocytes, or spleen CD4 cells (lower panel) were isolated on day 8 after induction of oxazolone colitis and either stimulated or not stimulated (unstimulated) for 48 hr with plate-bound anti-CD3 and soluble anti-CD28 (α CD3/CD28), untransfected L cells (LCD1) and vehicle (α GalCer Solvent), or CD1 transfected L cells and 100 ng/ml α GalCer (LCD1 and α GC). Each study group analyzed contained \geq ten mice. The data shown represent the mean values obtained from three independent experiments. IL-13 secretion levels induced by α GalCer in CD4 cells from mice without oxazolone colitis were below the limit of detection.

and then are deleted unless the mouse is systemically immunized with VZV-ovalbumin, in which case extensive cytolytic destruction of the epithelium occurs along with gut wall inflammation (Vezys et al., 2000). Thus, if potentially cytolytic cells in the gut wall (in this case NK-T cells) are appropriately activated, they can cause extensive tissue injury. Finally, in recent studies of Con A-induced hepatitis, a model of autoimmune hepatitis also mediated by NK-T cells, it was shown that tissue injury is induced by release of perforin and granzymes as well as induction of Fas-mediated apoptosis (Kaneko et al., 2000). Similar cytolytic mechanisms causing lysis of epithelial cells by NK-T cells are likely to exist in oxazolone colitis.

A second factor that has a possible role in tissue injury mediated by NK-T cells in oxazolone colitis is that NK-T cells produce IL-13. Thus, in the studies presented here, we show that while IL-4 is the initial cytokine produced in oxazolone colitis, IL-4 is rapidly superseded by IL-13 production and that indeed when IL-13 is neutralized, the administration of oxazolone does not induce any histological changes or other signs of inflammation. This strongly suggests that IL-13 acts as an effector cytokine that mediates tissue injury in the mucosa. Precedent for this possibility comes first from studies of animal models of asthma, in which IL-13 has been shown to induce increased mucus secretion and airway hyperresponsiveness (Grunig et al., 1998; Wills-Karp et al., 1998). In addition, it has recently been shown that infusion of IL-25, a newly discovered cytokine, causes epithelial cell hyperplasia, mucus production, and infiltration of the

gastrointestinal tract with granulocytes and that this pathologic effect is probably mediated by induction of IL-13 secretion (Fort et al., 2001). In oxazolone colitis, IL-13 can conceivably activate epithelial cells to secrete mucus and fluid; in addition, this cytokine as well as IL-4 can lead to disruption of epithelial cell tight junctions (Ceponis et al., 2000) which, in turn, opens the way to invasion of bacteria. Finally, NK-T cells may indirectly require an IL-13 signal to proliferate, as IL-13 may also increase CD1 antigen presentation on epithelial cells; thus, IL-13 as such may exert a positive feedback effect on NK-T cell-mediated tissue injury.

Given the resemblance of oxazolone colitis to human UC at the histopathological level, the data presented here lead to the question of whether the mechanisms resulting in oxazolone colitis also underlie the human disease. It is known that UC is not a Th1 T cell-driven inflammation as is CD, yet it cannot readily be classified as a Th2 disease since IL-4 secretion is not increased (Fuss et al., 1996). Our finding that a Th2 colitis resembling UC can be mediated by NK-T cells producing IL-13 leads to the possibility that a similar mechanism underlies the human disease. This possibility is strengthened by the fact that both in ulcerative colitis and in established oxazolone colitis IL-4 production from T cells is not evident. If indeed NK-T cell-producing IL-13 accounts for inflammation in UC, it could explain the fact that inflammation in this disease involves continuous areas of the bowel rather than discontinuous areas as in CD. Thus, in UC, the inflammation usually starts in the rectum where the bacterial load is highest, and where an

underlying dysregulation of the immune response might therefore lead to the stimulation of NK-T cells by antigen-presenting cells expressing CD1d. The IL-13 secreted by activated NK-T cells could then spread the inflammation to neighboring tissues by upregulating CD1d and causing injury to the epithelial cell barrier in that tissue.

The studies reported here revealed certain difficulties in identifying NK-T cells in active inflammatory lesions which may impede their study in human disease states. We found that, whereas only a relatively small fraction of the CD3⁺ cells extracted from inflamed mucosal tissues expressed NK-T cell markers, purified CD4⁺ cells obtained in the same way responded vigorously to α GalCer, an antigen that only activates invariant NK-T cells. In addition, we found that pretreatment with anti-NK1.1 antibodies prevented oxazolone colitis, indicating that pathogenic NK-T cells must have been NK1.1-positive prior to disease induction. These discrepancies between observable NK-T numbers and NK-T cell function can be explained by the observation that NK-T cells lose their expression of NK1.1 after activation and that CD3 and NK1.1 only poorly define activated NK-T cells in disease states. Currently, the only conclusive method for identifying NK-T cells is staining with CD1d tetramers, and indeed such staining has recently identified both CD4⁺ and CD4⁻CD8⁻ NK-T cell subpopulations in humans. In addition, in accord with the data presented here, CD1d-tetramer staining revealed that in humans only CD4⁺ NK-T cells produce IL-13 (Lee et al., 2002). Finally, it should be noted that a subset of human NK-T cells express the canonical T cell receptor V α 24 which could also be used as a NK-T cell marker in humans. That this marker may indeed prove useful is supported by our finding that J α 281 knockout mice are resistant to oxazolone colitis, suggesting that the V α 14-positive CD1-reactive T cells expressing the invariant TCR- α chain associated with CD1 reactivity are involved in colitis induction.

NK-T cells that act as effector cells in oxazolone colitis have been shown to play a regulatory role in other models of murine inflammation (Ishikawa et al., 2000; Kumar et al., 2000; Sonoda et al., 1999; Strober et al., 1996). This may be due to their potential to downregulate Th1 inflammation via the production of Th2 cytokines. These findings suggest that a possible treatment of human disease mediated by Th1 cytokines is the stimulation of NK-T cells. Indeed, such an approach has already been developed to treat murine experimental allergic encephalitis and has proved to be effective (Miyamoto et al., 2001). It should be noted, however, that this mode of treatment might also be very harmful since, as shown here, Th2-cytokine producing NK-T cells can cause colitis and since others have shown that these cells can cause hepatitis (Takeda et al., 2000).

In summary, our data indicate that CD4⁺ NK-T cells producing IL-13 induce a progressive colitis resembling human UC. This model of inflammation can be effectively blocked by neutralizing the IL-13, depleting NK-T cells, or inhibiting the activation of NK-T cells through CD1. If any of these mechanisms is involved in the pathogenesis of human UC, they might represent a possible treatment option for this disease.

Experimental Procedures

Mice and In Vivo Treatment Protocols

Male C57Bl/10 mice were obtained from a breeding facility maintained by the National Cancer Institute (NCI, Bethesda, MD) and were housed under specific pathogen-free (SPF) conditions. Five- to seven-week-old mice were used for all experiments. C57Bl/6-CD1^{-/-} mice were a generous gift from M. Exley (Spada et al., 1999); C57Bl/6-J α 281^{-/-} mice have been described previously (Cui et al., 1997) and were bred at the animal facility of Beth Israel Deaconess Medical Center, Harvard Medical School (Boston, MA) (kindly provided by Dr. M. Exley). B2m-deficient B6/SJL and wild-type B6/SJL mice were purchased from Taconic Farms animal facility. Oxazolone (4-ethoxymethylene-2-phenyl-2-oxazoline-5-one) was obtained from Sigma-Aldrich (St. Louis, MO). In order to presensitize mice, a 2 × 2 cm field of the abdominal skin was shaved, and 200 μ l of a 3% (w/v) solution of oxazolone in 100% ethanol was applied. 5 days after presensitization mice were rechallenged intrarectally with 150 μ l 1% oxazolone in 50% ethanol or only 50% ethanol (i.e., vehicle) under general anesthesia with isoflurane (Baxter, Deerfield, IL). Intrarectal injection was administered with a polyurethane umbilical catheter (Sherwood, St. Louis, MO). Neutralization of IL-13 in vivo was performed with IL-13R α 2-Fc (gift of Deb Donaldson, Wyeth Research, Cambridge, MA). Mice received 5 × 200 μ g of IgG2a (control Ig) or IL-13R α 2-Fc starting on the day before presensitization i.v. and then every other day i.p. Depletion of NK1.1⁺ cells was achieved by injecting 250 μ g anti-mouse NK1.1 monoclonal antibodies (clone PK136) i.v. 48 hr before and after sensitization. Control mice received mouse IgG2a. FACS analysis of splenocytes from treated animals showed that NK as well as NK-T cells were completely depleted (data not shown). Antigen presentation by CD1 molecules was blocked in vivo with anti-mouse CD1.1 (clone 20H2, gift from A. Bendelac). Mice were injected with 1 mg antibody every 2 days, starting from the time of intrarectal oxazolone challenge.

Histology

Mice were euthanized 5 days after induction of colitis. The colons were removed and segments were fixed in formalin (Fisher, Fair Lawn, NJ). After paraffin embedding 5 μ m sections were cut and stained with hematoxylin/eosin (Lerner, New Haven, CT).

Cell Isolation and Cytokine Production

Splenocytes (SPC), mesenteric lymph node cells (MLNC), or lamina propria cells were isolated at various time points after colitis induction. Cells were isolated as described in detail in Current Protocols of Immunology (Scheiffele and Fuss, 2002). In brief, LPMC were isolated after removal of epithelial cells by incubation of colon strips in HBSS/2.5 mM EDTA. Mononuclear cells were released by digesting the tissue in ISCOVES media supplemented with 10% FCS, 200 U/ml collagenase (Roche, Indianapolis, IN), 10 μ g/ml DNase 1 (Roche), and 1 μ g/ml gentamicin (BioWhittaker, Walkersville, MD). Finally, leukocytes were separated from epithelial cells by centrifugation in a Percoll gradient (Amersham, Piscataway, NJ) of 33% and 66%. MLN cells and splenocytes were isolated by grinding the tissue in a petri dish and filtering the cell suspension through a 40 μ m mesh. Spleen cells were treated with ACK lysis buffer to lyse red blood cells (Biosource, Camarillo, CA). Initially, CD3⁺ cells were isolated with mouse T cell selection columns (R&D, Minneapolis, MN) according to the manufacturer's instructions. Purified CD4 cells were positively selected with CD4-beads and MACS minicolumns (Miltenyi, Auburn, CA) according to the manufacturer's instructions. Cells were cultured in RPMI 1640 supplemented with 10% FCS, 20 mM HEPES, 5% NCTC, 2 mM Glutamine, 50 μ g/ml penicillin/streptomycin, 50 μ g/ml gentamicin, 50 μ M 2-mercaptoethanol, and 50 U rhu IL-2 ml. T cells were stimulated in vitro with plate-bound anti-CD3 (10 μ g/ml clone 2C11, Pharmingen, San Diego, CA) and soluble anti-CD28 (1 μ g/ml clone 37.51, Pharmingen). Stimulation of T cells with α GalCer was accomplished using a fibroblast cell line (L-929) transfected with mouse CD1 as antigen-presenting cells. CD1-transfected cells (LCD1) were a kind gift from Dr. W. Paul (Chen and Paul, 1997). L cells were treated for 1.5 hr with mitomycin C and seeded at 1 × 10⁵ cells/ml. α -galactosylceramide (α GalCer; Kirin, Tokyo, Japan) or vehicle was added at 100 ng/ml. Lymphocyte

concentrations were generally 1×10^6 cells/ml. After 48 hr culture, supernatants were harvested and stored at -20°C until further analysis. IL-4 and IL-5 were measured with OptEIA ELISA sets from Pharmingen. IL-13 was measured with a Quantikine M ELISA kit from R&D (Minneapolis, MN).

FACS Analysis

Cells were stained with antibodies to CD3 (2C11), CD4 (RM4-5), NK1.1 (PK136), Ly49C (5E6), Ly6C (AL-21), and DX5 after incubation with FcBlock (2.4G2) (all from BD Pharmingen, San Diego, CA). Surface staining was analyzed on a FACScanner (Becton-Dickinson, Mansfield, MA). Percentage values were calculated with CellQuest software after gating on living lymphocytes in the scatter diagram.

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