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The conversion of redox status of peritoneal macrophages during pathological progression of spontaneous inflammatory bowel disease in Janus family tyrosine kinase $3^{-/-}$ and IL-2 receptor $\gamma^{-/-}$ mice

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Abstract

The distinct thiol redox status in macrophages, either elevated or reduced intracellular content of glutathione (GSH), was confirmed during aging in IL-2 receptor (IL-2R)γ and Janus family tyrosine kinase (JAK)3 gene-disrupted mice. Oxidative macrophages (OMp) with reduced GSH dominated initially at a younger age in both mice. OMp-dominated JAK3 or IL-2Rγ chain-deficient mice showed shortened life longevity compared with wild-type littermates. These mice elicited spontaneous onsets of inflammatory bowel disease (IBD)-like symptoms accompanied with the conversion of the redox status of macrophages to reductive phenotypes with elevated intracellular GSH. Conversion of OMp to the reductive phenotype by GSH monoethyl ester or by a β-(1–3)-glucan accelerated the disease onset, concomitant with the skewing from T_h2 to T_h1 responses. On the contrary, *N,N′*-diacetyl cystine dimethylester, which is capable of inducing OMp, delayed the incidence of IBD-like symptoms and improved the survival rate. This implies that the conversion of OMp/T_h2 to reductive macrophages/T_h1 may be critical for the disease progression. The study of these mice may provide insight into the mechanisms underlying Crohn's disease and ulcerative colitis.

Introduction

The pathogenesis of inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC) remains poorly understood (1). Initiation and progression of the intestinal inflammation in IBD may be a result of the dysregulated cellular immune responses of the intestinal mucosa to a constituent of endogenous luminal bacterial flora (2). Accumulating evidence indicates that activated $CD4^+$ T lymphocytes are likely to play a central role in the pathogenesis of IBD (3). Several mouse models for IBD have already shown that alterations in cytokine propensities result from a failure of regulation by T_h cells (4–6). T_h1 cells produce pro-inflammatory cytokines such as IL-2 and IFN-γ, whereas T_h2 cells produce anti-inflammatory cytokines such as IL-4 and IL-10. In general, human CD is thought to be a T_h1-like disease, whereas UC is thought to be a T_h2-like disease (7–9). Moreover, crossbreeding IL-2 gene-disrupted mice with $RAG2^{-/-}$ mice prevents colitis, whereas cross-breeding with $JH^{-/-}$ mice has no effect (10).

Macrophages play diverse and relevant roles in host defense against invasive and noxious insults (11,12). Recently, the important role of innate immune responses has been re-evaluated based on the findings of the essential roles

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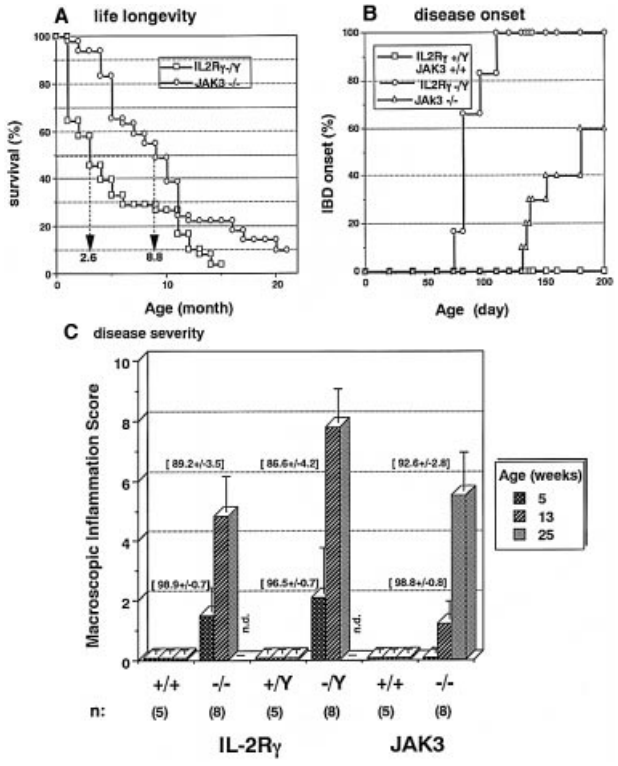

Fig. 1. Life longevity and onset of IBD-like diseases. (A) Life longevity of IL-2R$^{-/Y}$ and JAK3$^{-/-}$ male mice was followed using 48 and 49 mice respectively under normal breeding conditions. (B) IBD-like disease onset was evaluated by the occurrence of anal prolapse (n = 18 for IL-2R$^{-/Y}$ and n = 20 for the other three strains). (C) Macroscopic inflammation score. At the age indicated, macroscopically visible inflammation was assessed using the scoring criteria described by Grisham *et al.* (51), with a maximum score of 11. A macroscopic score was obtained by summation of scores on severity of colonic adhesion to surrounding tissues, strictures, mucosal ulceration, hyperemia, mesentery bleeding and bowel wall thickness. The number shown in square brackets indicates the relative body weight (percent) compared with the wild-type mice of the same age. n.d., not done.

of IFN-γ produced by macrophages and of the Toll-like receptor (TLR) family–MyD88–NF-κB system (13–15). Intestinal epithelial cell lines constitutively express several functional TLR, key regulators of the innate response system. Infiltrating macrophages and neutrophils are abundantly present in the inflamed gut of patients with IBD. The pivotal role of IL-12 produced by macrophages was demonstrated in chronic intestinal inflammation. There is a growing body of evidence that these cells expose the inflamed intestine to substantial oxidative stress by production of reactive oxygen species (ROS) and that ROS contribute to tissue injury in IBD (16–19). Intestinal inflammation induced by a ROS-generating agent could be reversed by the repletion of glutathione (GSH) (20). GSH is the most important intracellular anti-oxidant and is abundant in virtually all cells, playing significant roles in many biological processes (21). This has led to the proposal that IBD results from an imbalance between pro-oxidant and anti-oxidant mechanisms (22,23). Our group and others have demonstrated that the intracellular content of GSH in macrophages is critical for the secretion of IL-12 by regulating MAPK p38 activity (24–29). This suggests that GSH levels in macrophages play a crucial role in determining whether T_h1 or T_h2 cytokine responses predominate (27). Furthermore, the exposure of macrophages to IFN-γ increased the GSH:oxidized GSH (GSSG), ratio whereas exposure of macrophages to IL-4 decreased the GSH:GSSG ratio (27,30). Thus, the ability to generate a T_h1- or T_h2-type response has turned out to depend not only on T cells, but also on the intracellular thiol redox status of macrophages (27). Macrophages with a reduced intracellular GSH content are referred to as oxidative macrophages (OMp) and those with an elevated amount are referred to as reductive macrophages (RMp) (24,26–28). The OMp showed elevated IL-6 and IL-10 production, and reduced NO and IL-12 production, while the RMp elicited a reciprocal response, i.e. elevated IL-12 and NO production, and reduced IL-6 and IL-10 production. The cytokine propensities of OMp or RMp were inter-converted to each other in the absence of cell proliferation and protein synthesis. Accumulating evidence suggests that intracellular GSH:GSSG balance regulates transcription factor NF-κB or AP-1 activity relevant for the gene activation of cytokines (31). The cytosolic complex cascade of phosphorylation by kinase and dephosphorylation by phosphatase is modulated by intracellular redox states (32). A paradigm has been proposed based on whether the presence of the functional heterogeneity of macrophages (33–36) is pro-inflammatory or anti-inflammatory. Our discussions treat RMp as pro-inflammatory and OMp as anti-inflammatory (27). This paper describes the pathogenesis of IBD in Janus family tyrosine kinase (JAK)3- and IL-2 receptor (IL-2R)γ-deficient mice and provides evidence that the T_h1/T_h2 balance of these mice is also regulated by the intracellular thiol redox status of macrophages. The stepwise skewing of the redox status in macrophages (oxidative to reductive) generated first T_h2 and then T_h1 dominancy, resulting in the periodical infiltration of inflammatory cells into intestinal mucosa, followed by tissue injury and anal prolapse along with disease progression. The successful prophylactic/therapeutic intervention with either cysteine or GSH derivatives is also discussed.

Methods

Mice and reagents

C57BL/6 mice were obtained from Charles River (Atsugi, Japan). JAK3 gene-disrupted (JAK3$^{-/-}$) and IL-2Rγ chain gene-disrupted (IL-2Rγ$^{-/-}$) mice, both of the C57BL/6 background, were established as previously reported (37,38). Founders (F_0) of generated gene-disrupted mice were identified by PCR analysis of tail DNA. The primers used for gene-disrupted mouse screening were as follows: forward 5′-ACCCAGGTACTCCATGCCCT-3′ (JAK3), 5′-CTG-CTCAGAATGCCTCCAATTCC-3′ (IL-2Rγ); reverse 5′CCA-GACCAGCAGAGGGACTT-3′ (JAK3), 5′CCTGCGTGCAATC-CATCTTGTTCAAT-3′ (IL-2Rγ); and reverse primers for an inserted neomycin gene 5′-CGACCACCAAGCGAAACATC-3′ (JAK3), 5′-GATCCAGATTGCCAAGGTGAGTAG-3′ (IL-2Rγ). The gene-disrupted mice were bred with either C57BL/6 or with homo- or hetero-knockout mice. GSH monoethylester

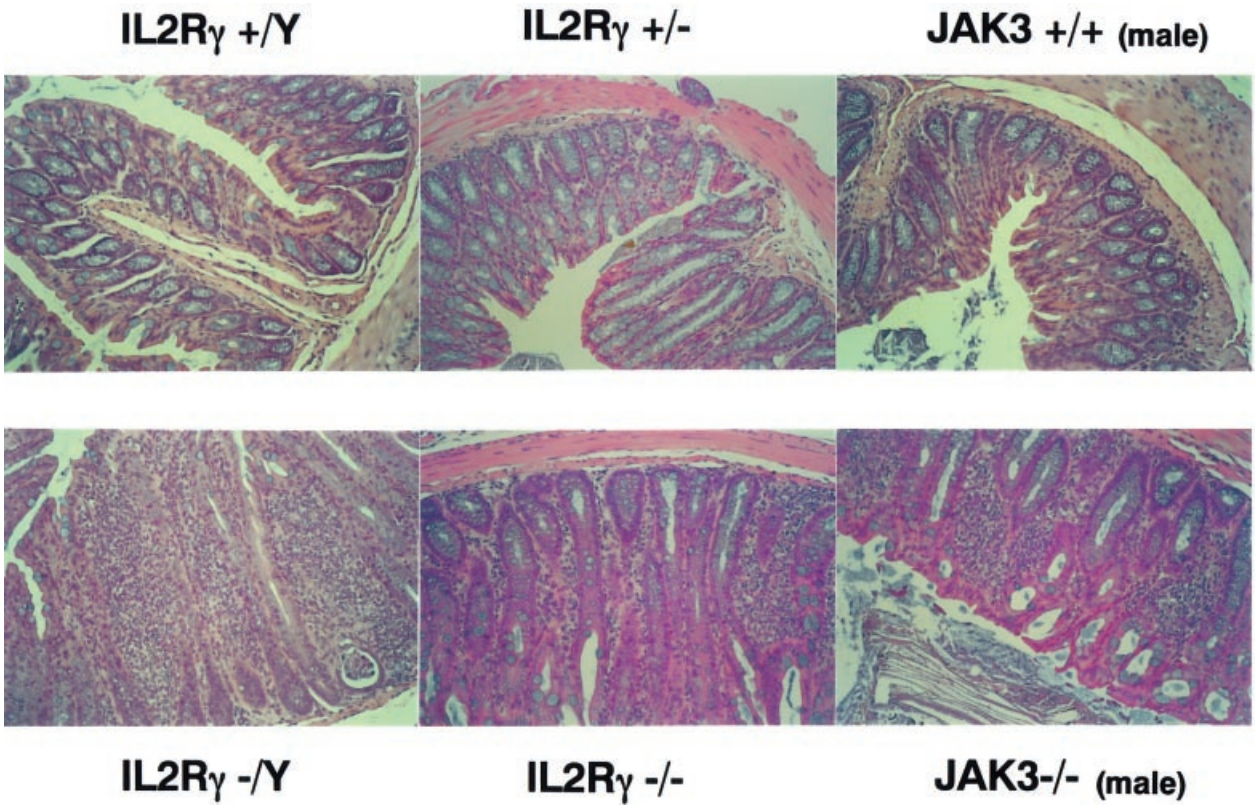

Fig. 2. Histology of colonic inflammation. The age of each mouse was 13 weeks for IL-2R$^{+/Y}$ and IL-2R$^{-/Y}$, 15 weeks for IL-2R$^{+/-}$ and IL-2R$^{-/-}$, and 23 weeks for JAK3$^{+/+}$ and JAK3$^{-/-}$ (male).

(GSH-OEt) was purchased from Sigma (St Louis, MO). *N,N'*-Diacetyl cystine dimethylester [$(NACOMe)_2$] was from Bachem (Bubendorf, Switzerland). Lipopolysaccharide (LPS) was derived from *Escherichia coli* 055:B5 (Difco, Detroit, MI). NO_2 was measured using a commercial Griess-Romijn reagent (Wako Pure Chemical, Tokyo, Japan). Monochlorobimane (MCB) was from Molecular Probes (Eugene, OR). Mouse IFN-γ was from R & D Systems (Minneapolis, MN). Mouse IL-10 was from Genzyme (Cambridge, MA). Human IL-12, anti-CD4 antibody and anti-CD3 antibody were from PharMingen (San Diego, CA). Human IL-2, IL-6 and lentinan [a β-(1–3)-glucan from edible mushroom, *Lentinus edodes* Berk. (Sing)] are the products of Ajinomoto (Tokyo, Japan).

Peritoneal macrophages

The procedures were published previously (27). Briefly, peritoneal cells were harvested by injecting 5 ml of an ice-cooled phenol red-free RPMI 1640 medium (Nikken Seibutsu, Kyoto, Japan) without antibiotics into the peritoneal cavity. The collected cells from five or more mice were cultured in a microplate (167008; Nunc, Roskilde, Denmark) at 1–3 × 10^5/200 μl in the same medium. Cells were plated in quadruplicate wells. The adherent cells were used as resident peritoneal macrophages. RMp or OMp were induced by administering 20 mg of GSH-OEt or 200 μg of $(NACOMe)_2$ i.p. 1 day before the collection. Macrophages were stimulated with 100 U/ml IFN-γ and/or 100 ng/ml LPS for cytokine and NO production for 48 h.

Nitrite and cytokine determination

Briefly, cell-free supernatants were incubated with Griess reagent for 10 min and absorbance at 550 nm was measured using an Arvo multilabel counter (Wallac, Turuk, Finland). The cytokine concentration was determined using an ELISA kit (Opt EIA; PharMingen) according to the manufacturer's instructions.

Qualitative determination of intracellular GSH with ACAS

First, 300 μl of peritoneal cells suspension adjusted to a cell density of 3 × 10^5 cells/ml medium was charged into a Lab-Tek chamber slide (136439; Nunc) and incubated for 3 h. After washing, 300 μl of 10 μM MCB was added and incubated for 30 min. Then the fluorescent intensity was monitored by argon-ion laser cytometry with a ACAS 570 work station (Meridian, Okemos, MI). Intracellular GSH levels were detected with excitation and emission wavelengths of 350 and 460 nm.

CD4$^+$ T cell culture and T_h1 polarization assay

Spleens from five to 10 mice were pooled, and CD8$^+$ T cells and B cells were first removed using magnetic microbeads (Polyscience, Warrington, PA) coated with anti-CD8 antibody (53-6.7) and anti-HSA antibody (M16/9). CD4$^+$ T cells were further positively selected with an auto-MACS (Miltenyi Biotech, Bergisch-Gladbach, Germany) using microbeads

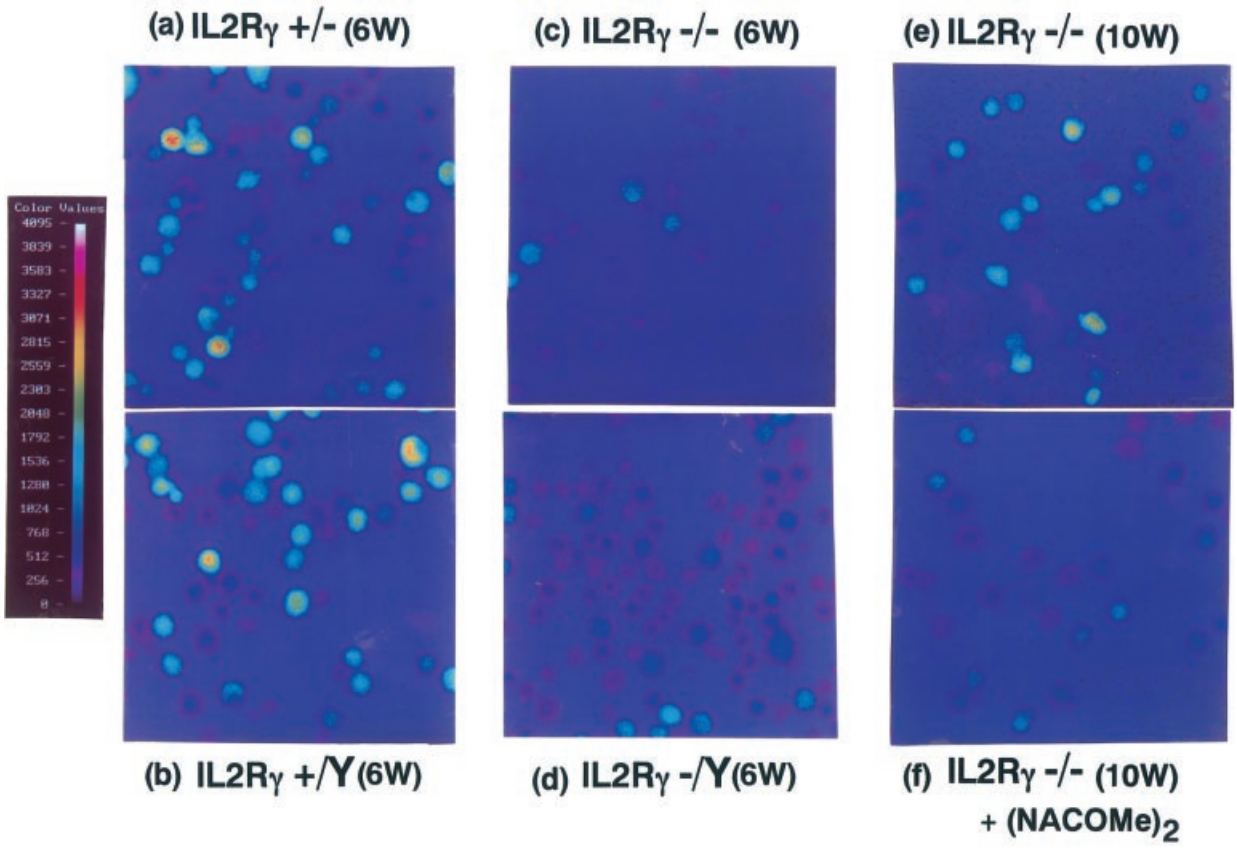

Fig. 3. Intracellular GSH content of RMp and OMp. The amount of intracellular GSH in adherent macrophages of the mice indicated (n = 5) was monitored by argon-ion laser cytometry. The yellowish to red or blue color indicates the presence of abundant intracellular GSH. Alternatively IL-2Rγ$^{-/Y}$ mice were injected i.p. with 20 μg $(NACOMe)_2$ 24 h before the collection of macrophages. Results shown are representative of two separate experiments.

coated with anti-CD4 antibody. The purity of the fractionated CD4$^+$ T cells was consistently confirmed to be >99% purity using FACScan (Becton Dickinson, Franklin Lakes, NJ). Macrophages were added at a cell density of 1×10^5/well onto the microplates (Becton Dickinson) pre-coated with anti-CD3 antibody, adhered for 3 h followed by washing and were then co-cultured together with purified CD4$^+$ T cells (5×10^5/well) for 48 h.

Statistical analysis

Results are expressed as means ± SE. Significant differences were calculated using Student's t-test. $P < 0.05$ was considered statistically significant.

Results

Life longevity and the onset of IBD-like diseases in IL-2Rγ$^{-/-}$ and JAK3$^{-/-}$ mice

The mortality and the extent of colitis were investigated in IL-2Rγ or JAK3 gene-disrupted mice. A significant reduction in survival rate was observed (IL-2R$^{-/Y}$ mice 2.6 months and JAK3$^{-/-}$ male mice 8.8 months, Fig. 1a) compared with normal C57BL/6 male mice (21.5 months). Whereas the wild-type littermates never manifested the IBD-like symptoms spontaneously, both IL-2Rγ$^{-/-}$ and IL-2Rγ$^{-/Y}$ spontaneously displayed the symptoms within 4 months. Half of the IL-2Rγ$^{-/-}$ mice exhibited anal prolapse by 12 weeks and JAK3$^{-/-}$ (male) by 26 weeks (Fig. 1b). The features of colitis in IL-2Rγ$^{-/-}$, IL-2Rγ$^{-/Y}$ and JAK3$^{-/-}$ were characterized by intestinal shortening, colonal hypertrophy, diarrhea, loose passage, bloody stool and anal prolapse. Pathological analysis was conducted on specimens of the large intestine between 2 and 3 cm proximal to the anus. The colon was opened longitudinally and fixed in 4% formaldehyde. After embedding in paraffin, 4-μm serial sections were prepared and stained with hematoxylin. Neither infiltration of inflammatory cells nor aberrant changes in goblet cells and mucosal epithelium cells were observed in wild-type. In contrast, the hyperplasia of the mucosal epithelium was 2–3 times thicker than those of wild-type, and the shortening of colon length became evident in IL-2Rγ$^{-/-}$ and IL-2Rγ$^{-/Y}$ by 13 weeks. Similar changes were also detected in JAK3$^{-/-}$ by 24 weeks, although the disease severity indexed by macroscopic inflammation score was milder than that in IL-2Rγ$^{-/-}$ and IL-2Rγ$^{-/Y}$ (Fig. 1c). Infiltration of macrophages and lymphocytes as a local cluster was observed only in the lamina propria mucosa, and the infiltration of neutrophils was not observed. The cluster was only observed at a low level in the bottom of

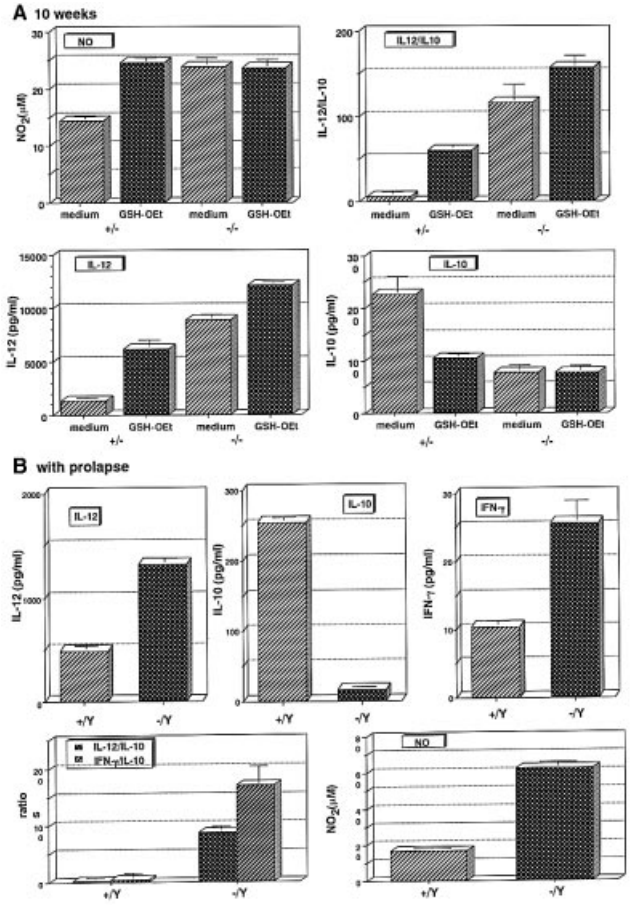

Fig. 4. Skewing to RMp phenotypes in γ_c gene-disrupted mice. (a) Macrophages collected from IL-2R$\gamma^{+/-}$ or IL-2R$\gamma^{-/-}$ mice at the age of 10 weeks were evaluated for their NO-producing capability, IL-12 and IL-10. RMp were induced *in vitro* by treating with 10 mM GSH-OEt 24 h. macrophages were stimulated for 48 h with 100 U/ml IFN-γ and 100 ng/ml LPS for IL-12 and NO production, and with 100 ng/ml LPS for IL-10. (b) IL-2R$\gamma^{+/Y}$ (23 weeks old) and IL-2R$\gamma^{-/Y}$ mice with anal prolapse (17–23 weeks old) were analyzed. The procedures were the same as those in (a). IFN-γ production was also confirmed with a culture stimulated with 100 ng/ml LPS. Macrophages were collected from at least six mice for each experiment. Results shown are representative of two separate experiments.

the lamina propria mucosa (Fig. 2). When autopsies were conducted immediately before the occurrence of anal prolapse, crypt distortion, degeneration and loss of goblet cells, and metaplasia and degeneration of mucosal epithelium were identified at high levels (Fig. 2).

Periodical change of thiol redox status in macrophages at the early stage of IBD

Interestingly, macrophages of IL-2R$\gamma^{-/-}$ of 6 weeks elicited functional phenotypes of OMp with reduced NO, IL-12 and IFN-γ, and elevated IL-10 production (data not shown), and intracellular GSH (icGSH) was reduced compared with those of wild-type and IL-2R$\gamma^{-/-}$ (Fig. 3c and d versus a and b). However, the OMp phenotypes varied later to those of RMp with increased icGSH at the age of 10 weeks (Fig. 3e) and the functional phenotypes became comparable to macrophages of IL-2R$\gamma^{-/-}$ treated with GSH-OEt (Fig. 4a). The conversion to RMp phenotypes was functionally evident also for IL-2R$\gamma^{-/Y}$ of 13 weeks, with the anal prolapse (Fig. 4b).

Conversion of RMp to OMp and a prophylactic intervention

RMp of IL-2R$\gamma^{-/-}$ of 10 weeks could be converted to OMp by *in vitro* treatment with 1 mM $(NACOMe)_2$ for 24 h (Fig. 3f). $(NACOMe)_2$ is a cystine derivative which efficiently reduces the intracellular GSH content in macrophages and is possible to apply in the clinic (27,28). Thus, converted OMp elicited a reduced potential to produce IL-12 and NO (Fig. 5a and b). The conversion to OMp by i.p. administration of 20 μg $(NACOMe)_2$ (5 times, twice a weeks starting at the age of 9.5 weeks) delayed the onset of anal prolapse and increased the survival rate (Fig. 5c and d). This indicates that the blockade of conversion from OMp to RMp by $(NACOMe)_2$ may interfere with the occurrence of IBD-like symptoms, which was also confirmed by histological studies on specimens of the large intestine.

Conversion of T_h2 skewing to T_h1, concomitant with the onset of IBD symptoms

As expected, the IL-2R$\gamma^{-/Y}$ that survived for 23 weeks and JAK3$^{-/-}$ for 14 weeks without IBD-like symptoms maintained the phenotypes of OMp with reduced NO, IL-12 and IFN-γ, and elevated IL-10 production (data not shown). Consistent with this observation, the T_h1/T_h2 balance was skewed to T_h2 in these OMp prone mice, and they also showed lowered IFN-γ:IL-4 and IFN-γ:IL-10 ratios, which was deduced from the amount of cytokines produced by purified CD4$^+$ T cells (Fig. 6a and b). Considering the late onset of IBD symptoms in JAK3$^{-/-}$ (Fig. 1b), the skewing to T_h1 was expected to be later than in IL-2Rγ gene-disrupted mice. Accordingly the skewing became evident initially at the age of 5.5 months (Fig. 7c), when the occurrence of the anal prolapse was detected in 40% of JAK3$^{-/-}$ (Fig. 1b). *In vivo* application of GSH-OEt converted OMp in JAK3$^{-/-}$ of 20 weeks to RMp and endowed the potential to produce IL-12 (Fig. 8a and b). GSH-OEt is a cell-permeable GSH derivative which efficiently induces RMp *in vivo* (27,28). In parallel with this conversion, GSH-OEt accelerated the onset of the IBD of OMp prone JAK3$^{-/-}$ leading to 90% mortality in 4 months when injected 3 times a week for 2 weeks from 20 to 22 weeks (Fig. 8c). A β-(1–3)-glucan, lentinan, a potent inducer of RMp/T_h1 (24,28), structurally far removed from GSH-OEt, also accelerated the onset comparable to GSH-OEt when injected at a dose of 20 μg on a similar schedule.

T_h2 cytokines augmented the effector function of macrophages depending on the redox status

The impact of T_h2 cytokines on the function of OMp was investigated to provide a preliminary clarification of the mechanism underlying the conversion of OMp/T_h2 to RMp/T_h1. T_h2 cytokines, IL-4, IL10 and IL-13, are well known to be anti-inflammatory or immunosuppressive cytokines, and the action is mainly mediated by the suppressive effect on the effector functions of macrophages, including the reduction of the potential to produce NO. Correspondingly, macrophages of JAK3$^{+/+}$ or IL-2R$\gamma^{+/Y}$ elicited a reduced potential to produce NO when exposed to either of IL-4, IL10 or IL-13 for 24 h, and

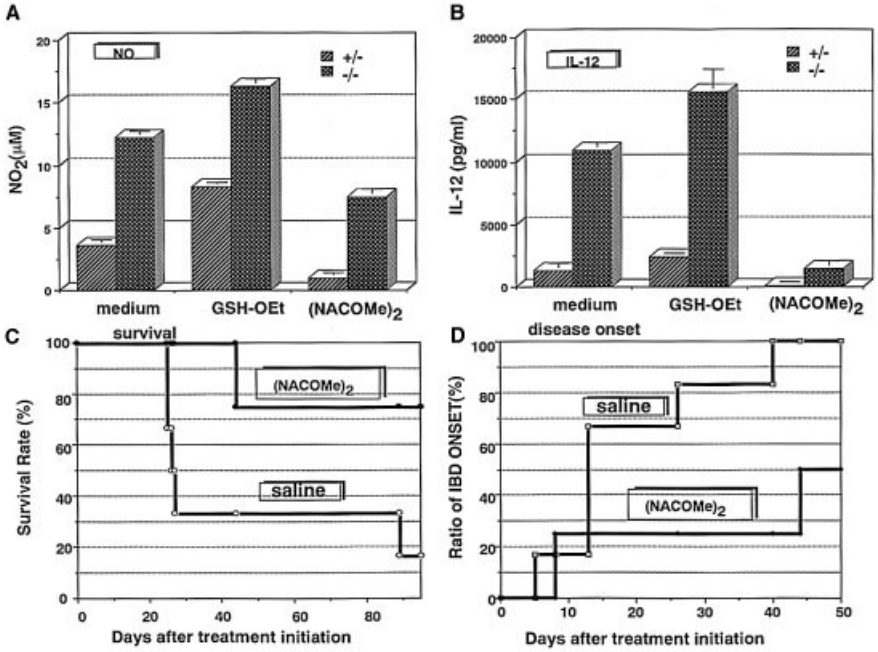

Fig. 5. Conversion of RMp to OMp and a prophylactic intervention. RMp of IL-2Rγ$^{-/-}$ of 10 weeks of age were skewed to OMp by *in vitro* treatment with 1 mM $(NACOMe)_2$ for 24 h, and the culture was stimulated with LPS + IFN-γ to measure the produced NO (a) and IL-12 (b), as well as the production by non-treated or 10 mM GSH-OEt-treated macrophages [a and b; $P < 0.01$; medium versus $(NACOMe)_2$]. Results shown are representative of two separate experiments. The skewing to OMp by i.p. administration of 20 μg $(NACOMe)_2$ (5 times, twice a week starting at the age of 9.5 weeks) delayed the onset of anus prolapse (c, $n = 12$) and increased the survival rate (d, $n = 12$).

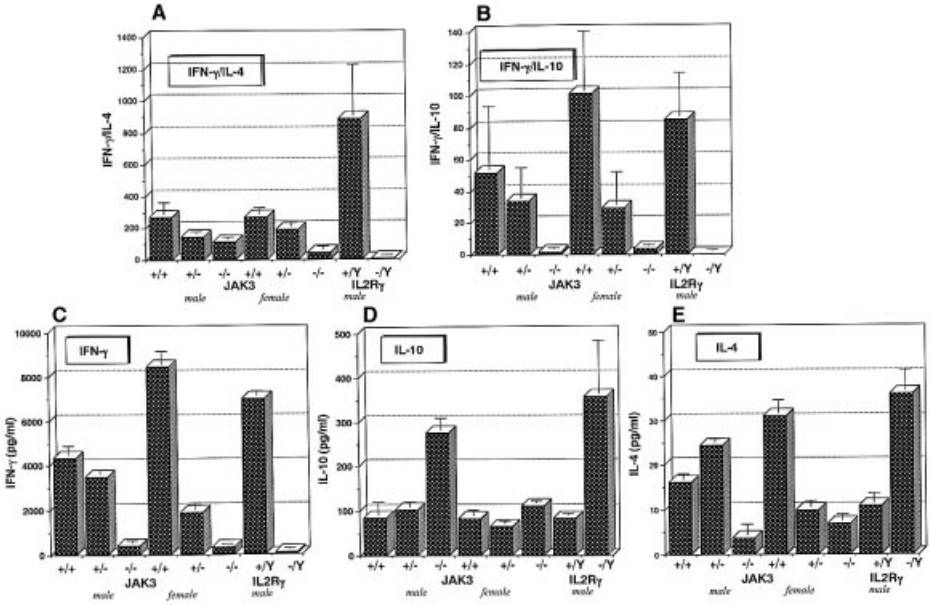

Fig. 6. The skewing of the T_h1/T_h2 balance in IL-2Rγ gene-disrupted mice. CD4$^+$ T cells were purified from spleen cells of IL-2Rγ$^{+/Y}$ and IL-2Rγ$^{-/Y}$ mice surviving for 23–29 weeks without anal prolapse, and of JAK3$^{+/+}$, JAK3$^{+/-}$ and JAK3$^{-/-}$ mice between 12 and 16 weeks of age. After stimulation with anti-CD3 for 48 h the supernatants were assayed for IFN-γ, IL-4 and IL-10, and IFN-γ:IL-4 (a) and IFN-γ:IL-10 (b) ratios were deduced. The amounts of secreted cytokines are also indicated (c–e). Results shown are representative of two separate experiments.

the extent of reduction was comparable to transforming growth factor (TGF)-β exposure. On the contrary, however, macrophages of JAK3$^{-/-}$ or IL-2Rγ$^{-/Y}$ (OMp phenotype) behaved in a contrasting manner, elevating the NO production potential (Fig. 9a). These T_h2 cytokines share the potential to regulate bilaterally the thiol redox status of macrophages, depending on the pre-existing redox status (data not shown). OMp were converted to RMp with either IL-4, IL10 or IL-13,

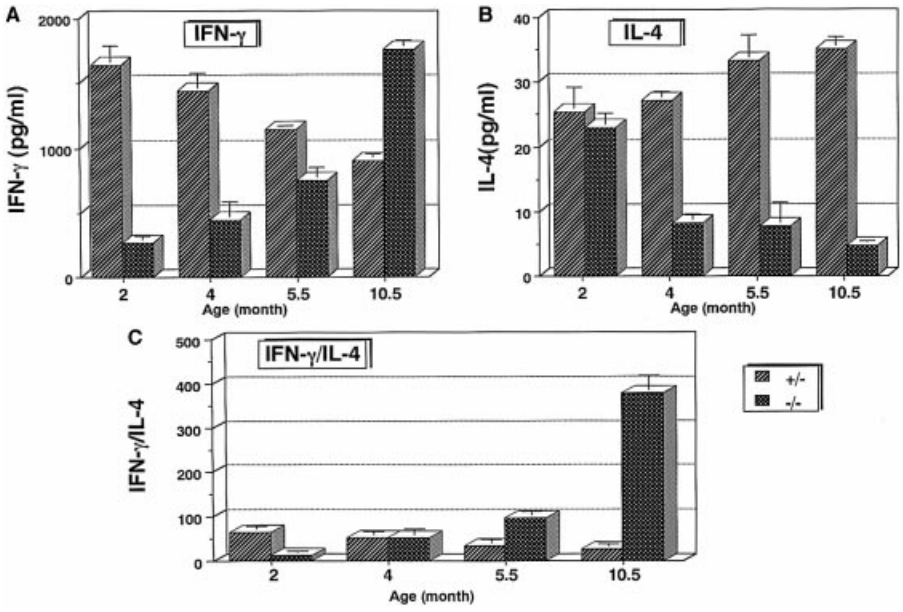

Fig. 7. The skewing of the T_h1/ T_h2 balance in JAK3 gene-disrupted mice. $CD4^+$ T cells were purified from spleen cells of $JAK3^{+/-}$ and $JAK3^{-/-}$ mice between 2 and 10.5 months of age were investigated (c: $P < 0.01$; 2, 4, 5.5 versus 10.5 month). IFN-γ (a), IL-4 (b) and IFN-γ:IL-4 ratio (c) were deduced as shown in Fig. 5. Results shown are representative of two separate experiments.

while RMp were converted to OMp, whereas TGF-β consistently induced OMp, irrespective of the pre-existing redox status.

Discussion

The spontaneous onsets of IBD-like diseases in IL-2Rγ chain (γ_c)-deficient mice with a truncated mutation (Fig. 2) coincides well with the reported observation describing inflammatory changes in the intestine from 8 weeks of age onwards (39). JAK3 is an essential transducer of γ_c-dependent signals, so it is not surprising that $JAK3^{-/-}$ elicited similar symptoms to IL-$2R\gamma^{-/-}$ and IL-$2R\gamma^{-/Y}$. It is of note that the T_h1/T_h2 balance was skewed to T_h2 (Figs 6 and 7) and macrophages to OMp in IL-$2R\gamma^{-/-}$, IL-$2R\gamma^{-/Y}$ and $JAK3^{-/-}$ at an early stage of disease. The skewing of macrophages to OMp may be due to the impact of luminal antigens on the intracellular redox status of macrophages, although the pathological meaning of the skewing to OMp at an early stage of disease remains unclear. One possibility is the forced infiltration of macrophages into intestinal mucosa by modulating the chemotaxis of macrophages. A certain subset of macrophages selectively produces CC chemokines (40) and the thiol redox status affects the expression of adhesion molecules critical for transendothelial migration of the inflammatory cells (41).

IL-12 and IL-10 play a pivotal role in maintaining the *in vivo* balance between the T_h1 and T_h2 response (42–45). Concurrent with the conversion to RMp, IL-12 produced by RMp may participate in the disease progression through the induction of T_h1 skewing. T_h1-type inflammation has a more pivotal role in CD, whereas T_h2-type responses are more pivotal in UC, although the relative relevance of both responses is still controversial (7,8,46–50). Our findings that OMp/T_h2 skewing shifted to RMp/T_h1 skewing along with disease progression (Fig. 4) and that $(NACOMe)_2$ prevented the onset of IBD-like symptoms (Fig. 5), together with the accelerated disease progression by RMp/T_h1 skewing by GSH-OEt or a β-(1–3)-glucan (Fig. 8c), support the concept that T_h1-type inflammation has a relevant role in the later pathological progression of colitis, at least in our models. Initial skewing to OMp/T_h2 prior to later conversion to RMp/T_h1 is the pre-requisite for disease onset, because RMp/T_h1 skewing by GSH-OEt or a β-(1–3)-glucan in wild-type did not cause any sign of colitis at all (data not shown). The combined affect of genetic factors and luminal antigens on the redox status of macrophages may trigger the initial priming to OMp/T_h2. RMp may also participate in the disease progression through the production of NO by RMp (Fig. 4). It is becoming increasingly apparent that chronic inflammation in IBD is associated with the sustained overproduction of NO (51,52). The mechanism underlying the observed conversion of OMp/T_h2 to RMp/T_h1 is unclear. In this context it is of interest that T_h2 cytokines elicited a bilateral effect to convert OMp to RMp and augmented NO production (Fig. 9). T_h2 cytokine's function as either an anti-inflammatory or pro-inflammatory mediator may depend on the redox status of macrophages. Infiltration of inflammatory cells in inflamed sites may be triggered initially by OMp, resulting in the release of anti-inflammatory cytokines, which in turn may trigger the conversion of OMp to RMp. Subsequent tissue injury is ascribed to RMp. As such, depending on the redox status of macrophages, anti-inflammatory cytokines may elicit a paradoxical pro-inflammatory action. The sequential change of the redox status of macrophages has also turned out to be responsible for other autoimmune diseases, such as type I diabetes and hepatitis (Y. Murata, in preparation). The bilateral functions of T_h2 cytokines have also been reported (34). The role of the intracellular thiol redox status of macrophages in regulating

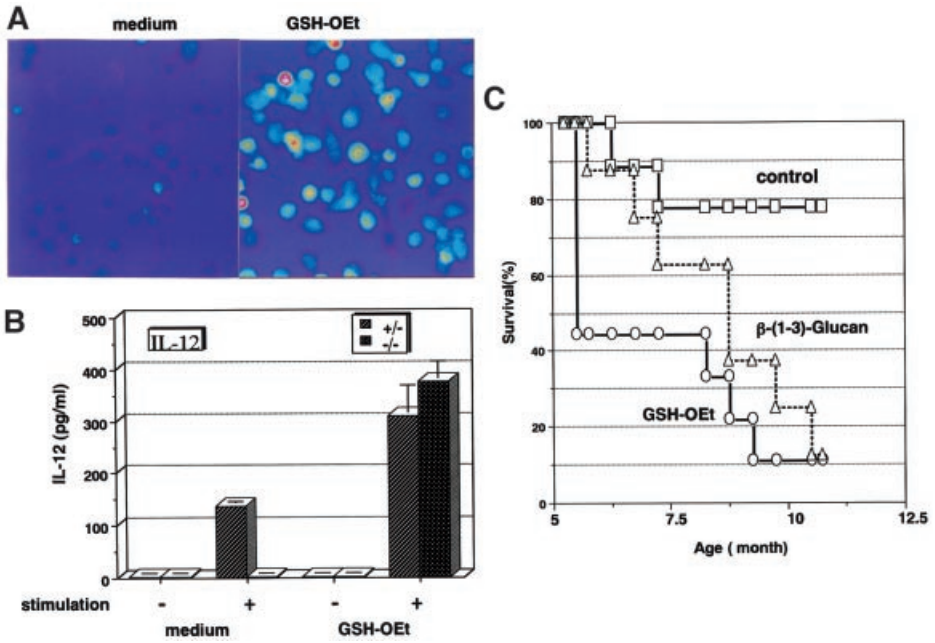

Fig. 8. Conversion of OMp to RMp accelerated the onset of IBD symptoms. *In vivo* application of GSH-OEt (i.p., 20 mg) converted OMp in JAK3$^{-/-}$ of 20 weeks to RMp (a) and the macrophages collected were investigated for their potential to produce IL-12 (b, $P < 0.01$) according to the procedures described. GSH-OEt injected 3 times a week for 2 weeks from 20 to 22 weeks and lentinan injected at a dosage of 1 mg/kg at the same schedule accelerated the onset of IBD of JAK3$^{-/-}$ (c, $n = 10$).

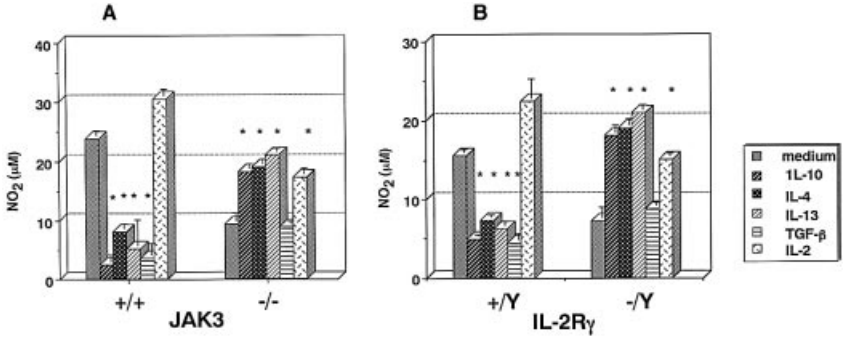

Fig. 9. T_h2 cytokines augmented NO production by OMp. Macrophages of JAK3$^{+/+}$, JAK3$^{-/-}$, IL-2R$\gamma^{+/Y}$ or IL-2R$\gamma^{-/Y}$ mice were exposed to either IL-4 (10 ng/ml), IL-10 (50 ng/ml), IL-13 (10 ng/ml), TGF-β (20 ng/ml) or IL-2 (1000 U/ml) for 20 h, and then stimulated with 100 U/ml IFN-γ and 100 ng/ml LPS for 48 h to measure the production of NO. Results shown are representative of two separate experiments. *$P < 0.01$ for medium control.

functional phenotypes of macrophages producing either NO, IFN-γ and IL-12 or IL-10 has also been proposed (24,26–28). The intestinal epithelium is likely to be exposed to oxidants derived from bacterial constituents or endogenously generated ROS by infiltrating macrophages and neutrophils within the lamina propria. GSH, being the major intracellular thiol, provides protection against oxidative injury (53). Clinical and experimental studies revealed that intestinal mucosa showed deficiency in GSH content. Accordingly, *in vivo* administration of *N*-acetyl-L-cysteine attenuates the acute colitis through increasing mucosal GSH levels, suggesting that GSH precursors may be of relevance in the acute relapse of IBD (54,55). Further study is required to define the interplay between OMp or RMp and mucosal epithelium, in the context of the influence of icGSH on the counteraction of infiltrating cells and intestinal mucosa. Lamina propria macrophages lack the capacity to elaborate cysteine and thereby secure the physiological unresponsiveness of lamina propria T cells to nominal antigen exposure (56). The local recruitment of blood monocytes in IBD may endow hyper-responsiveness with T cells (56). The proposed hypothesis may well be compatible with our notion that the conversion of OMp to RMp might underlie the hyper-responsiveness of T cells in IBD.

The most crucial and urgent issue to address is the analysis of the redox status of locally recruited macrophages into inflamed sites of the intestinal tract. GSH is present in virtually

all cells, which makes it difficult to demonstrate directly the frequency of RMp/OMp in small and large intestines. The procedures needed for multi-step purification of locally recruited macrophages also accompany the artificial changes of redox status of macrophages due to the plasticity (conversion) of the redox status. To explore the relevant issue to compare the macrophages isolated from disease and normal sites, further elaboration of the new technology to define directly the content of GSH will be necessary. The further study will be needed to define the biological significance of the new insight for the potential role of RMp/OMp in the development of IBD. In this context the cytokine propensity of the intestinal T cells should be also analyzed in relation to the disease progression. Finally, it should be noted that the up-regulation of TLR4 in both UC and CD (57) indicates the necessity of studies on the influence of the redox status of macrophages on the TLR–MyD88–NF-κB system, considering the recruitment of macrophages to intestinal mucosa, accessible to luminal bacterial constituents.

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Abbreviations

CD	Crohn's disease
GSH	glutathione
GSH-OEt	glutathione monoethylester
GSSG	oxidized glutathione
IL-2R	IL-2 receptor
JAK	Janus family tyrosine kinase
icGSH	intracellular GSH
LPS	lipopolysaccharide
MCB	monochlorobimane
OMp	oxidative macrophages
RMp	reductive macrophages
ROS	reactive oxygen species
TGF	transforming growth factor
TLR	Toll-like receptor
UC	ulcerative colitis

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