Contributions of mucosal immune cells to methotrexate-induced mucositis

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Keywords: innate and adaptive immune system, intestinal damage, LPS, MTX

Abstract

The use of high doses of the anti-cancer drug methotrexate (MTX) is associated with intestinal damage. As a result, mucosal immune cells become increasingly exposed to a vast amount of microbial stimuli. We aimed at determining whether these cells are still functional during MTX treatment. Furthermore, we assessed if activation of the mucosal immune system would play a role in the pathogenesis of mucositis. A contributive role to mucositis for the adaptive immune system was established by showing that mucosal lymphocytes from MTX-treated mice secreted enhanced amounts of cytokines upon ex vivo polyclonal stimulation. Next, in vitro experiments revealed that macrophages were not affected by MTX in the capacity to produce tumor necrosis factor-α (TNF-α) and IL-10 after LPS exposure. Moreover, peritoneal macrophages from MTX-treated mice produced more IL-10 and TNF-α upon LPS stimulation, compared with cells derived from control mice. These data indicate a persistence of both innate and adaptive immune responses in this model. The clinical relevance of these findings was further established by the fact that LPS exposure prior to MTX treatment aggravated the course of mucositis. Furthermore, LPS-responsive mice recovered more slowly compared with LPS-unresponsive mice from MTX treatment. Finally, we found an increase in weight loss and intestinal damage upon MTX treatment in IL-10-deficient mice in comparison to wild-type controls, suggesting a protective role for IL-10 in mucositis. We conclude that mucosal immune responses remain resilient during MTX-induced mucositis. Whereas TNF-α production may contribute to mucosal damage, IL-10 may regulate by restricting excessive mucositis.

Introduction

One of the most severe side effects of chemotherapy is the damage induced to the gastrointestinal mucosa, often referred to as mucositis (1). Mucositis is characterized by severe pain, diarrhea and weight loss. Patients suffering from mucositis experience a decreased quality of life (2) and an enhanced risk on developing infections with micro-organisms originating from the oral cavity and intestinal lumen (3–6). These side effects may lead to delays in scheduled chemotherapy courses, thereby potentially reducing the efficacy of anti-cancer treatment (1, 3). Thus far, there is no definitive prophylaxis or treatment for mucositis, partly caused by a lack of insight into the complex pathophysiology.

Besides digestive absorption of dietary nutrients, the main task of the intestine is to form a barrier against micro-organisms and food antigens that are present within the lumen. Methotrexate (MTX) damages rapidly dividing cells, such as epithelial cells in the intestinal crypts, thereby causing diminished cell renewal and decreased cell replacement. Ultimately, this leads to ulceration and a decreased barrier function (7–9). As a consequence, the mucosal immune system is exposed to an increased amount of microbial stimuli. In healthy individuals, the homeostasis at the mucosal surfaces of the gastrointestinal tract is maintained by a balanced release of anti- and pro-inflammatory cytokines. Emerging
knowledge on mucosal immune regulation comes from animal models of colitis (10). As such, it has been established that the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) is associated with epithelial damage (11, 12). Furthermore, chronic intestinal inflammation is highly associated with increased mucosal TNF-α production. A causative role for TNF-α in the pathogenesis of intestinal inflammation is further confirmed by the fact that these patients experience dramatic improvement upon anti-TNF-α treatment (13). Under physiological conditions these pro-inflammatory responses are under the control of anti-inflammatory processes. As such, IL-10 knockout (IL-10 KO) mice develop spontaneous intestinal inflammation. Corollarily, treatment with an IL-10-producing Lactococcus appears to exert a potent anti-inflammatory effect both in mice models as well as in man (14, 15). In parallel with the expanding knowledge on the pathogenesis of chronic inflammatory diseases, recent data suggest that pro-inflammatory cytokines may also play a role in the pathophysiology of mucositis (16). In adult leukemia patients receiving chemotherapy, mucositis development is correlated with increased serum levels of TNF-α (17). Moreover, various interventions that limit mucositis are frequently found to result from direct or indirect inhibition of the TNF-α release (16, 18, 19).

Thus far, it is not known to what extent the mucosal immune system is still functional despite therapy with MTX. Subsequently, it is not clear whether the mucosal immune cells actively contribute to the course of mucositis.

To address these questions, we analyzed the effects of MTX treatment on various components of the mucosal immune system.

Methods

Animals

Specific pathogen-free (SPF) 10-week-old female BALB/c mice were purchased from Charles River (Saint Aubin Lees Elbeuf, France). SPF 10-week-old female LPS-unresponsive C3H/HeJ mice and LPS-responsive C3H/HeN mice (20, 21) were purchased from CLEA Japan, Inc. (Tokyo, Japan). SPF 8- to 10-week-old IL-10-deficient C57BL/6 mice, formally designated ‘IL-10tm1Cgn’ (I. J. Bristol, M. Mähler, E. H. Leiter, J. P. Sundberg: IL-10tm1Cgn, an IL-10 gene-targeted mutation; JAX notes 471, The Jackson Laboratory, Bar harbor, ME, USA, 1997) (obtained from M. Mähler, Hanover, Germany), and their wild-type (WT) littermates were bred at the animal facility of the Erasmus MC (Rotterdam, the Netherlands) and maintained in isolator cages with water and a standard pellet diet (Hope Farms, Woerden, the Netherlands) ad libitum.

Induction of mucositis by MTX in different mouse models

All animal procedures and protocols were performed with the approval of local institutional animal studies ethics committee. For the in vivo studies, we have adapted a rat MTX-induced mucositis model to a mouse system (22, 23). The dosages of MTX [Emethexate (PF) Pharmacie B.V., Haarlem, the Netherlands] used in this model were optimized for each mouse strain in order to obtain full recovery after severe damage of the small intestine (characterized by villus atrophy, crypt loss and epithelial flattening). All mice were weighed daily and euthanized by CO₂ administration at indicated time points.

LPS pre-treated mice. BALB/c mice were treated with an intra-peritoneal (i.p.) injection of 5 μg LPS (from Escherichia coli serotype O55:B5, Sigma-Aldrich, Zwijndrecht, the Netherlands) or saline on day –2. Subsequently, on day –1 and day 0, mice were injected i.p. with MTX dosages of 100 and 50 mg kg⁻¹ body weight, respectively, or equivalent volumes of saline for controls. Mid-jejunum segments were collected on days 3 and 5 from 4 to 5 animals per treatment group (described in Histology).

LPS-responsive versus -unresponsive mice. On days –1, 0 and 4, C3H/HeJ and C3H/HeN mice were injected i.p. with MTX dosages of 200, 100 and 100 mg kg⁻¹ body weight, respectively, or equivalent volumes of saline for controls. Each treatment group consisted of 8 animals per group. All mice were sacrificed on day 7 after MTX treatment.

Treatment of mice for isolation of lymphocytes and peritoneal macrophages. BALB/c mice were injected i.p. with a single MTX dose of 100 mg kg⁻¹ body weight or an equivalent volume of saline for controls. At 42 h after injection, mononuclear cells of lamina propria (LPMCs), mesenteric lymph nodes (MLNs) and Peyer’s patches (PPs) were isolated (described in Isolation of Lymphocytes). Each study group consisted of at least 4 mice.

IL-10-deficient C57BL/6 (IL-10 KO) versus WT littermates. On day –1 and day 0, mice were treated i.p. with MTX dosages of 100 and 50 mg kg⁻¹ body weight, respectively, or equivalent volumes of saline for controls. On days 1, 3 and 7, segments of mid-jejunum were collected from 6 MTX-treated animals and 3 controls of both WT and KO mice (described in Histology).

Isolation and culture of macrophages

Macrophage cell line. Cells of a murine macrophage cell line (RAW264.7) were obtained from the American Type Culture Collection. Cells were grown in DMEM (Life technologies, Breda, the Netherlands) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Integro, Leuvenheim, the Netherlands), 1% non-essential amino acids (Bio Whitaker, Verviers, Belgium), 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin (Life technologies) and incubated in a humidified incubator at 37°C with 5% CO₂. Cells were exposed to LPS in the presence of various quantities of MTX as indicated. A total of 1 × 10⁶ cells per well were seeded in 96-well plates in the presence of 0 and 1 ng ml⁻¹ LPS. Serial dilutions of MTX ranging from 0 to 12.5 μg ml⁻¹ were added per well. After 24 h of incubation, supernatants were assayed by ELISA for IL-10 and TNF-α (described in Immunoassays for Cytokines).

In vitro MTX treatment of peritoneal macrophages. Naive 10-week-old female BALB/c mice were sacrificed. Resident peritoneal macrophages were collected by flushing the peritoneal cavity with 5 ml of RPMI 1640 medium (Life technologies) containing 10% FBS, 0.015 mol l⁻¹ HEPES,
0.002 mol l⁻¹ L-glutamine (Bio Whittaker), 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin. Cells were centrifuged for 10 min at 1400 revolutions per minute (r.p.m.) and re-suspended in medium. Next, macrophages were counted with Türk solution and 1 × 10⁶ macrophages per well were seeded in 96-well plates in the presence of 10 ng ml⁻¹ LPS. After 24 h of incubation, supernatants were assayed by ELISA for IL-10 and TNF-α.

In vivo MTX treatment of peritoneal macrophages. Ten-week-old female BALB/c mice were injected i.p. with a single MTX dose of 100 mg kg⁻¹ body weight or an equivalent volume of saline for controls. At 42 h after injection, peritoneal macrophages were isolated and cultured as described above. Peritoneal macrophages were stimulated with 0, 1 or 10 ng ml⁻¹ LPS and supernatants were assayed by ELISA for IL-10 and TNF-α at 24 h.

Isolation of lymphocytes

The small intestine from stomach to the ileocecal valve was resected for the isolation of LPMCs, PPs lymphocytes and MLN lymphocytes. Viability of the isolated lymphocytes was determined by trypan blue exclusion.

LPMCs. Mononuclear cells from the small intestinal lamina propria were isolated according to the procedures described by Colgan et al. (24) with some minor modifications.

The intestine was cut open longitudinally, thoroughly washed in Ca²⁺- and Mg²⁺-free (CMF) HBSS (Life technologies) with 0.015 mol l⁻¹ HEPES (Merck/VWR, Amsterdam, the Netherlands) pH 7.2 and cut into small pieces. To remove epithelial cells and intra-epithelial lymphocytes, the intestinal pieces were incubated twice for 20 min in a shaker (250 r.p.m.) at 37°C in 20 ml HBSS–CMF–HEPES supplemented with 10% FBS, 0.005 mol l⁻¹ dithiothreitol (DTT; Sigma-Aldrich), 0.005 mol l⁻¹ EDTA (Merck/VWR), 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin. LPMCs were isolated by incubating the remaining intestinal pieces during two subsequent periods of 1 h at 37°C in a shaker (250 r.p.m.) in 20 ml complete RPMI pH 7.2, containing RPMI 1640 (Life technologies), 10% FBS, 0.005 mol l⁻¹ DTT, 0.015 mol l⁻¹ HEPES, 0.002 mol l⁻¹ L-glutamine (Bio Whittaker), 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin supplemented with 100 U ml⁻¹ collagenase type VIII (Sigma-Aldrich) and 10 µg ml⁻¹ DNAse I (Sigma-Aldrich). Mononuclear cells were harvested by sieving the cell suspension through a 70 µm cell strainer (Micronic, Lelystad, the Netherlands), washed with HBSS–CMF–HEPES and purified by Percoll (Amersham Biosciences, Roosendaal, the Netherlands) density gradient centrifugation (40/90%) at the interface. After washing, cells were suspended in complete Iscove’s Modified Dulbecco’s Medium (IMDM) containing 50 µM β-mercaptoethanol (Merck/VWR).

PPs lymphocytes. The small intestine segment from the stomach to the ileocecal valve was isolated and PPs were excised. Excised PPs were incubated in HBSS–CMF–HEPES supplemented with FBS, EDTA, DTT, penicillin and streptomycin for 20 min in a shaker at 250 r.p.m. at 37°C to remove epithelial cells. Lymphocytes were recovered in HBSS–CMF–HEPES by sieving the PP through a 70 µm cell strainer. After washing with HBSS–CMF–HEPES, lymphocytes were suspended in complete IMDM containing 50 µM β-mercaptoethanol.

MLN lymphocytes. MLNs were placed in HBSS–CMF–HEPES and sieved through a 70 µm cell strainer. After washing with HBSS–CMF–HEPES, lymphocytes were suspended in complete IMDM containing 50 µM β-mercaptoethanol.

Culture and stimulation of lymphocytes

Lymphocyte suspensions were stimulated with hamster anti-mouse CD3α-chain mAbs and hamster anti-mouse CD28 mAb (145-2C11 and 37.51, respectively, BD Pharmingen, Alphen a/d Rijn, the Netherlands). A 96-well plate was pre-coated overnight with 100 µl anti-CD3c (2 µg ml⁻¹) in PBS. Hundred microliters of lymphocyte suspension (10⁶ cells ml⁻¹) in complete IMDM containing 50 µM β-mercaptoethanol was added to the wells combined with 100 µl anti-CD28 (4 µg ml⁻¹) in complete IMDM containing 50 µM β-mercaptoethanol. Cell cultures were maintained in a humified incubator at 37°C with 5% CO₂. After 48 h of incubation, the supernatant was collected for further analysis.

Immunoassays for cytokines

Supernatants of macrophage cultures were assayed by ELISA using the mouse IL-10 BD OptEIA™ ELISA Set (BD Biosciences, Alphen a/d Rijn, the Netherlands) and the mouse TNF-α (Mono/Poly) BD OptEIA™ ELISA Set (BD Biosciences). Measurements were analyzed by a VERSAmax Microplate reader (Molecular Devices Ltd, Wokingham, UK).

Supernatants of LPMCs, PP lymphocytes and MLN lymphocytes were assayed using a Cytometric Bead Array kit for IL-10, TNF-α and IFN-γ according to instructions of the manufacturer (BD Biosciences). IL-2 levels were determined with ELISA (BD Biosciences).

Fluorescent antibody staining of isolated LPMCs

After polyclonal stimulation, cytopsins of 25 000 LPMCs per slide were prepared according to standard procedures. After fixation in methanol, slides were blocked for 1 h at room temperature with 10% normal mouse serum in Tung-T (0.01 M Tris, 0.005 M EDTA, 0.15 M NaCl, 0.25 % gelatin, 0.05 % Tween-20), rinsed with PBS and incubated overnight in the dark at 4°C with antibodies diluted in PBT (1 % BSA, 0.1 % Triton-X100 in PBS): anti-CD3ε-FITC, 1:50 (clone 145-2C11); anti-CD11b-FITC, 1:50 (clone M1/70) (Biologend, San Diego, CA, USA) and anti-TNF-α, 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Texas Red-labeled donkey anti-goat was applied for 1 h at room temperature to detect TNF-α. All slides were mounted with a 4,6-diamidino-2-phenylindole, dihydrochloridehydraat/Mowiol solution. As a control, double and single incubations of the antibodies were included.

Histology

For histology, 5-mm segments of mid-jejunum were fixed in 4% PFA in PBS and paraffin embedded. 4-µm sections were routinely stained with hematoxylin (Vector Laboratories, Burlingame, CA, USA) and eosin (Sigma-Aldrich) to study morphological alterations in crypts and villi. We have developed a histology score in order to quantitate the severity of...
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mucositis. The histology score ranged from 0 to 13 and was subdivided in the following categories: villus aspect (0 = normal, 1 = short, 2 = extremely short), villus tops (0 = normal, 1 = damaged, 2 = severely damaged), epithelium (0 = normal, 1 = flattened, 2 = damaged, 3 = severely damaged), inflammation (0 = no infiltration, 1 = mild infiltration, 2 = severe infiltration), crypts (0 = normal, 1 = mild crypt loss, 2 = severe crypt loss), cryptabcesses (0 = none, 1 = present) and bleeding (0 = none, 1 = present).

Finally, villus lengths were measured (10 villi per histology section). Histology was scored blinded by an independent pathologist.

Statistical analysis

Changes in weight and levels of cytokines were statistically analyzed using the Student’s t-test. A P < 0.05 was considered statistically significant. Data are presented as the mean ± standard error of the mean and n = 4 to 8 mice per group as indicated. All experiments have been performed at least twice. One representative experiment is shown. The IL-10 KO versus WT experiment (as shown in Fig. 5) was performed once (n = 6 mice per treatment group for each selected time point).

Results

LPS pre-treatment is associated with enhanced weight loss and increased signs of mucositis

We sought to determine whether microbial stimuli contribute to MTX-induced mucositis. BALB/c mice, pre-treated with LPS prior to MTX treatment, showed a more profound weight loss compared with saline pre-treated mice. These differences reached statistical significance on days 3 and 4 (Fig. 1A). The severity of weight loss upon MTX treatment corresponded with the intestinal damage as determined by histological analysis on days 3 and 5. Morphological analysis of saline pre-treated mice that were treated with MTX revealed mild epithelial flattening in the crypt and mild crypt loss but no villus atrophy on day 3 (Fig. 1B and C). In contrast, LPS pre-treated mice showed enhanced intestinal damage compared with saline pre-treated mice at this time point as characterized by severe villus atrophy, massive crypt loss and increased cellular infiltration of the lamina propria (Fig. 1B and C). Finally, regeneration occurred in saline pre-treated MTX-treated mice on day 5. However, LPS pre-treated mice regenerated less well since they still displayed villus atrophy and a higher histology score compared with controls on day 5 (Fig. 1B and C). Notably, no morphological changes on any of the assessed days were found in control mice that were treated with LPS or saline only (Fig. 1B and C).

To further elucidate the role of LPS in the pathogenesis of MTX-induced mucositis, we exposed LPS-responsive and LPS-unresponsive mice to MTX. In general, mice with the C3H background appeared to be less susceptible for the MTX-induced mucosal damage when compared with the previously tested mouse strains. In this set of experiments, LPS-responsive mice recovered more slowly compared with LPS-unresponsive mice, which reached significance on days 2, 5, 6 and 7 (Fig. 2). These experiments establish a contributive role for LPS to the severity of MTX-induced mucositis.

MTX does not inhibit LPS-induced TNF-α and IL-10 release by macrophages

To elucidate the nature of the innate immune responses upon MTX treatment, we exposed a macrophage cell line to increasing concentrations of MTX and LPS (1 ng ml−1). Upon LPS stimulation, these cells release both TNF-α and IL-10. As depicted in Fig. 3(A), increasing concentrations of MTX did not affect LPS-induced TNF-α and IL-10 release. Next, we stimulated peritoneal macrophages from naïve mice with LPS (10 ng ml−1) in the presence of increasing concentrations of MTX. Again, MTX did not suppress LPS-induced TNF-α and IL-10 production (Fig. 3B). To establish whether in vivo MTX treatment would affect cytokine production by macrophages, peritoneal macrophages from MTX-treated mice were stimulated ex vivo with LPS (at 1 and 10 ng ml−1). After 24 h of LPS stimulation, peritoneal macrophages that were isolated from MTX-treated mice produced a significantly higher amount of both TNF-α and IL-10 in comparison to peritoneal macrophages isolated from untreated mice (Fig. 3C). Notably, no differences were found in the viability of macrophages after MTX treatment either in vivo or in vitro.

Alterations in cytokine production by lymphocytes from lamina propria, MLNs and PPs derived from MTX-treated mice

Responses of the adaptive intestinal immune system were assessed by determining the production of TNF-α, IFN-γ, IL-2 and IL-10 by re-stimulated mononuclear cells derived from various intestinal locations. Upon polyclonal stimulation with anti-CD3ε and anti-CD28 mAb, lymphocytes from MLN, PP and LPMCs from both MTX-treated and untreated mice produced TNF-α, IFN-γ, IL-10 and IL-2. The production of none of the cytokines studied was significantly inhibited by in vivo MTX treatment (Fig. 4A). Moreover, LPMCs from MTX-treated mice produced significantly more TNF-α, IFN-γ and IL-10 versus LPMCs from non-treated mice. Also, there was a significant increase in TNF-α production in MLN cultures of MTX-treated mice compared with untreated controls. PP lymphocytes from MTX-treated mice produced significantly more IL-2 than PP lymphocytes from untreated controls (Fig. 4). The fact that lymphocytes from all intestinal locations produce IL-2 confirms that adaptive immune cells are functional despite MTX treatment since IL-2 is specifically produced by lymphocytes. However, within LPMCs it is possible that non-lymphocytes contribute to the cytokine production observed. To address this question TNF-α/CD3 and TNF-α/CD11b immunohistochemistry of polyclonally stimulated LPMCs was performed. These stainings show that both CD3+ lymphocytes as well as CD11b+ monocytes/macrophages are the source of the TNF-α (Fig. 4B). These experiments show that both innate and adaptive mucosal immune cells are still capable of producing pro-inflammatory cytokines such as TNF-α and IFN-γ as well as the anti-inflammatory cytokine IL-10 during mucositis.
IL-10 restricts MTX-induced mucositis

To gain further insight into the role of IL-10 in mucositis development in vivo, we treated IL-10 KO mice and their WT littermates with MTX. MTX-treated IL-10 KO mice lost profoundly more weight in comparison to MTX-treated WT controls that reached significance on days 1 and 2 (Fig. 5A). On days 1 and 3 after MTX treatment, analysis of the intestinal morphology of IL-10 KO mice revealed strongly increased symptoms of mucositis in comparison to WT mice (Fig. 5B and C). On day 7, both groups appeared to regenerate equally well (Fig. 5B and C). Notably, at this age, we did not detect any pathological change in the intestinal morphology of non-treated IL-10 KO mice, which has been described in IL-10 KO mice at later time points (Fig. 5B and C).

Discussion

In this study, we showed that during MTX-induced mucositis the mucosal immune system is still able to respond to bacterial stimuli, which is remarkable considering the immunosuppressive capacity of MTX. In healthy individuals, mucosal homeostasis of the gastrointestinal tract requires the involvement of innate cells, such as macrophages, and adaptive cells, such as B and T cells. The typical innate responsiveness of macrophages to LPS is not suppressed either by in vivo or in vitro MTX treatment. Notably, in our experimental set-up these cells expressed an enhanced susceptibility to LPS stimulation that indicates that MTX treatment may rather prime
instead of suppress these cells. The adaptive immune responses are also intact as reflected by the capacity of lamina propria, MLN and PP lymphocytes from MTX-treated mice to respond to anti-CD3$\varepsilon$ and anti-CD28 mAb stimulation.

MTX is effective as chemotherapy through the inhibition of cellular proliferation. As a result, rapidly dividing intestinal epithelial cells are strongly affected, leading to severe intestinal barrier dysfunction. Subsequently, the innate immune cells are increasingly exposed to microbial immunogens such as LPS that is associated with activation of the mucosal immune system. We have established a contributive role for LPS in the pathogenesis of mucositis by showing that LPS pre-treatment enhances MTX-induced intestinal damage. These data are substantiated by our observations that LPS-responsive mice regained weight more slowly compared with LPS-unresponsive mice after MTX treatment.

In order to establish whether disruption of the intestinal barrier would also lead to activation of adaptive immune responses, we isolated LPMCs, PP lymphocytes and MLN lymphocytes of mice treated with MTX. Indeed, upon ex vivo re-stimulation, these cells produced enhanced levels of cytokines. This finding indicates that instead of MTX-induced immune suppression, endogenous priming of these lymphocytes may have occurred as a consequence of MTX-induced mucositis.

As activation of the mucosal immune system can lead to pro-inflammatory as well as anti-inflammatory processes, we determined the production of TNF-$\alpha$ and IL-10 by these mucosal T cells.

Intriguingly, besides the anticipated pro-inflammatory TNF-$\alpha$ response, we also detected a robust IL-10 release by LPMCs. As IL-10 is strongly implicated in protection from intestinal inflammatory processes such as IBD, this prompted us to specifically investigate the potential protective role for IL-10 in MTX-induced mucositis. Indeed, we established that mice deficient in IL-10 experience more weight loss and enhanced intestinal damage on histology compared with WT controls. To a certain extent, these data imply that the pathogenesis of mucositis has strong similarities to that of inflammatory bowel diseases.

The pivotal finding of this study is that both innate and adaptive immune responses remain intact during MTX treatment. In response to enhanced exposure to microbial-derived stimuli, a combined pro- and counter-inflammatory response is elicited. How and to what extent these responses contribute to the damage and repair that is associated with mucositis is difficult to establish.

Based on these data, we support that selective targeting of the pro-inflammatory response during mucositis may become a beneficial strategy (18, 19, 25). As such, this may facilitate ongoing protective anti-inflammatory responses. In line with this view, other strategies such as the use of antibiotics aiming at neutralizing specific microbial stimuli (i.e. LPS) appear...
Fig. 4. Increased cytokine release ex vivo, by re-stimulated LPMCs, cells from MLNs and cells from PPs derived from MTX-treated mice in comparison to controls. (A) We determined the production of TNF-α, IFN-γ, IL-10 and IL-2 by stimulated T cells derived from various intestinal locations. Upon polyclonal stimulation (anti-CD3/anti-CD28), LPMCs from MTX-treated mice produced significantly more TNF-α, IFN-γ and IL-10 versus LPMCs from non-treated mice. MLN-derived lymphocytes from MTX-treated mice produced significantly more TNF-α and PP-derived lymphocytes from MTX-treated mice produced significantly more IL-2 compared with lymphocytes derived from untreated mice (P-values are indicated). (B) TNF-α/CD3 and TNF-α/CD11b double stainings of polyclonally stimulated LPMCs from MTX-treated (right panel) or untreated (left panel) mice. Red-stained cells are TNF-α-producing cells. Green-stained cells are either CD3+ (upper panel) or CD11b+ (lower panel). Yellow cells indicate double positivity for CD3 and TNF-α (upper panel) or CD11b and TNF-α (lower panel). These figures indicate that both innate (CD11b+) and adaptive (CD3+) immune cells within the lamina propria are capable of producing TNF-α despite MTX treatment.
crucial. Finally, novel experimental therapies for IBD patients such as the application of IL-10-producing Lactococci may provide attractive means to prevent mucositis (14, 15).

Acknowledgements

We thank Prof. H. A. Büller for critically reading the manuscript. This work was supported by a grant from Numico Research BV, Wageningen, the Netherlands.

Abbreviations

CMF Ca\(^{2+}\) and Mg\(^{2+}\) free
DTT dithiothreitol
FBS fetal bovine serum
i.p. intra-peritoneal
KO knockout
LPMCs lamina propria mononuclear cells
MLNs mesenteric lymph nodes
MTX methotrexate
PP Peyer’s patch
r.p.m. revolutions per minute
SPF specific pathogen-free
TNF-\(\alpha\) tumor necrosis factor-\(\alpha\)
WT wild type

References


Fig. 5. IL-10 restricts severity of MTX-induced mucositis. (A) IL-10 KO mice and their WT littermates were treated with MTX. MTX-treated IL-10 KO mice lost more weight in comparison to MTX-treated WT controls. (Arrows indicate the timing of MTX or saline treatment i.p. and asterisks indicate statistically significant difference). (B) Increase of histopathological changes in intestinal samples (increased crypt and epithelial loss and complete villus atrophy) derived from IL-10 KO mice in comparison to samples from their WT littermates on day 3. (C) Increase of histopathological score and reduced villus lengths displayed by IL-10 KO mice in comparison to WT littermates on days 1 and 3 (P-values are indicated).


