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Decreased numbers of FoxP3-positive and TLR-2-positive cells in intestinal mucosa are associated with improvement in patients with active inflammatory bowel disease following selective leukocyte apheresis

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Background. Impaired immunological tolerance to commensal enteric flora is considered one possible pathogenic mechanism of inflammatory bowel disease (IBD). Given that regulatory T cells and Toll-like receptor (TLR)-positive cells are key actors in mucosal immune regulation, we aimed to identify the dynamics of these actors in the intestinal mucosa in relation to clinical improvement following selective leukapheresis treatment. ***Methods.*** Ten patients with active IBD despite treatment with corticosteroids, immunomodulators, or anti-tumor necrosis factor therapy were assessed by immunohistochemical staining of colorectal mucosal biopsies obtained before and after five sessions (week 7) of granulocyte and monocyte adsorption apheresis (GCAP). The presence of FoxP3-positive regulatory T cells, macrophages, dendritic cells, and TLR-2 and -4 positive cells was determined in relation to short- (week 7) and long-term (week 52) clinical outcome data. ***Results.*** Following GCAP, the number of FoxP3- ($P = 0.012$) and TLR-2 ($P = 0.008$)-positive cells significantly decreased in biopsies after 7 weeks, in parallel with both clinical improvement at week 7 and a longstanding response after 12 months. ***Conclusions.*** Downregulation of FoxP3 and TLR-2 cells in the colorectal mucosa mirrors both short- and long-term improvement in patients with active IBD responding to GCAP. This observation suggests a potential role of these cells in the pathogenesis of IBD and the induction of immunological tolerance in the mucosa.

Key words: FoxP3, TLR, IBD, selective leukocyte apheresis

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Introduction

One hypothesis regarding the etiopathogenesis of inflammatory bowel disease (IBD) is a loss of immunological tolerance, manifested by increased humoral and cellular immune responses toward commensal enteric flora.[1] A variety of cells participate in this inflammatory response, including regulatory T cells, Toll-like receptor (TLR)-bearing dendritic cells, macrophages, and epithelial cells.

A balance between effector cells and regulatory T cells is pivotal for immunological homeostasis in the intestinal mucosa, and a breakdown of this balance may be partly responsible for the mucosal inflammation that is characteristic of IBD patients.[2,3] Already 10 years ago, Duchmann et al.[4] demonstrated that mononuclear effector cells in the intestine proliferate when exposed to unfamiliar bacterial flora, and regulatory T cells have recently been shown to suppress the proliferation and cytokine secretion of colonic effector CD4+ T cells.[5] Moreover, the number of regulatory T cells increases with disease activity in patients with ulcerative colitis,[5] which is in line with data from an animal model of IBD.[2] A specific marker for regulatory cells described recently is the forkhead transcription factor FoxP3.[6] FoxP3 correlates with suppressor activity irrespective of CD25 expression and acts as a determinant of regulatory T-cell lineage.[7]

Intestinal dendritic cells have been proposed to constitute key initiators and perpetuators of mucosal inflammation, and they display upregulated expression of microbial recognition receptors such as TLR-2 and -4 in IBD patients.[3] Recently, it was reported that TLR-2 polymorphisms may affect disease severity in IBD patients, further supporting a role for TLR expression in this disease entity.[8]

We have recently demonstrated long-term improvement in this group IBD patients following granulocyte and monocyte adsorption apheresis, which was paral-

leled by significant downregulation of interferon γ-producing cells in the mucosa and in circulation.[9] Earlier GCAP studies included mainly ulcerative colitis (UC) patients, and the clinical part of our study was the first European pilot study to include mostly patients with Crohn's disease (CD). Given that regulatory T cells and TLR expression constitute additional key actors in mucosal immune regulation, in this study we aimed to identify the dynamics of these actors in the intestinal mucosa in relation to clinical improvement following GCAP treatment.

Materials and methods

Patients

Ten patients (seven with CD, three with UC; median age, 31 years; three men and seven women) with mild to moderate chronic active IBD were studied. All patients were refractory to or dependent on corticosteroids, and all had been treated previously with azathioprine but were either intolerant or refractory. Seven patients had received infliximab; patients 2, 8, 9, and 10 ceased to respond to infliximab, and patients 1, 3, and 7 stopped infliximab treatment because of severe side effects. However, no patient received infliximab during the 6 months preceding inclusion in the present study.

All anti-inflammatory drugs were continued with unchanged doses, but the oral prednisolone dose was allowed to taper by 2.5 mg/week if the patient's condition improved.

The clinical activity index (CAI) in UC patients was calculated according to Rachmilewitz;[10] a CAI reduction of 3 points or more was considered to indicate a clinical response, and a score of 4 or less to indicate clinical remission. A clinical response in CD patients was defined as a reduction of 70 points or more in the Crohn's disease activity index (CDAI), and a reduction to less than 150 was considered clinical remission.[11]

Eight of ten patients improved clinically with good long-term effect after Adacolumn leukocyte apheresis (GCAP) was done once weekly over 5 consecutive weeks.[9]

Immunohistochemical staining of colonic biopsy materials, taken from the area most affected by inflammation before the start of treatment and 2 weeks after the last treatment (7 weeks after the start of treatment), was performed as described previously.[9] The patient data were presented by us earlier,[9] and we performed other immunostainings at that time.

Biopsies and immunostaining

The biopsy samples were snap-frozen immediately. The samples were section and fixed in acetone (CD25 and CD163) or formalin (FoxP3, BDCA-2, -3, -4, and TLR-2 and -4) before staining. The following specific monoclonal antibodies (mAbs) were used for immunostaining: FoxP3 (clone 236A/E7; a generous gift from Dr. Alison H. Banham, Oxford, UK[12]); TLR-2 (clone 14-9024, T2, 5) and TLR-4 (clones 14-9917, HTA 125) (Biosite, San Diego, CA, USA); markers for lymphoid DC BDCA 2 (MACS 130-90-690, mouse IgG1 clone; Ac 144) and BDCA4 (MACS 130-090-693, mouse IgG1 clone) (Miltenyi Biotec, Bergisch Gladbach, Germany); marker for myeloid DC BDCA 3 (MACS 130-090-694, mouse IgG1 clone; AD5-14H12; Miltenyi Biotec); macrophage marker CD163 (MO794, lot 030(401), BerMac; Dako, Glostrup, Denmark); T-cell marker CD3 (Leu-4, 347340, lot 35104; PharMingen, Becton Dickinson, San Diego, CA, USA); interleukin (IL)-2 receptor marker (activated T-cell marker) CD25 (MO731 clone, ACT-1; Dako).

The staining procedure was briefly as follows. First, the slides were immersed in a mixture of 1% H_2O_2 and 2% NaN_3 in phosphate-buffered saline (PBS) for 1 h in the dark at room temperature to block endogenous peroxidase activity. Primary monoclonal antibodies (mAbs) in a mixture of PBS, bovine serum albumin (BSA), NaN_3, and saponin were used for the formalin-fixed slides. The primary antibodies were incubated overnight in a humid chamber. Incubation with normal horse serum (NHS) (Vector Laboratories, Burlingame, CA, USA) was used in the blocking procedure. The secondary antibody was diluted in a mixture of PBS and NHS for incubation. Sections were incubated in a solution of avidin–biotin–horseradish peroxidase (Vectastain, ABC-HP-standard kit or ABC-HP elite kit, Vector Laboratories) diluted in PBS or PBS–saponin. The staining reaction was developed with a Peroxidase Substrate Kit (Vector Laboratories) containing 3,3′-diaminobenzidine tetrahydrochloride (DAB).

The sections were assessed on two different occasions by two independent observers, in all cases with concordant results. All examinations were done in a blinded manner with regard to patient response, biopsy site, and time point. The assessments were done by semiquantitative evaluation with a grading scale of 0 to 3 as follows: 0, no visible staining; 1, 1–10 cells stained; 2, 10–50 cells; and 3, >50 cells. Mean values are reported in the results.

Statistics

Statistical analyses were done by with Wilcoxon nonparametric tests. P values < 0.05 were considered significant.

Ethical considerations

The study was approved by the local ethics committee at the Karolinska Institutet in Stockholm.

Results

Clinical results

Clinical improvement was defined as described in the Methods section. Eight of ten patients had clinically improved at 12 months;[9] four CD patients and two UC patients were in clinical remission, and one CD patient and one UC patient showed a clinical response (Table 1).

Immunostaining

The number of FoxP3-positive cells in the mucosa (Fig. 1a and b, and Table 2) decreased significantly. The FoxP3 pattern staining was similar to that reported previously.[6] Furthermore, the number of TLR-2-

Table 1. Clinical activity index

Patient no.	Sex	IBD type	Age (years)	CAI[a]			CDAI[b]		
				W0	W7	W52	W0	W7	W52
1	M	CD	27				374	186	133
2	F	CD	29				295	315	243
3	M	CD	31				218	122	64
4	F	UC	51	16	12	1			
5	F	UC	31	13	10	8			
6	F	CD	27				345	204	203
7	F	CD	32				285	182	47
8	F	UC	28	12	6	4			
9[c]	M	CD	31				291	—	
10	F	CD	51				191	171	124

CAI, clinical activity index; CDAI, Crohn's disease activity index; IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; W, week
[a] UC patients
[b] CD patients
[c] Patient 9 did not improve and had to undergo a colectomy before week 7

Table 2. Immunohistochemical staining[a] in colonic biopsy samples before and 2 weeks after treatment (week 7)

Patient no.	IBD	FoxP3 T regulatory cells		TLR-2[b] expression		CD163 macrophages		TLR-4 expression		BDCA3 DCs		CD25-activated T cells	
		W0	W7	W0	W7	W0	W7	W0	W7	W0	W7	W0	W7
1	CD	1	1	3	2.5	3	2	2	2	1	1	1	1
2[c]	CD	2	1	3	0	2	2	0	0	1	1	1	0
3	CD	1	0	2.5	2	2	2	1	1	2	1	1	1
4	CD	3	0	3	1	2	2	2	0	2	1	3	1
5	UC	2	1	3	1	2.5	2	2	0	1	1	1	1
6	UC	2	0	3	1	2.5	1.5	2	0.5	2	1	2	1
7	CD	2.5	1	3	0	2	1	1	0	2	2	1	2
8	UC	2	1	3	0	3	1	0	0	2	2	2	1
9[c]	CD	1	Op[d]	3	Op[d]	2.5	Op[d]	0	Op[d]	2	Op[d]	1	Op[d]
10	CD	3	1	3	1	1.5	1.5	0	1	2	1	2	1
P value		0.012		0.008		0.046 ($n = 8$[e]) 0.076 ($n = 9$[g])		NS		NS		NS	

Week 0 values are those before the first treatment
DCs, dendritic cells; TLR, Toll-like receptor; Op, operation; NS, not significant
[a] Semiquantitative grading scale: 0, no visible staining; 1, 1–10 cells stained; 2, 10–50 cells; 3, > 50 cells
[b] Lamina propria only
[c] No clinical improvement or response at week 52
[d] Patient 9 was referred for colectomy before the final visit
[e] Excluding patients 2 and 9
[g] Excluding patient 9

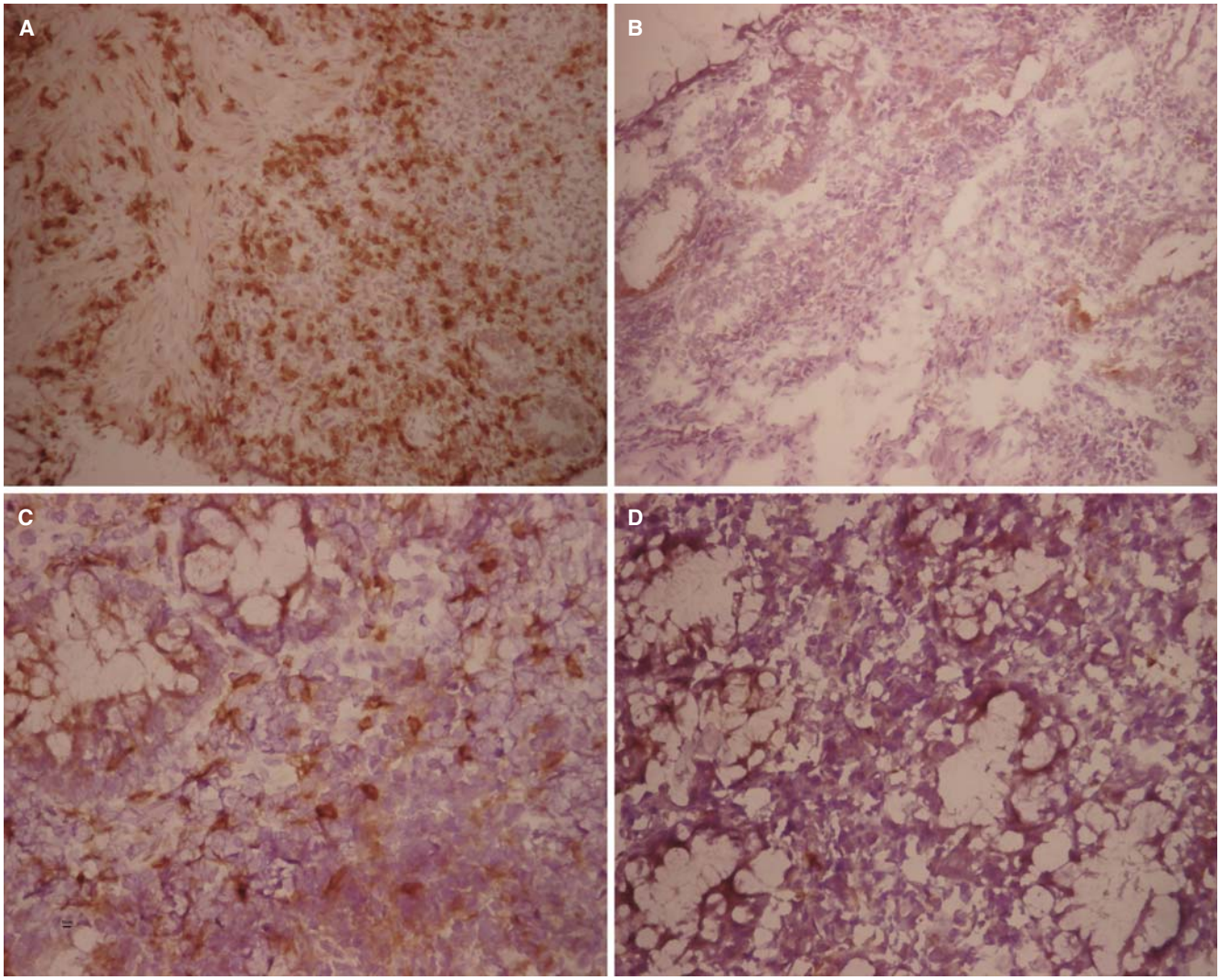

Fig. 1. Immunohistochemical staining of biopsy specimens from inflammatory bowel disease patients. **a** FoxP3-positive staining before the treatment start. **b** FoxP3-positive staining 2 weeks after the fifth treatment. **c** Toll-like receptor (TLR)-2-positive staining before the treatment start. **d** TLR-2-positive staining 2 weeks after the fifth treatment. Magnification, **a**, **b**, ×200; **c**, **d**, ×400

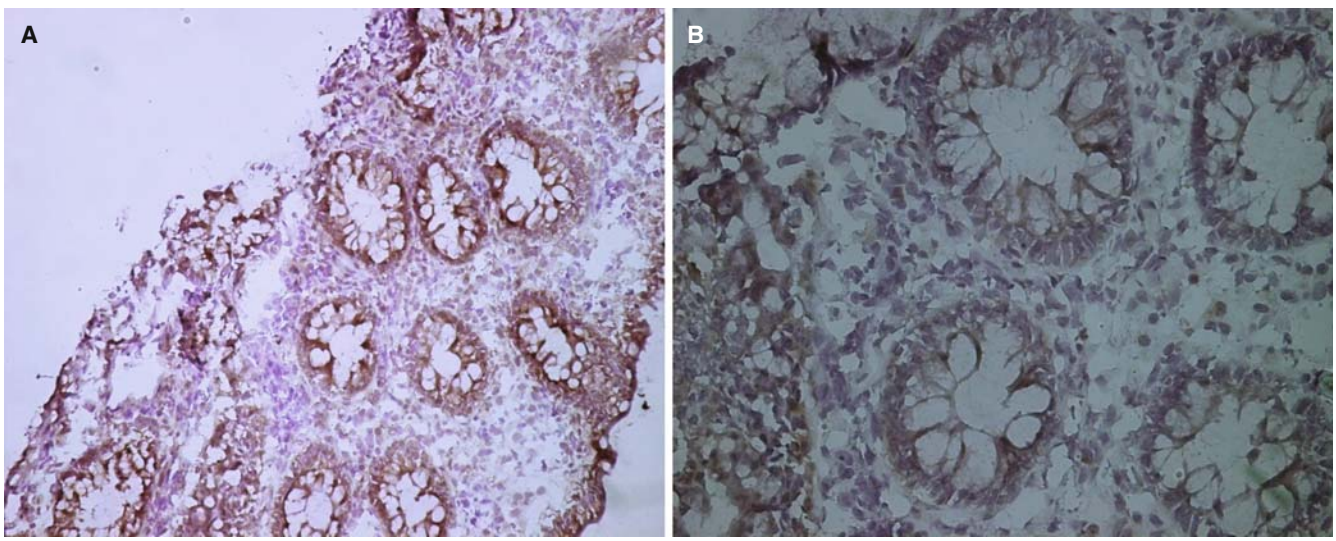

Fig. 2. **a** FoxP3 and **b** TLR-2 staining pattern in colorectal mucosal biopsy specimens from healthy subjects without inflammatory bowel disease. Magnification, **a**, ×200; **b**, ×400

positive cells decreased significantly in the lamina propria, but not in the epithelium (Fig. c and d, and Table 2). TLR-4-positive cells were unchanged. No significant differences in BDCA-2, -3, or -4 or in CD3 or CD25 (Table 1) were observed. In the patients who experienced a long-term treatment effect ($n = 8$), the number of CD163-positive cells decreased significantly ($P = 0.046$), but when all patients were included ($n = 9$), the decrease was not significant ($P = 0.076$) (Table 2). The UC and CD groups analyzed separately showed equivalent responses in the measured parameters. In the CD group, FoxP3 expression was significantly downregulated ($P = 0.043$) and TLR-2 expression was also diminished significantly ($P = 0.027$). Similarly, expression of both FoxP3 and TLR-2 was downregulated in the three UC patients (Table 2).

Discussion

The main observation of this study is that the clinical improvement after GCAP parallels downregulation of mucosa-dwelling FoxP3- and TLR-2-positive cells. The clinical response was similar in both CD and UC patients and in keeping with the results of other studies.[12]

Initially, regulatory T cells were identified as controlling autoimmune inflammation, and they have also been shown to regulate inflammation caused by microorganisms and allergens.[13] Emerging data indicate that compartmentalization and trafficking of regulatory T cells are strictly regulated by specific adhesion molecules and chemokine receptor expression.[14] A role of regulatory FoxP3+ T cells in IBD has been indicated by data demonstrating that these cells may prevent and even abolish established colitis in an animal IBD model.[15] That mucosal-dwelling FoxP3+ T cells are functional is shown by the observation that their regulatory properties are retained when they are cultured from colonic mucosa of IBD patients.[16]

A protective role of regulatory T cells in IBD is suggested by their dynamics in different locations. It was recently shown that peripheral circulating regulatory T cells are decreased in patients with active IBD but increased in patients in remission.[2] In parallel, the regulatory T-cell population is increased in inflamed mucosa compared with in nonaffected mucosa.[2,17] Additionally, functional characterization of regulatory T cells isolated from mesenteric lymph nodes of UC patients has recently shown that these cells preserve their suppressive features.[18] Given the proposed protective role of regulatory T cells, we suggest that the decreased numbers of FoxP3-positive cells in the colonic mucosa after successful selective leukocyte apheresis reflects a more balanced immunological homeostasis in the intestinal mucosa. However, further studies are warranted to confirm a causative role of these cells in the pathogenesis of IBD.

We did not find a strict relationship between FoxP3 and CD25 staining, and FoxP3+ cells were slightly more numerous than CD25+ cells at the start of treatment. It is not clear how these data should be interpreted, but they suggest that FoxP3 expression is not exclusively linked to CD25 expression, which is supported by recent studies.[19] Even though the assessments were done on two different occasions by two independent observers, in all cases with concordant results, one should interpret small differences with caution.

Attention has recently been paid to the innate immune response and the interaction between the bacterial flora and the immune cells, especially macrophages and dendritic cells. We noted a marked decrease in TLR-2-positive, but not TLR-4-positive, cells after leukocyte apheresis. This decrease occurred exclusively in the lamina propria; no change in TLR-2 or -4-positive cells was noted in the epithelium. The selective decrease in TLR-2 expression may reflect differences in the regulation of TLR-2 and TLR-4 expression. For example, TLR-2 expression is induced by bacterial infections, whereas TLR-4 is constantly expressed.[20] The number of macrophages decreased in parallel with the downregulation of TLR-2-positive cells in clinically improved patients.

Several lines of evidence underscore that bacterial flora play a key role in the pathogenesis of IBD. TLR-2 has specificity for Gram-positive bacteria and lipopolysaccharide derived from *Bacteroides fragilis*, *Chlamydia trachomatis*, *Pseudomonas aeruginosa*,[21] and protozoan parasites such as *Treponema*,[22] and a clinical response to treatment with antibiotics has been reported in IBD patients.[23] Tolerance or hyporesponsiveness to bacterial lipoproteins may therefore represent a protective mechanism and may be associated partly with downregulation of TLR-2 expression.[24] The dynamics of TLR-2 expression observed in our study may support the view that IBD results from a breakdown of tolerance and a subsequent immune response toward the commensal enteric flora, in which upregulation of microbial recognition receptors may play a potential pathological role.

In summary, we demonstrated that clinical improvement in IBD patients following GCAP is paralleled by downregulation of FoxP3- and TLR-2-expressing cells, suggesting a potential role of these cells in the pathogenesis of IBD and the induction of immunological tolerance in the mucosa.

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References

1. Ohkusa T, Nomura T, Sato N. The role of bacterial infection in the pathogenesis of inflammatory bowel disease. Intern Med 2004;43: 534–9.
2. Maul J, Loddenkemper C, Mundt P, Berg E, Giese T, Stallmach A, et al. Peripheral and intestinal regulatory CD4+CD25^{high} T cells in inflammatory bowel disease. Gastroenterology 2005;128:1868–78.
3. Hart AL, Al-Hassi HO, Rigby RJ, Bell SJ, Emmanuel AV, Knight SC, et al. Characteristics of intestinal dendritic cells in inflammatory bowel diseases. Gastroenterology 2005;129:50–65.
4. Duchmann R, Schmitt E, Knolle P, Meyer zum Buschenfelde KH, Neurath M. Tolerance towards resident intestinal flora in mice is abrogated in experimental colitis and restored by treatment with interleukin-10 or antibodies to interleukin-12. Eur J Immunol 1996;26:934–8.
5. Holmen N, Lundgren A, Lundin S, Bergin AM, Rudin A, Sjövall H, et al. Functional CD4+CD25^{high} regulatory T cells are enriched in the colonic mucosa of patients with active ulcerative colitis and increase with disease activity. Inflamm Bowel Dis 2006;12:447–56.
6. Roncador G, Brown PJ, Maestre L, Hue S, Martinez-Torrecuadrada JL, Ling KL, et al. Analysis of FoxP3 protein expression in human CD4+CD25+ regulatory T cells at the single-cell level. Eur J Immunol 2005;35:1681–91.
7. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. Immunity 2005;22:329–41.
8. Pierik M, Joossens S, Van Steen K, Van Schuerbeek N, Vlietink R, Rutgeers P, et al. Toll-like receptor-1, -2, and -6 polymorphisms influence disease extension in inflammatory bowel diseases. Inflamm Bowel Dis 2006;12:1–8.
9. Muratov V, Lundahl J, Ulfgren AK, Elvin K, Fehrman I, Ahlborg N, et al. Downregulation of interferon-γ parallels clinical response to selective leukocyte apheresis in patients with inflammatory bowel disease. A 12-month follow-up study. Int J Colorectal Dis 2006;21:493–504.
10. Rachmilewitz D. Coated mesalazine (5-amino-salicilic acid) versus sulphasalazine in treatment of active ulcerative colitis: a randomised trial. BMJ 1989;298:82–6.
11. Best WR, Becktel JM, Singleton JW. Rederived values of eight coefficients of the Crohn's Disease Activity Index (CDAI). Gastroenterology 1979;77(4 Pt 2):843–6.
12. Ljung T, Thomsen OØ, Vatn M, Karlén P, Karlsen LN, Tysk C, et al. Granulocyte, monocyte/macrophage apheresis for inflammatory bowel disease: the first 100 patients treated in Scandinavia. Scand J Gastroenterol 2007;42:221–7.
13. Suri-Payer E, Fitzsching B. Regulatory T cells in experimental autoimmune disease. Springer Semin Immunopathol 2006;28:3–16.
14. Huehn J, Siegmund K, Lehmann JCU, Siewert C, Haubold U, Feuerer M, et al. Developmental stage, phenotype, and migration distinguish naive- and effector/memory-like CD4^{+} regulatory T cells. J Exp Med 2004;199:303–13.
15. Denning TL, Qi H, König R, Scott KG, Naganuma M, Ernst PB. CD4+ T cells resembling regulatory T cells that inhibit chronic colitis differentiate in the absence of interactions between CD4 and class II MHC. J Immunol 2003;171:2279–86.
16. Kelsen J, Agnholt J, Hoffmann HJ, Rømer JL, Hvas CL, Dahlerup JF. FoxP3+CD4+CD25+ T cells with regulatory properties can be cultured from colonic mucosa of patients with Crohn's disease. Clin Exp Immunol 2005;141:549–57.
17. Uhlig HH, Coombes J, Mottet C, Izcue A, Thompson C, Fanger A, et al. Characterization of FoxP3+ CD4+ CD25+ and IL-10-secreting CD4+ CD25+ T cells during cure of colitis. J Immunol 2006;177:5852–60.
18. Yu QT, Satura M, Avanesyan A, Fleshner PR, Banham AH, Papadikis KA. Expression and functional characterization of FOXP3+CD4+ regulatory T cells in ulcerative colitis. Inflamm Bowel Dis 2006;13:191–9.
19. Lundgren A, Strömberg E, Sjöling Å, Lindholm C, Enarsson K, Edebo A, et al. Mucosal FoxP3-expressing CD4+ CD25^{high} regulatory T cells in *Helicobacter pylori*-infected patients. Infect Immun 2005;73:523–31.
20. Matsuguchi T, Musikacharoen T, Ogawa T, Yoshikai Y. Gene expressions of Toll-like receptor 2, but not Toll-like receptor 4, is induced by LPS and inflammatory cytokines in mouse macrophages. J Immunol 2000;165:5767–72.
21. Erridge C, Pridmore A, Eley A, Stewart J, Poxton IR. Lipopolysaccharides of *Bacteroides fragilis*, *Chlamydia trachomatis* and *Pseudomonas aeruginosa* signal via toll-like receptor 2. J Med Microbiol 2004;53:735–40.
22. Opitz B, Schröder NWJ, Spreitzer I, Michelsen KS, Kirschning CJ, Hallatschek W, et al. Toll-like receptor-2 mediates *Treponema* glycolipid and lipoteichoic acid-induced NF-κB translocation. J Biol Chem 2001;276:22041–7.
23. Gionchetti P, Rizzello F, Lammers KM, Morselli C, Tambasco R, Campieri M. Antimicrobials in the management of inflammatory bowel disease. Digestion 2006;73 Suppl 1:77–85.
24. Wang JH, Doyle M, Manning BJ, Di Wu Q, Blankson S, Redmond HP. Induction of bacterial lipoprotein tolerance is associated with suppression of toll-like receptor-2 expression. J Biol Chem 2002;277:36068–75.