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Preparation of functionally preserved $CD4^+$ $CD25^{high}$ regulatory T cells from leukapheresis products from ulcerative colitis patients, applicable to regulatory T-cell transfer therapy

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Background

Ulcerative colitis (UC) is an intractable disease, therefore new therapies need to be developed. $CD4^+$ $CD25^{high}$ regulatory T cells (Treg) significantly ameliorate colitis in animal models. In active UC patients, although Treg are functionally preserved, their proportion in peripheral blood decreases. Thus Treg transfer therapy is expected to be efficacious for UC. During leukapheresis for UC, Treg are depleted, as well as colitogenic effector leukocytes. We therefore designed a leukapheresis/Treg transfer therapy in which Treg are isolated from leukapheresis products and transfused to patients, and studied large-scale germ-free methods of Treg preparation.

Methods

Using the CliniMACS cell selection system, we conducted Treg isolation experiments from leukapheresis products in which B and $CD8^+$ T cells were depleted, followed by positive selection of $CD25^+$ cells. In some experiments, isolated Treg or non-Treg were expanded with interleukin-2 (IL-2) ± transforming growth factor (TGF)-β1. Expression of a Treg-specific marker, FOXP3, and gut-homing receptors, and suppressor activity of isolated or cultured cells, were analyzed.

Results

$CD4^+$ $CD25^{high}$ T cells were collected and efficiently enriched with a good recovery rate. Isolated cells preferentially expressed FOXP3 and significantly suppressed T-cell proliferation in vitro. *In addition, isolated Treg could be efficiently expanded, and Treg could be induced from non-Treg with TGF-β1* in vitro. *TGF-β1 significantly up-regulated αEβ7 and α4β7 integrins.*

Discussion

We have established a method of Treg isolation from leukapheresis products that can be used clinically, therefore, Treg transfer therapy is feasible in combination with leukapheresis for UC. Expansion or induction of Treg in vitro *may be another approach to Treg-based immunotherapy.*

Keywords

adoptive transfer, leukapheresis, regulatory T cell, ulcerative colitis.

Introduction

Ulcerative colitis (UC), a form of inflammatory bowel disease (IBD), is a chronic inflammatory disorder that causes persistent colonic inflammation. It mainly affects adolescents and young adults and deeply impairs their quality of life for a long period of time. Its etiology remains unknown; therefore, immunomodulators, including corticosteroids, which suppress immune responses in a non-specific manner, have been the mainstay of treatment for this disease. However, the development of innovative therapy is desperately needed because, despite conventional therapy, the majority of patients experience repeated recurrence of the disease following remission, and a considerable number of patients with severe UC ultimately undergo colectomy.

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Through much research, a gradual understanding of the pathophysiologic process of this disease has been gained, and the evidence suggests that an imbalance between colitogenic effector and the regulatory function of the intestinal immune system is responsible for colonic inflammation.

T cells with immunosuppressive capability play an important role in the maintenance of homeostasis by actively regulating immune reactions. One such subset of T cells, naturally occurring $CD4^+$ $CD25^+$ regulatory T cells (Treg), is characterized by their constitutive expression of CD25, cytotoxic T-lymphocyte antigen-4 (CTLA-4), glucocorticoid-induced tumor necrosis factor (TNF) receptor, and the transcription factor forkhead box P3 (FOXP3) [1]. Treg exhibit a hyporesponsiveness to stimuli through T-cell receptors, and possess the ability to suppress $CD4^+$ $CD25^-$ T-cell and $CD8^+$ T-cell proliferation *in vitro*. Treg are essential for the prevention of intestinal inflammation and thus for the maintenance of gut homeostasis. The transfer of naive $CD4^+$ $CD45RB^{high}$ T cells into immunodeficient recipient mice leads to the development of chronic persistent colitis, which resembles human IBD. This phenomenon is the result of the absence of Treg in the transferred cell population, and the co-transfer of Treg into such recipient mice clearly prevents and even cures colonic inflammation [2,3]. The existence of Treg was first demonstrated in mice [4–7] and they have also been identified in human peripheral blood [8,9]. In mice, Treg constitute a typically distinct population of $CD25^+$ cells that comprise 5–10% of thymic, lymph node and splenic $CD4^+$ T cells. In humans, however, up to 40% of peripheral blood $CD4^+$ T cells express CD25 to some extent. It has been shown that only a minority of human $CD4^+$ T cells that express the highest levels of CD25 ($CD25^{high}$) have suppressor activity [8]. The function of Treg seems to be qualitatively preserved in UC patients, as isolated Treg from peripheral blood or intestinal lamina propria in IBD patients reveal suppressor activity *in vitro*, similar to those in healthy subjects [10–12]. However, Treg seem to be quantitatively reduced, as the percentage of Treg is significantly reduced in peripheral blood of active UC patients, in inverse correlation with disease activity [13]. Thus Treg-based cell therapy is expected to be efficacious for the treatment of UC.

Apheresis that depletes large numbers of leukocytes from circulating peripheral blood has been developed for the treatment of UC [14–17]. It is now considered a standard therapy for patients with refractory UC who are steroid-resistant or -dependent in Japan, and its use is spreading to other countries. There are three methods of extracorporeal circulation for leukapheresis: (1) leukapheresis using a centrifugal cell separator; (2) leukapheresis by an adsorptive membrane filter (Cellsorba™); and (3) granulocyte and monocyte/macrophage apheresis by a column filled with cellulose diacetate beads (Adacolumn™). Each method removes different cell populations and thus seems to work through different mechanisms. The former two methods deplete a large number of lymphocytes. As the depletion of lymphocytes by these methods is non-selective, numerous Treg are removed during leukapheresis, together with colitogenic effector lymphocytes. We have considered a possible new therapy in which Treg are isolated from the lymphocytes removed during leukapheresis, and retransfused into UC patients, and named it leukapheresis/Treg transfer therapy. By such therapy, selective depletion of colitogenic effector T cells from UC patients and an increase in the Treg/effector ratio are theoretically possible. To conduct such adoptive transfer therapy with Treg, a large-scale Treg isolation method, which is clinically applicable, needs to be established. We chose the CliniMACS cell selection system from Miltenyi Biotec for this purpose. CliniMACS is a magnetic bead separation method developed for large-scale sterile isolation of cells for clinical application, and has already been applied clinically for the enrichment of $CD34^+$ cells for peripheral blood stem cell transplantation [18,19]. The aim of this study was to establish an appropriate method for *in vitro* Treg-enrichment from apheresis products from UC patients, which is applicable to Treg transfer therapy.

Methods

Patients

Four patients with moderate to severe UC, admitted to Kyushu University Hospital, Fukuoka, Japan, between 2006 and 2007, were enrolled in this study. The protocol of this study was approved by the institutional ethical committee at the Graduate School of Medical Sciences, Kyushu University. Written informed consent was obtained from all patients. Patients underwent centrifugal leukapheresis therapy weekly for 5–11 weeks. Clinical characteristics of the patients were summarized in Table 1. Patient 1 was given two separate courses of leukapheresis because she relapsed after remission induced by the first treatment. An isolation experiment of Treg was conducted

Table 1. Patient characteristics

CliniMACS run number	Patient number	Age (years)	Sex	Type of disease	Clinical activity index‡ at the initiation of the treatment	Concomitant treatment
1	1*	70	Female	Left-sided colitis	10	Prednisolone, 5-aminosalicylic acid
2	2	59	Female	Pancolitis	12	Total parenteral nutrition, prednisolone
3	1†	70	Female	Left-sided colitis	6	Prednisolone, 5-aminosalicylic acid
4	3	44	Male	Left-sided colitis	12	Total parenteral nutrition, prednisolone, 5-aminosalicylic acid
5	4	18	Male	Pancolitis	16	Total parenteral nutrition, prednisolone, azathioprine, cyclosporine A

**First treatment for patient 1.*
†Second treatment for patient 1.
‡Rachmilewitz criteria.

in each course of the leukapheresis for each patient. Concomitant pharmacotherapy included 5-aminosalicylates and peroral and intravenous prednisone and azathioprine. Patient 4 underwent leukapheresis after intravenous cyclosporine A.

Isolation of Treg from leukapheresis products using CliniMACS

Five large-scale cell-separation experiments with clinical-grade CliniMACS reagents were performed. The CliniMACS system (Miltenyi Biotec, Bergisch-Gladbach, Germany) includes an instrument, circuit tubes, clinical-grade magnetic beads reagents and computer programs. All of the procedures were done in sterile closed circuits. The leukapheresis product (LP) cells were washed, adjusted to 88 mL with phosphate-buffered saline (PBS)/ethylenediaminetetra-acetic acid (EDTA) buffer (Miltenyi Biotec), supplemented with 2% human serum albumin (HSA; Mitsubishi Pharma, Osaka, Japan) and one vial each of clinical-grade magnetic beads bearing anti-CD8 and anti-CD19 monoclonal antibodies (MAb) (CliniMACS CD8 MicroBeads and CliniMACS CD19 MicroBeads; Miltenyi Biotec), and incubated for 30 min at room temperature with frequent manual agitation in a cell-preparation bag. Then cells were centrifuged at 300 *g*, washed again, and resuspended in 60 mL PBS/EDTA/HSA. B cells and $CD8^+$ T cells were depleted with the CliniMACS^{plus} instrument (Miltenyi Biotec) by using the LS tubing set (Miltenyi Biotec) and the depletion program 2.1. B cell/$CD8^+$ T-cell depleted cells were suspended in 190 mL PBS/EDTA/HSA, labeled with 7.5 mL magnetic beads bearing anti-CD25 MAb (CliniMACS CD25 MicroBeads; Miltenyi Biotec) for 30 min at room temperature, washed, and resuspended in 100 mL PBS/EDTA/HSA. $CD25^+$ cells were isolated by three automatic cycles of positive selection using the enrichment program 3.1 of the CliniMACS device. Upon completion of the enrichment program, CD25-enriched (target cell fraction; TCF) and CD25-depleted cell fractions (non-target cell fraction; NTCF) were collected in separate collection bags. Aliquots before and after each step were taken for flow cytometric analysis.

Flow cytometry

Cells were resuspended in fluorescence-activated cell sorter (FACS) buffer, which consisted of PBS with 0.5% bovine serum albumin (BSA) at 10^7 cells/mL. For analysis of CD25 expression after magnetic cell separation, 1×10^6 cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (Miltenyi Biotec) and biotin-conjugated anti-human CD25 (4E3; Miltenyi Biotec) at 4°C in the dark for 15 min. Then the cells were washed and incubated with phycoerythrin (PE)-labeled anti-biotin antibody (Ab)

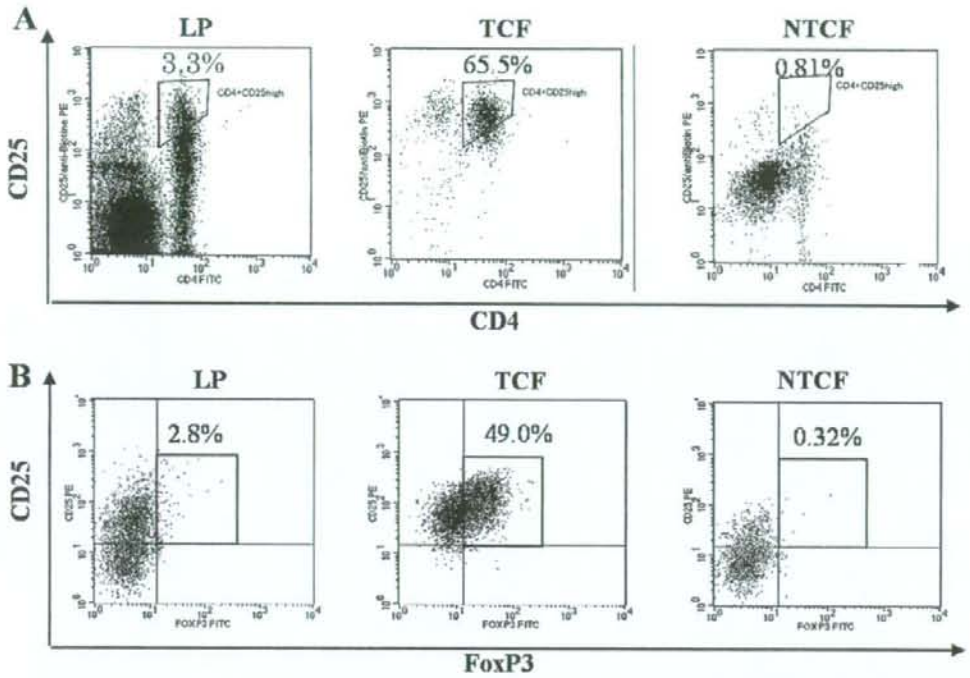

Figure 1. Flow cytometry of isolated cells by CliniMACS from LP from UC patients. LP were depleted of $CD8^+$ T and B cells and separated into $CD25^+$ TCF and NTCF. Before and after isolation, aliquots were taken and analyzed by flow cytometry. Representative data of five independent experiments are shown. (A) Cells were stained with FITC-conjugated anti-CD4 and biotin-conjugated anti-CD25 plus PE-conjugated anti-biotin Ab. Data in the quadrant indicate the percentage of $CD4^+$ $CD25^{high}$ T cells among the total number of cells. (B) Cells from each fraction were stained with Cy-Chrome-conjugated anti-CD4 and biotin-conjugated anti-CD25/PE-conjugated anti-biotin Ab, washed, fixed, permeabilized and stained with FITC-conjugated anti-FOXP3. The $CD4^+$ gate is shown. The percentages of $CD25^+$ $FOXP3^+$ among $CD4^+$ T cells are shown.

FOXP3, which showed that Treg were truly concentrated. In summary, these data revealed a preferential enrichment of $CD4^+$ $CD25^{high}$ $FOXP3^+$ T cells from LP by the two-step isolation strategy using the CliniMACS system.

Suppressor activity of enriched Treg by CliniMACS

To determine whether selective separation of functional Treg was done successfully by the CliniMACS cell selection, we assessed the suppressor activity of the isolated TCF cells. $CD4^+$ $CD25^-$ T cells and/or TCF were stimulated with anti-CD3 MAb and antigen-presenting cells (APC). As shown in Figure 2, cultures containing $CD4^+$ $CD25^-$ T cells alone proliferated vigorously, whereas cultures containing TCF alone were hypoproliferative, a characteristic of Treg. When $CD4^+$ $CD25^-$ T cells were co-cultured with equal numbers of TCF cells, T-cell proliferation was suppressed. This suppressive effect was enhanced by increasing the number of TCF cells (data not shown). These data demonstrated that successful enrichment of functional Treg in the TCF was achieved by CliniMACS cell separation. The isolated cells were, therefore, considered to be suitable for Treg transfer therapy.

In vitro expansion of Treg using anti-CD3/anti-CD28-coated beads

The intestine is not only the digestive organ but also the biggest lymphoid tissue in the body, containing numerous lymphocytes. Thus the question arises of how many Treg need to be transferred to regulate colonic inflammation. Although fairly large numbers of Treg could be recovered by one session of CliniMACS cell separation from LP, it may be necessary to transfer more Treg. Therefore, we conducted further experiments to investigate whether isolated Treg can be expanded *in vitro* by retaining their

Table 3. Percentages of FOXP3-expressing cells among $CD4^+$ T cells before and after CliniMACS selection

CliniMACS run number		1	2	3	4	5	Average
LP	$CD4^+$ $FOXP3^+/CD4^+$ T cell (%)	0.4	6.9	10.4	4.2	5.7	5.5
TCF	$CD4^+$ $FOXP3^+/CD4^+$ T cell (%)	54.3	66.6	51.3	52.4	57.3	56.4

immunoregulatory function. Poor proliferative potential upon stimulation through T-cell receptors is a well-known characteristic of Treg, and might be an obstacle for their use in sufficient numbers as an immunotherapeutic modality. Nevertheless, the anergic phenotype of Treg is not absolute and can be broken by synergistic stimulation through T-cell receptors with appropriate co-stimulatory signals or growth factors, such as CD28 signaling and IL-2 [5,6,20]. Therefore, we decided to use magnetic beads coupled with anti-CD3 and anti-CD28 Ab as artificial APC [21–23] in the presence of IL-2. As TGF-β has been reported to be an inducer of Treg development [24,25], we also analyzed the stimulation conditions in the presence of TGF-β1. We stimulated TCF cells and $CD4^+$ $CD25^-$ T cells as a control; the results are summarized in Table 4. TCF proliferated with anti-CD3/anti-CD28-coated beads and 500 U/mL IL-2 to a level comparable with $CD4^+$ $CD25^-$ T cells (Figure 3 and Table 4). Similarly,

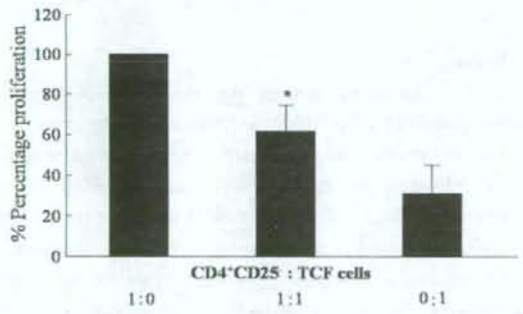

*Figure 2. Suppressor function of Treg isolated by CliniMACS from LP from UC patients. The TCF isolated by CliniMACS were hypoproliferative and showed suppressor activity. Autologous $CD4^+$ $CD25^-$ T cells and/or the TCF cells were co-cultured and stimulated with anti-CD3, in the presence of autologous APC for 4 days. Cells were pulsed with [^{3}H]thymidine (1 μCi) and the level of the proliferation is shown as the percentage proliferation, compared with the count with culture of $CD4^+$ $CD25^-$ T cells alone. Data represent the means ± SE of four independent experiments. *$P < 0.05$ for co-culture of $CD4^+$ $CD25^-$ and TCF cells compared with $CD4^+$ $CD25^-$ alone.*

in the presence of TGF-β1, TCF and $CD4^+$ $CD25^-$ T cells proliferated to a comparable level (Figure 3 and Table 4).

FACS analysis revealed that TCF cells expanded *in vitro* maintained higher levels of FOXP3 expression than cultured $CD4^+$ $CD25^-$ T cells (Figure 4, left and right center). When stimulated in the presence of TGF-β1, FOXP3 expression was up-regulated in both TCF and $CD4^+$ $CD25^-$ populations (Figure 4, left center and right). These results suggest that Treg can be expanded and maintained in cell culture in the presence of IL-2 and CD28 signaling, and that TGF-β1 induces the development of Treg from non-Treg.

We analyzed the function of expanded cells. Expanded TCF cells did not proliferate in response to stimulation in the same manner as freshly isolated Treg. In contrast, $CD4^+$ $CD25^-$ T cells proliferated well under these conditions, and their proliferation was suppressed in co-culture with *in vitro*-expanded TCF cells (Figure 5, left panel). The suppressor activity of bead-expanded TCF cells was stronger than that of freshly isolated TCF cells from the same patient (data not shown). In contrast, *in vitro*-expanded $CD4^+$ $CD25^-$ T cells proliferated vigorously and did not suppress the proliferation of $CD4^+$ $CD25^-$ T cells upon co-culture (Figure 5, right center). When cultured in the presence of TGF-β1, not only TCF cells but also $CD4^+$ $CD25^-$ cells revealed a regulatory capability. Cultured $CD4^+$ $CD25^-$ T cells became hyporesponsive to stimulation and suppressed the proliferation of $CD4^+$ $CD25^-$ T cells upon co-culture to a similar extent as TCF cells (Figure 5, left center and right). *In vitro* culture with TGF-β1 did not further enhance the regulatory activity of TCF (Figure 5, left and left center). Similar results were obtained with allogeneic stimulation (data not shown). Hence, Treg in the TCF expanded *in vitro* retained their characteristic phenotype of Treg, as well as their suppressive function. In addition, it is suggested that TGF-β1 induces the development of Treg *in vitro* from non-Treg.

Table 4. Results of expansion of $CD4^+$ $CD25^{high}$ Treg from LP from UC patients using the CliniMACS system

CliniMACS run number	TCF			TCF+TGFβ1			$CD4^+$ $CD25^-$			$CD4^+$ $CD25^-$+TGFβ1		
	Fold-expansion	$FOXP3^+$ $CD25^+$/ $CD4^+$ T cells, (%)		Fold-expansion	$FOXP3^+$ $CD25^+$/ $CD4^+$ T cells, (%)		Fold-expansion	$FOXP3^+$ $CD25^+$/ $CD4^+$ T cells, (%)		Fold-expansion	$FOXP3^+$ $CD25^+$/ $CD4^+$ T cells, (%)	
2	15.8	53.3		10.8	73.3		5.5	11.9		7.6	59.0	
3	21.0	23.9		19.5	47.0		16.3	15.1		15.0	16.3	
4	20.8	49.8		32.0	59.1		16.0	4.8		24.4	66.1	
5	17.1	67.6		18.1	91.7		10.9	9.8		18.3	68.1	
Average	18.7	48.7		20.1	67.8		12.2	10.4		16.3	52.4	

For adoptive cell therapy for UC, it is hoped that expanded cells strongly express gut-homing receptors, such as α4(CD49d) β7 and αE(CD103)β7 integrins. As it has been previously reported that the expression of αE and β7 integrins is regulated by TGF-β1 [26,27], we investigated whether 10-day cultures in the presence or absence of TGF-β1 could up-regulate these integrins on the cell surface by flow cytometry. α4β7 and αEβ7 integrins were expressed only in the small percentage of expanded TCF cells and $CD4^+$ $CD25^-$ T cells without TGF-β1 (Figure 6, left and right center). The expression of αE and β7 integrins was up-regulated by TGF-β1 on both cell populations. The expression of α4 integrin was not altered significantly. In consequence, the expression of αEβ7 integrin and also α4β7 integrin on the surface of TCF cells and $CD4^+$ $CD25^-$ T cells was significantly increased by addition of exogenous TGF-β1 (Figure 6, left center and right). These results suggest that TGF-β1 enhances the expression of not only αEβ7 but also α4β7 integrin on the cell surface, and that treatment with TGF-β1 may be useful to enhance the Treg immunoregulatory capability to suppress intestinal inflammation by increasing the expression of gut-homing receptors and thus migration of injected Treg to the mucosal sites of inflammation.

Discussion

UC is an intractable disease that develops into chronic persistent colitis. As there are many patients who are refractory to conventional therapy, the generation of new, more effective therapies is desperately needed. Treg comprise a subset of $CD4^+$ T cells that possess regulatory activity of a broad range of immune reactions. As a result of their potent immunosuppressive capability, Treg are considered to be an attractive therapeutic tool for inflammatory human disorders. Extensive studies of animal models of IBD have revealed a potent and essential role of Treg for the prevention of colonic inflammation [2,3]. On account of their suppressive effect on experimental models of colitis, not only for prevention but also for therapeutic use, Treg are expected to be an attractive tool for the treatment of IBD. In humans, we and others have demonstrated that the proportion of Treg in the peripheral blood is significantly reduced in active UC [11,13]. As Treg are functionally preserved [10–12] and only quantitatively decreased in UC patients, Treg transfer

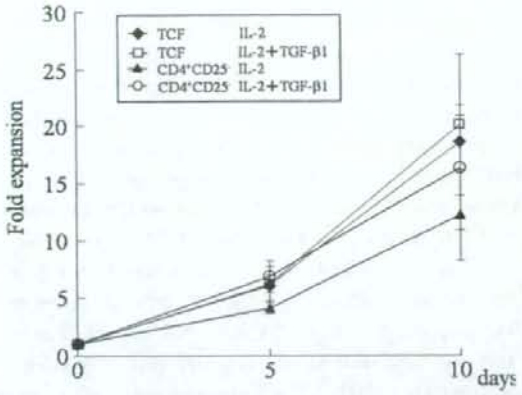

Figure 3. In vitro *expansion of Treg isolated by CliniMACS. Cells in the TCF of the CliniMACS selection and CD4$^+$ CD25$^-$ cells were activated with anti-CD3/anti-CD28-coated beads at a ratio of four beads per cell and 500 U/mL IL-2 in the presence or absence of 2 ng/mL TGF-β1. On days 5 and 10, cells were counted in aliquots of the cultures. The expansion of cells was demonstrated as a fold-increase compared with the original cell number at day 0 (1×10^6). Values represent means ± SE of four independent experiments.*

immunotherapy that enhances regulatory activity is thus expected to be efficacious for the treatment of UC.

Leukapheresis therapy is one of the standard therapies for UC in Japan and is mainly applied to steroid-resistant patients. During leukapheresis, it is considered that Treg, as well as colitogenic effector cells, are removed from the peripheral blood. It is, therefore, reasonable to expect better efficacy when Treg are isolated from LP and transferred to the patient. Thus, we chose a strategy to isolate and transfuse Treg following leukapheresis. For the application of leukapheresis, there are adsorptive and centrifugal methods [14–17]. The former utilizes columns to which leukocytes adhere. We chose the latter because intact leukocytes can be collected by this method. Centrifugal leukapheresis for patients with UC manages *c.* 2000–2500 mL peripheral blood per session, which is *c.* 40–45% of the total blood volume. In the present study, one session of centrifugal leukapheresis removed on average 9.67×10^8 CD4$^+$ T cells from the body and 6.0×10^7 Treg were isolated from LP. When isolated Treg are returned to patients just after leukapheresis, it is possible to achieve an increase of 1–3% in the ratio of Treg/total CD4$^+$ cells in the peripheral blood, which could add a suppressive effect of Treg to the conventional leukapheresis effect.

For clinical application, it is essential to isolate Treg safely and hygienically after leukapheresis. Although magnetic enrichment of CD4$^+$ CD25$^+$ Treg using anti-CD25 Ab-coated beads has already been applied in many laboratories for experimental purposes [9,28], it is unclear

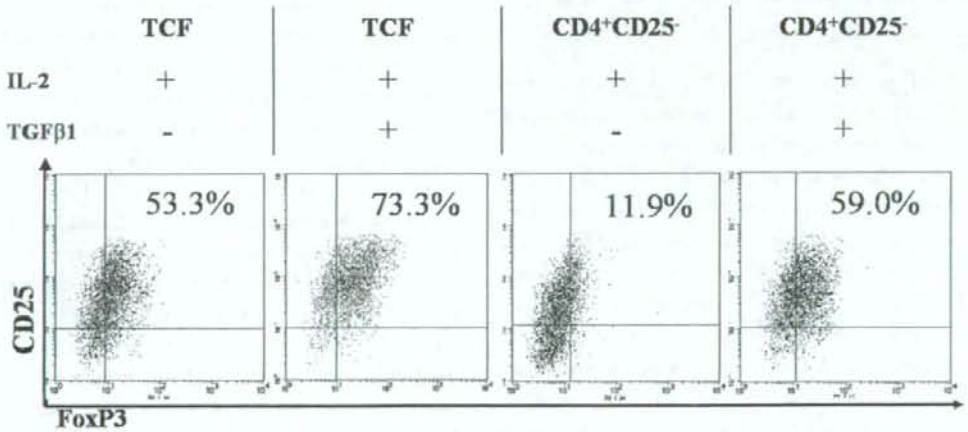

Figure 4. FOXP3 expression in the in vitro*-expanded cells. Cells in the TCF of the CliniMACS selection and CD4$^+$ CD25$^-$ cells were stimulated with anti-CD3/CD28 beads and IL-2 in the presence or absence of TGF-β1. Flow cytometry for FOXP3 expression was performed on day 24 (after resting for 2 weeks). Cells were stained with PE–anti-CD25 and PE-Cy5–anti-CD4, fixed, permeabilized, stained with FITC–anti-FOXP3 and analyzed by flow cytometry. Expression of CD25 and FOXP3 on the CD4$^+$ gate is shown. Data are representative of four independent experiments.*

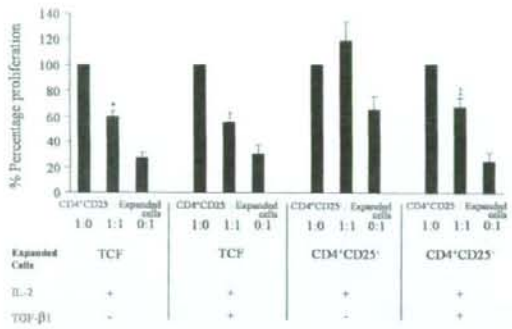

Figure 5. Suppressor function of the in vitro*-expanded cells. Cells in the TCF of the CliniMACS selection and CD4$^+$ CD25$^-$ cells were stimulated with anti-CD3/CD28 beads and IL-2 in the presence or absence of TGF-β1. After 10 days, cells were separated from beads by a magnet and rested for 2 weeks.* In vitro*-expanded TCF cells, TCF cells in the presence of TGF-β1, CD4$^+$ CD25$^-$ cells and CD4$^+$ CD25$^-$ cells in the presence of TGF-β1 were mixed with CD4$^+$ CD25$^-$ T cells and stimulated with anti-CD3 and mitomycin-C-treated APC. Proliferation was determined by [^{3}H]thymidine uptake, and the percentage proliferation was calculated by comparing with cultures of CD4$^+$ CD25$^-$ T cells alone. Data represent the means ± SE of four independent experiments.* *$P < 0.05$ *for co-culture of CD4$^+$ CD25$^-$ and each expanded cell compared with CD4$^+$ CD25$^-$ alone,* †$P < 0.05$ *for co-culture of CD4$^+$ CD25$^-$ and each expanded cell compared with CD4$^+$ CD25$^-$ alone,* ‡$P < 0.05$ *for co-culture of CD4$^+$ CD25$^-$ and each expanded cell compared with CD4$^+$ CD25$^-$ alone.*

what would be suited to the large-scale purification of Treg under sterile conditions for clinical applications. To this end, we sought to develop a strategy that ensures the efficient and reliable enrichment of human CD4$^+$ CD25^{high} T cells from LP. We chose the CliniMACS cell selection system for this purpose. This system is a magnetic bead-based cell isolation system developed by Miltenyi Biotec for clinical purposes, and has been used clinically for such procedures as CD34$^+$ stem cell transplantation [18,19]. Our protocol comprised one cycle of CD8$^+$ T/B-cell depletion with anti-CD8 and anti-CD19 beads, followed by three repetitive enrichment cycles for CD25$^+$ cells. Pre-depletion was included to eliminate activated CD25$^+$ B cells and CD8$^+$ CD25$^+$ T cells. These cells would contaminate Treg-enriched cells to various degrees, depending on the blood composition of each patient. Therefore, after almost all CD4$^-$ CD25$^+$ cells were deleted, the TCF could include Treg at high purity. In fact, B cells and CD8$^+$ T cells were almost undetectable in the cell products after depletion, and a low percentage of CD4$^-$ CD25$^+$ cells was only present in the TCF. It has recently been shown that a single magnetic cell-separation step efficiently depletes human Treg from LP but is insufficient for their enrichment [29]. Hoffmann *et al.* [30] have reported a protocol for Treg enrichment that comprises B-cell depletion with anti-CD19 beads followed by enrichment for CD25$^+$ cells. However, the low percentage of CD8$^+$ T cells still contaminates the TCF by their method. We consider that it is safer to deplete CD8$^+$ CD25$^+$ T cells because such cells may act as colitogenic factors. In our protocol, the enrichment for CD25 ensured not only the preferential retention of cells with high CD25 expression but also an almost-complete elimination of CD4$^-$ CD25$^+$ cells. As expected from their CD25 expression profile, TCF contained a high percentage of FOXP3-expressing Treg, and potently suppressed non-Treg proliferation in functional assays. In consequence, Treg could be isolated in fairly good numbers and with good recovery rates and, more importantly, isolated Treg retained their immunoregulatory activity. Thus Treg can be isolated from LP in large numbers that are clean enough for clinical use, and Treg transfer therapy is feasible in combination with centrifugal leukapheresis for UC.

The mechanism of Treg-mediated suppression of intestinal inflammation is not fully understood. Most *in vitro* studies have failed to identify a soluble suppressor cytokine for CD4$^+$ CD25$^+$ Treg activity [5,6]. *In vivo*, however, an experimental model of colitis revealed that suppression of colonic inflammation depends on immunosuppressive cytokines, IL-10 and TGF-β, as administration of Ab-neutralizing TGF-β [2] and IL-10 [31] to recipient mice abrogated the protective effect of Treg. We have previously demonstrated that Treg express a high level of TGF-β1 [32]. We have shown that Treg-produced TGF-β1 is essential for the suppression of colitis, as Treg isolated from TGF-β1-deficient mice fail to ameliorate the disease [33]. In contrast, it is not always required for the inhibition of T-cell proliferation *in vitro* [33]. Recently, Li *et al.* [34] have reported that Treg from T-cell specific TGF-β1 knockout mice failed to suppress a CD4$^+$ CD45RB^{high} T-cell transfer model of colitis, which demonstrates that TGF-β1 produced from Treg is essential for gut homeostasis. It is therefore of interest whether

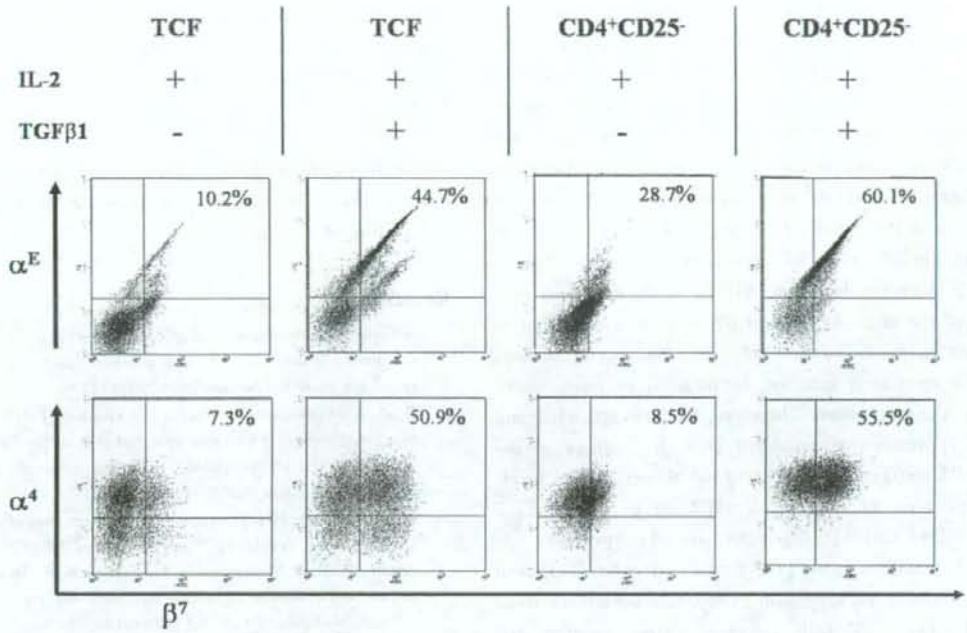

Figure 6. αEβ7 and α4β7 expression on the in vitro*-expanded cells. Cells in the TCF of the CliniMACS selection and CD4$^+$ CD25$^-$ T cells were stimulated with anti-CD3/CD28 beads and IL-2 in the presence or absence of TGF-β1. αEβ7 and α4β7 expression was analyzed by FACS after a 10-day culture. Cells were stained with PE–Cy5-anti-CD4, PE–anti-CD103 (αE) or PE–anti-CD49d (α4) and FITC–anti-β7 and analyzed by flow cytometry. Expression of αEβ7 and α4β7 on the CD4$^+$ gate is shown. Data are representative of two independent experiments.*

Treg mobilized to the intestine in IBD patients express high levels of TGF-β1 and IL-10.

In Treg transfer therapy for UC, the number of Treg that are considered sufficient for amelioration of colonic inflammation is unclear. Collection of 1×10^8 CD4$^+$ CD25^{high} cells per session of CliniMACS separation from LP is fairly good. However, more Treg may be needed to suppress intestinal inflammation as the intestine is the organ that contains the most immune cells in the body. Thus we conducted *in vitro* expansion of Treg. Treg isolated using CliniMACS could be expanded *in vitro* up to 18-fold in 10 days. Treg retained their immunosuppressive activity during *in vitro* culture. These results suggest that more Treg can be transferred to patients after *in vitro* expansion, at least theoretically. However, we need to pay much more attention to the safety of the transfer of such cultured cells than that for simple transfusion of isolated cells.

Treg develop in the thymus and were originally considered to be of a different lineage from conventional CD4$^+$ T cells [1]. Recently, however, TGF-β1 has been found to induce development of Treg from non-Treg *in vitro* [24,25]. Thus we tested whether CliniMACS-isolated Treg can be further enriched during cultivation in the presence of TGF-β1, and whether Treg can be induced from non-Treg by TGF-β1. Culture with TGF-β1 increased the percentage of CD4$^+$ FOXP3$^+$ cells in the TCF, although TGF-β1-treated TCF cells did not show a significant increase in suppression. Cultivation of CD4$^+$ CD25$^-$ T cells in the presence of TGF-β1 *in vitro* significantly increased the proportion of CD4$^+$ FOXP3$^+$ cells, and such cells showed suppressor activity to reduce T-cell proliferation. Thus, as reported previously in healthy subjects [24,25], TGF-β1 can induce Treg differentiation *in vitro* from non-Treg obtained from UC patients. Induction of Treg *in vitro* may be an alternative approach to conduct Treg-based immunotherapy.

Lymphocyte trafficking is mainly regulated by adhesion molecules, L-selectin and integrins. The integrin α4β7 is a homing receptor for cellular migration of T lymphocytes to mucosal sites [35,36]. The α4β7 integrin binds to

vascular addressins, selectively expressed by venules in mucosal tissues. Expression of the other β7 integrin, αEβ7, on T cells is thought to be important for intestinal intraepithelial T-lymphocyte (IEL) entry into the epithelial compartment [37,38]. It has been postulated that lymphocytes entering the gastrointestinal tract from the peripheral blood do so via an interaction of α4β7 on their surface with the mucosa-associated cell adhesion molecule on endothelial cells. Subsequent to migration, the αE subunit is up-regulated by TGF-β1 in the microenvironment of the intestine. The αEβ7 integrin is suggested to interact with E-cadherin on the enterocyte surface, thereby mediating selective localization or retention of IEL in the epithelium. Therefore, we analyzed α4β7 and αEβ7 expression of expanded cells. In contrast to the TGF-β1 untreated cells, we could detect higher levels of expression of αEβ7 and α4β7 on expanded TCF and $CD4^+ CD25^-$ T cells in the presence of TGF-β1, and each of TGF-β1-treated cells (Treg and non-Treg) showed suppressive properties for conventional $CD4^+ CD25^-$ T cells *in vitro*. Taken together, the treatment with TGF-β1 may support clinical cell therapy for mucosal inflammation using *in vitro*-induced or -expanded human Treg by up-regulating the expression of gut-homing receptors and thus the trafficking of infused Treg to the gut.

In summary, we have established a protocol to isolate Treg from LP from UC patients in clinically relevant numbers under sterile conditions, and this enables clinical trials of Treg transfer therapy for UC patients, in combination with leukapheresis. Successful expansion or induction of Treg *in vitro* demonstrates the future possibility of transfer therapy of cultivated Treg.

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Class-specific Regulation of Pro-inflammatory Genes by MyD88 Pathways and IκBζ*

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Toll-like receptors trigger the induction of primary response genes via MyD88-mediated activation of NF-κB and other transcription factors. These factors then act in concert with primary response gene products to induce secondary response genes. Although the MyD88 pathway is important for the expression of both primary and secondary response genes, we show that the recruitment of NF-κB, RNA polymerase, and the TATA-binding protein is MyD88-dependent only at secondary response genes. This selective dependence correlates with the fact that MyD88 is required for nucleosome remodeling and histone H3K4 trimethylation at secondary response promoters, whereas rapidly induced primary response promoters are assembled into poised MyD88-independent chromatin structures. At a subset of secondary response promoters, IκBζ was identified as a selective regulator of H3K4 trimethylation and preinitiation complex assembly after nucleosome remodeling. These mechanistic distinctions advance our understanding of the diverse molecular cascades that underlie the differential regulation of pro-inflammatory genes.

Toll-like receptor (TLR)[4]-dependent recognition of microbial components controls immune responses through the activation of innate immunity and the subsequent development of antigen-specific adaptive immunity (1–3). Excessive activation of innate immunity has been shown to be associated with several immune disorders (4, 5). Therefore, TLR-mediated innate immune responses are finely controlled through the regulation of signaling cascades and the modulation of gene induction (5–7). TLR-mediated signaling consists of at least two pathways, a MyD88-dependent pathway and a TRIF-dependent pathway. In contrast to the selective role of TRIF in TLR3- and TLR4-mediated responses, MyD88 acts downstream of almost all TLRs to promote the activation of a broad range of pro-inflammatory and anti-microbial genes (8).

One gene that is induced in response to TLR signaling is *Nfkbiz*, which encodes a nuclear IκB family member, IκBζ (9–11). Because *Nfkbiz* expression is induced rapidly in the absence of new protein synthesis, it is considered to be a primary response gene. Newly synthesized IκBζ protein then triggers the induction of a subset of TLR-dependent secondary genes through the modulation of NF-κB activity (12). Thus, in IκBζ-deficient mice, rapidly induced primary response genes, including *Cxcl2*, *Cxcl1*, and *Il23a*, were activated normally (13). In contrast, impaired expression was observed with a subset of secondary response genes that, in wild-type mice, are induced at relatively late times after TLR stimulation, including *Il12b*, *Il6*, and *Lcn2* (13).

Although the mechanism by which IκBζ regulates secondary response genes is not known, accumulating evidence has demonstrated that chromatin structure plays a critical role in gene activation and suppression in cells of the immune system (14–18). Two main mediators, ATP-dependent nucleosome remodeling complexes and histone-modifying enzymes, help regulate chromatin structure (19–23). ATP-dependent chromatin remodeling complexes use the energy of ATP hydrolysis to disrupt histone-DNA interactions, whereas histone-modifying enzymes alter the N-terminal tails and core domains of histones to regulate the activation and suppression of transcription. Among these histone modifications, which include acetylation, methylation, ubiquitination, and sumoylation of lysine residues, methylation of specific lysine residues of histones H3 and H4 is well associated with gene activation or suppression. Of particular relevance to this study, di- and tri-methylation of H3 Lys-4 (H3K4) are generally found at genes that are competent for activation, with H3K4 trimethylation often linked to active transcription (24, 25).

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[4] The abbreviations used are: TLR, toll-like receptor; TBP, TATA-binding protein; Ab, antibody; LPS, lipopolysaccharide; pol II, polymerase II; ChIP, chromatin immunoprecipitation; IL, interleukin; PBS, phosphate-buffered saline.

Initial evidence that chromatin structure may be critical for the differential regulation of primary and secondary response genes following TLR stimulation was provided in an influential study by Saccani *et al.* (26). Specifically, chromatin immunoprecipitation (ChIP) experiments revealed that NF-κB associates rapidly with rapidly induced primary response genes but much more slowly with genes induced with delayed kinetics. Saccani and co-workers (26, 27) hypothesized that the association of NF-κB was delayed because changes in chromatin structure at this latter class of genes must precede NF-κB binding and transcriptional activation. More recently, this hypothesis received support from studies of the SWI/SNF family of ATP-dependent nucleosome remodeling complexes (28). SWI/SNF-dependent nucleosome remodeling was found to be important for the activation of secondary response genes and a subset of primary response genes induced with delayed kinetics. However, nucleosome remodeling by SWI/SNF complexes was not necessary for induction of rapidly induced primary response genes. Further analysis of chromatin structure using a restriction enzyme accessibility assay revealed inducible nucleosome remodeling at the promoters of secondary response and late primary response genes, with constitutively accessible chromatin observed at the promoters of early primary response genes. However, the contributions of specific signaling pathways and transcription factors to the differential regulation of primary and secondary response genes were not examined.

The importance of TLR signaling through MyD88 for the induction of a broad range of genes raises the intriguing question of whether the MyD88-dependent pathway makes similar or different contributions to the activation of primary and secondary response genes. The selective role of the primary response gene product IκBζ in regulating a subset of secondary response genes is equally intriguing, as, *a priori*, it must carry out a function that is not required for the activation of primary response genes. IκBζ could therefore be essential for nucleosome remodeling at a subset of secondary response genes or could catalyze another chromatin-related event that is not necessary for primary response gene activation.

In this study, we found that, in murine macrophages responding to LPS through TLR4, MyD88 is required for the recruitment of NF-κB p65, RNA polymerase II (pol II), and the TATA-binding protein (TBP) to secondary response promoters. However, because of redundancy with the TRIF-dependent pathway, MyD88 was not required for the recruitment of these factors to primary response promoters, although it is essential for efficient induction of primary response gene transcription. At the secondary response promoters, MyD88 was also essential for nucleosome remodeling and histone H3K4 trimethylation, whereas primary response promoters were assembled into constitutively open chromatin structures in unstimulated cells, with pre-existing H3K4 trimethylation. Surprisingly, although the function of IκBζ was restricted to secondary response genes, it was not necessary for nucleosome remodeling at these genes, but rather was important for H3K4 trimethylation and preinitiation complex assembly downstream of the remodeling event.

EXPERIMENTAL PROCEDURES

Antibodies and Mice—Antibodies against NF-κB p65 (C-20) (sc-372), pol II (H-224) (sc-9001), and TFIID (TBP) (SI-1) (sc-273) were purchased from Santa Cruz Biotechnology. Antibodies to trimethyl histone H3 (Lys-4) (07-473) and SNF2β/BRG1 (07-478) were purchased from Upstate Biotechnology, Inc. Polyclonal anti-IκBζ Ab was obtained by immunizing rabbit with a recombinant protein containing the N-terminal region of murine IκBζ (1–380 amino acids).

Myd88$^{-/-}$, *Trif*$^{-/-}$, and *Nfkbiz*$^{-/-}$ mice were generated as described previously (13, 29). All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Kyushu University and Osaka University.

Stable Cell Lines—RAW264.7 cells were transfected with pcDNA 3.1 (+)-FLAG-IκBζ. The cells resistant to G418 were selected in the presence of 0.4 mg/ml G418 and cloned. Expression of IκBζ mRNA was determined by real time reverse transcription-PCR, and expression of FLAG-IκBζ protein was monitored by Western blotting using anti-M2 monoclonal Ab (Sigma).

Cell Culture—For isolation peritoneal macrophages, mice were intraperitoneally injected with 2 ml of 4% thioglycollate medium (Sigma). Peritoneal exudate cells were isolated from the peritoneal cavity 3 days post-injection. Cells were incubated overnight and washed with PBS. Remaining adherent cells were used as peritoneal macrophages for the experiments. To prepare bone marrow-derived macrophages, bone marrow cells were prepared from femora and passed through nylon mesh. Then cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 μM 2-mercaptoethanol, and 30% supernatants of cultured L cells. After 6 days, the cells were used as macrophages for experiments. Macrophage cell line RAW264.7 cells and J774 cells were maintained in RPMI 1640 medium containing 10% fetal calf serum, 100 μM 2-mercaptoethanol. Peritoneal macrophages, bone marrow-derived macrophages, and RAW264.7 cells were stimulated with *Escherichia coli* O55:B5 LPS (Sigma).

Quantitative Real Time Reverse Transcription-PCR—Total RNA was isolated with TRIzol reagent (Invitrogen), and 1–2 μg of RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Promega) and random primers (Toyobo) after treatment with RQ1 DNase I (Promega). Quantitative real time PCR was performed on an ABI 7000 (Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems). All data were normalized to the corresponding gene *Eef1a1* encoding elongation factor-1α or 18 S rRNA expression, and the fold difference relative to the elongation factor-1α or 18 S rRNA level was shown. Amplification conditions were 50 °C (2 min), 95 °C (10 min), 40 cycles of 95 °C (15 s), and 60 °C (60 s). Primers of 18 S ribosomal RNA, *Cxcl2*, *Il23a*, *Tnf*, *Lcn2*, and *Nfkbiz* were purchased from Assay on Demand (Applied Biosystems). Sequence for *Eef1a1*, *Il12b*, *Il6*, and *Cxcl1* are follows: *Eef1a1*probe, 5′-gcacctgagcagtgaagccagctgct-3′, forward primer 5′-gcaaaaacgacccaccaatg-3′ and reverse primer 5′-ggcctggatggttcaggata-3′; *Il12b* probe, 5′-ctgcagggaacacatgcccacttg-3′, forward primer 5′-gctcaggatcgctat-

tacaat-3′ and reverse primer 5′-tcttccttaatgtcttccact3′; *Il6* probe, 5′-ccttcttgggactgatgctggtgaca-3′, forward primer 5′-ctgcaagagacttccatccagtt-3′ and reverse primer 5′-aagtagggaaggccgtggtt-3′; *Cxcl1* probe, 5′-ttgccctcagggccccactg-3′, forward primer 5′-caagaacatccagagcttgaaggt-3′ and reverse primer 5′-gtg gctatgacttcggtttgg-3′.

Chromatin Immunoprecipitation Assay—Peritoneal macrophages, bone marrow-derived macrophages, or RAW264.7 cells were stimulated with 100 ng/ml LPS for the indicated periods. Chromatin was cross-linked by 1% formaldehyde at room temperature for 10 min. The cells were scraped after washing with PBS and centrifuging at 3000 rpm, and then the pellet was resuspended in SDS buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% SDS). Chromatin was sonicated eight times with 30-s pulses, centrifuged at 14,000 rpm to remove debris, diluted 5-fold with ChIP dilution buffer (16.7 mM Tris-HCl, 167 mM NaCl, 1.2 mM EDTA, 1.1% X-100) supplemented with protease inhibitor, and precleared with salmon sperm DNA/protein A-agarose (Upstate). Diluted chromatin was immunoprecipitated at 4 °C overnight, and immune complexes were absorbed with salmon sperm DNA/protein A-agarose beads, and washed one time with low salt buffer (20 mM Tris-HCl (pH 8.1), 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA), high salt buffer (20 mM Tris-HCl (pH 8.1), 500 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA), LiCl buffer (10 mM Tris-HCl (pH 8.1), 0.25 M LiCl, 1 mM EDTA, 1% deoxycholic acid, 1% Nonidet P-40), and two times with TE buffer (10 mM Tris-HCl (pH 8.1), 1 mM EDTA). Immune complexes extracted in elution buffer (1% SDS, 100 mM $NaHCO_3$) were incubated for 4 h at 65 °C to revert DNA-protein cross-links. Then the DNA was extracted by incubation in proteinase K (final 50 μg/ml) buffer for 1 h at 45 °C. The purified DNA was used in PCR to assess the presence of target sequences. Promoter-specific primer was designed to include NF-κB-binding site. Sequence of primers are as follows: 5′-caacagtgtacttacgcagacg-3′ and 5′-ctagctgcctgcctcattctac-3′ in the *Cxcl2* promoter; 5′-ctgagcactggagactctgaag-3′ and 5′-gctgggatcatggtgctgtgtt-3′ in the *Cxcl1* promoter; 5′-gccactcctccaagaac-3′ and 5′-tttggaaagttggggacacc-3′ in the *Tnf* promoter; 5′-atccaaagccctgggaatgtc-3′ and 5′-gggtagtccatcctttaccaa-3′ in the *Lcn2* promoter; 5′-agtatctctgcctccttcctt-3′ and 5′-gcaacactgaaaactagtgtc-3′ in the *Il12b* promoter; 5′-agaagagtgctcatgcttc-3′ and 5′-agctacagacatccccagtctc-3′ in the *Il6* promoter; and 5′-gagatggccttgcatgaggat-3′ and 5′-gccagagtctcagtcttcaac-3′ in the *iNOS* promoter. Chromatin immunoprecipitation using J774 cells with reduced expression of BRG1/BRM was performed essentially as described (28). In brief, J774 cells (7.5×10^5/well) were seeded in 6-well plates and were transduced with either empty vector or BRG1/BRM short hairpin RNA vector. The BRG1/BRM short hairpin RNA targets a conserved region between BRG1 and BRM mRNAs (TGGAGAAGCAGCAGAAGAT). The cells were infected via spin infections on consecutive days at 2500 rpm for 1.5 h and at 30 °C. After the second spin infection, puromycin (3 μg/ml) selection was started. The enrichment of transduced cells was followed by flow cytometry, and RNA interference-mediated depletion was monitored by Western blot. For chromatin immunoprecipitation experiments, BRG1/BRM RNA interference-depleted cells and control cells were stimulated and cross-linked 5 days after the first spin infection.

Nuclei Preparation—Peritoneal macrophages or bone marrow-derived macrophages were stimulated with 10 μg/ml LPS for the indicated periods. Cells were scraped and pelleted at 1500 rpm. Cells were washed once with PBS. The cell pellet was resuspended in Nonidet P-40 lysis buffer (10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40, 0.15 mM spermine, and 0.5 mM spermidine) and incubated on ice for 5 min. Nuclei were pelleted at 1000 rpm, followed by washing with RE buffer (10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 10 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM EGTA, 1 mM β-mercaptoethanol, 0.15 mM spermine, 0.5 mM spermidine).

Restriction Enzyme Accessibility Assay—Restriction enzyme accessibility assay was performed essentially as described (30, 31). Isolated cell nuclei and restriction enzyme (100 units) (*Il12b* promoter and enhancer, SpeI; *Il6* promoter, AflII) were incubated for 15 min at 37 °C. Reactions were stopped by adding proteinase K buffer (100 mM Tris-HCl (pH 8.5), 200 mM NaCl, 5 mM EDTA, 0.2% SDS, 100 ng/ml proteinase K), incubated overnight at 56 °C, followed by genomic DNA isolation. Purified DNA (10–15 μg) was digested to completion to generate reference cleavage products using the following restriction enzymes: KpnI and SphI for the *Il12b* promoter and enhancer and XbaI and SpeI for *Il6*. Samples were analyzed by Southern blotting with ^{32}P-labeled gene-specific probes designed at the following regions, *Il12b* promoter (+64 to +437), *Il12b* enhancer (−8711 to −9113), and *Il6* promoter (−544 to −1043).

RESULTS

Different Roles of MyD88 at Primary and Secondary Response Genes—To understand how the MyD88 pathway contributes to the regulation of primary and secondary response genes, we compared LPS-stimulated wild-type and *Myd88*$^{-/-}$ macrophages. In the mutant cells, the expression of a large number of primary and secondary response genes is known to be severely reduced, despite an intact TRIF-dependent pathway (1). For this study, the *Cxcl2*, *Cxcl1*, and *Tnf* genes (encoding MIP2, GRO1, and tumor necrosis factor-α, respectively) were monitored as examples of primary response genes, which are induced rapidly (supplemental Fig. S1*A*) in the absence of a requirement for new protein synthesis (based on resistance to cycloheximide) (28). The *Lcn2*, *Il12b*, and *Il6* genes (encoding lipocalin 2, IL-12 p40, and IL-6, respectively) were used as examples of secondary response genes, which are induced with delayed kinetics (supplemental Fig. S1*B*) in a cycloheximide-sensitive manner (28). Although expression of all seven genes was greatly reduced in *Myd88*$^{-/-}$ macrophages, residual induction was observed with the four primary response genes. This induction was largely eliminated in *Myd88*$^{-/-}$*Trif*$^{-/-}$ macrophages (supplemental Fig. S1*C*).

To determine how the absence of MyD88 signaling alters the cascade of events leading to transcription initiation, ChIP experiments were performed. At the *Lcn2* secondary response promoter, the recruitment of the NF-κB p65 subunit, pol II, and TBP was greatly reduced in LPS-stimulated peritoneal macrophages from *Myd88*$^{-/-}$ mice, when compared with macro-

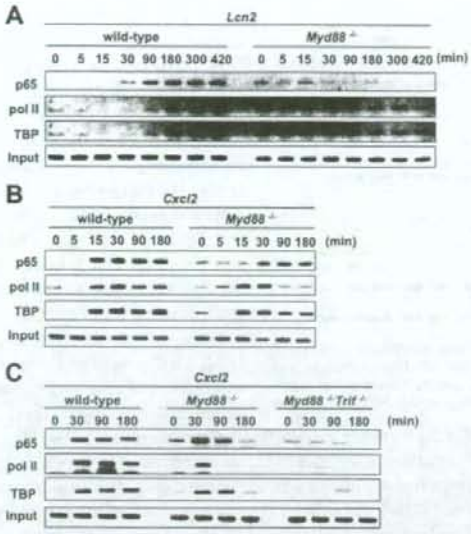

FIGURE 1. **MyD88-dependent regulation of primary and secondary response genes.** Peritoneal macrophages from wild-type, *Myd88*$^{-/-}$, and *Myd88*$^{-/-}$*Trif*$^{-/-}$ mice were stimulated with 100 ng/ml LPS for the indicated periods, and ChIP assay was performed with antibodies to NF-κBp65, pol II, or TBP. The immunoprecipitated *Lcn2* promoter (*A*) or *Cxcl2* promoter (*B* and *C*) was analyzed by PCR with promoter-specific primers. PCR amplification of the total input DNA in each sample is shown (*Input*). This is representative of five independent experiments. The same result was obtained when bone marrow-derived macrophages were used.

phages from wild-type mice (Fig. 1*A*). Remarkably, these same factors were recruited normally to the *Cxcl2* primary response promoter in *Myd88*$^{-/-}$ macrophages, with only a modest delay in p65 recruitment and perhaps more transient association of pol II (Fig. 1*B*). Consistent with previous findings (26), factor recruitment was observed at earlier time points at the primary response promoter than at the secondary response promoter. Similar results were obtained using bone marrow-derived macrophages (data not shown). Importantly, recruitment of p65, pol II, and TBP to the *Cxcl2* promoter was eliminated in *Myd88*$^{-/-}$*Trif*$^{-/-}$ macrophages (Fig. 1*C*), consistent with previous evidence that NF-κB activation by LPS is only modestly delayed in *Myd88*$^{-/-}$ macrophages but eliminated in *MyD88*$^{-/-}$*Trif*$^{-/-}$ macrophages (29).

These findings suggest a hypothesis in which MyD88 is essential for a change in chromatin structure at secondary response promoters that must precede the binding of NF-κB and the assembly of a transcription preinitiation complex. However, at primary response promoters, preinitiation complex assembly is relatively unperturbed because of the following: 1) these genes possess a poised MyD88-independent chromatin structure in unstimulated cells, and 2) the TRIF-dependent pathway can support NF-κB activation in the absence of MyD88. It is important to emphasize that *Cxcl2* transcription is severely reduced in *Myd88*$^{-/-}$ macrophages (supplemental Fig. 1*A*), despite the efficient recruitment of p65, pol II, and TBP to the *Cxcl2* promoter. Possible reasons primary response genes require the MyD88 pathway for efficient induction are considered below (see under "Discussion").

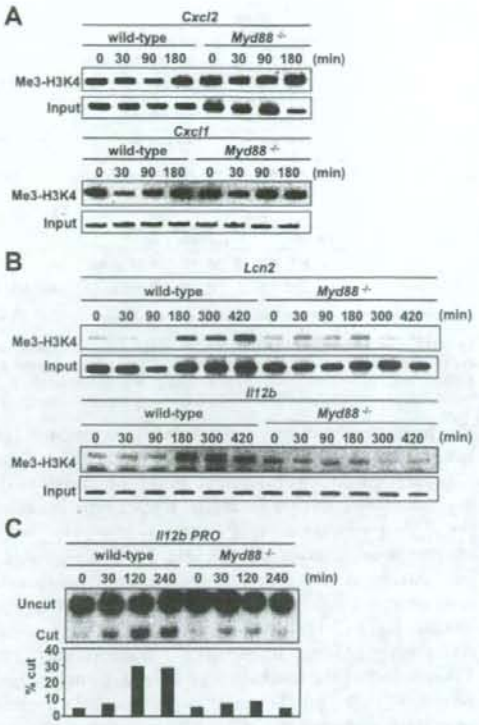

FIGURE 2. **MyD88-dependent nucleosome remodeling of secondary response promoters.** Peritoneal macrophages from wild-type and *Myd88*$^{-/-}$ mice were stimulated with 100 ng/ml LPS for the indicated periods, and chromatin immunoprecipitation (*ChIP*) assay was performed with anti-trimethyl histone H3 (Lys-4) Ab (Me3-H3K4). Precipitated DNA for the *Cxcl2* promoter, *Cxcl1* promoter (*A*), *Lcn2* promoter, or *Il12b* promoter (*B*) was analyzed by PCR. This is representative of two independent experiments. *C*, bone marrow macrophages from wild-type and Myd88$^{-/-}$ mice were stimulated with 10 μg/ml LPS for the indicated periods. Restriction enzyme accessibility assay at *Il12b* promoter region used nuclei from bone marrow macrophages.

MyD88-dependent H3K4 Trimethylation and Nucleosome Remodeling at Secondary Response Promoters—To test the above hypothesis, we evaluated the importance of MyD88 for the chromatin changes that accompany gene activation in LPS-stimulated macrophages. Trimethylation of histone H3K4 was first examined because of its close association with transcriptionally active genes (23, 32, 33). At the promoters of two representative primary response genes, *Cxcl2* and *Cxcl1*, ChIP experiments revealed constitutively high H3K4 trimethylation in unstimulated wild-type macrophages, with no significant change following LPS stimulation (Fig. 2*A*). These results are consistent with previous evidence that early primary response promoters possess constitutively acetylated histones and constitutively open chromatin structures (28). Importantly, similar H3K4 trimethylation levels were observed at these promoters in *Myd88*$^{-/-}$ macrophages (Fig. 2*A*).

In contrast to the constitutive H3K4 trimethylation observed at the primary response promoters, this modification was strongly induced in LPS-stimulated wild-type macrophages at

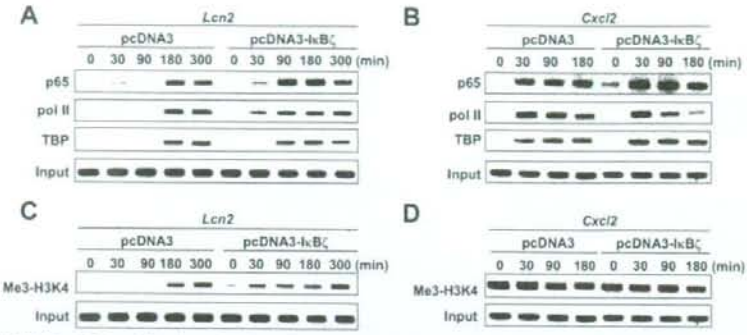

FIGURE 3. **IκBζ-mediated activation of secondary response gene promoters.** RAW264.7 cells stably expressing IκBζ were treated with 100 ng/ml LPS for the indicated periods and then used for ChIP assay with antibodies to NF-κBp65, pol II, or TBP (*A* and *B*) or anti-trimethyl-H3 (Lys-4) Ab (*C* and *D*). The immunoprecipitated *Lcn2* promoter (*A* and *C*) or *Cxcl2* promoter (*B* and *D*) was analyzed by PCR with promoter-specific primers.

the promoters for two representative secondary response genes, *Lcn2* and *Il12b* (Fig. 2*B*). Significantly, the LPS-induced H3K4 trimethylation observed at these promoters was MyD88-dependent. We further examined nucleosome remodeling at the *Il12b* promoter using a Southern blot-based restriction enzyme accessibility assay (28, 31). As demonstrated previously, strong increases in restriction enzyme cleavage efficiency were observed following LPS stimulation in wild-type macrophages (Fig. 2*C*) (28). However, restriction enzyme cleavage was greatly reduced in *Myd88*$^{-/-}$ macrophages (Fig. 2*C*). Taken together, the results in Figs. 1 and 2 strongly suggest that NF-κB, TBP, and pol II cannot associate with the promoters of secondary response genes in LPS-stimulated macrophages from *Myd88*$^{-/-}$ mice because the MyD88 pathway is required for LPS-induced H3K4 trimethylation and nucleosome remodeling at these promoters. In contrast, the recruitment of NF-κB, TBP, and pol II to primary response promoters does not depend on MyD88 because these promoters are assembled into poised chromatin structures in unstimulated cells.

IκBζ Mediates the Activation of a Subset of Secondary Response Genes—The above results suggest that, although the TRIF pathway can support NF-κB activation in LPS-stimulated macrophages from *Myd88*$^{-/-}$ mice, TRIF cannot support the activation of one or more factors that act prior to nucleosome remodeling and H3K4 trimethylation at MyD88-dependent secondary response promoters. Thus, an MyD88-specific target is essential for nucleosome remodeling and H3K4 trimethylation at these promoters. It is noteworthy that LPS-induced remodeling at a collection of secondary response promoters was previously found to require new protein synthesis (28). This previous finding suggests that the MyD88 target of interest may be a primary response gene product, as opposed to a transcription factor whose activity is induced post-translationally in response to TLR4 signaling.

One primary response gene product that is an attractive candidate for contributing to the activation of a subset of secondary response genes is the nuclear IκB protein, IκBζ, encoded by the *Nfkbiz* gene. As described previously, expression of a subset of secondary response genes is impaired in *Nfkbiz*$^{-/-}$ macrophages, whereas primary response genes are expressed normally (supplemental Fig. S2, *A* and *B*) (13).

To gain further insight into the importance of IκBζ for the expression of secondary response genes, IκBζ was constitutively overexpressed in the RAW264.7 macrophage line. When IκBζ was present at the time of LPS stimulation, three IκBζ-dependent secondary response genes, *Lcn2*, *Il12b*, and *Il6*, were induced more rapidly than in control RAW264.7 cells (supplemental Fig. S2C). Consistent with the more rapid induction in the presence of constitutively expressed IκBζ, ChIP assays revealed that the association of p65, pol II, and TBP reached a detectable level at the *Lcn2* promoter more rapidly than in control cells (Fig. 3*A*). In contrast, the kinetics of factor recruitment to the *Cxcl2* primary response promoter was unchanged (Fig. 3*B*). Importantly, histone H3K4 trimethylation was also induced more rapidly at the *Lcn2* promoter following LPS stimulation of the IκBζ-expressing cells (Fig. 3*C*), whereas the constitutive H3K4 trimethylation observed at the *Cxcl2* promoter remained unchanged (Fig. 3*D*). These findings are consistent with a model in which IκBζ plays a major role in the changes in chromatin structure that are associated with the induction of IκBζ-dependent secondary response genes.

IκBζ-dependent H3K4 Trimethylation at Secondary Response Promoters—To complement the IκBζ gain-of-function experiments, loss of function experiments were performed with bone marrow-derived macrophages from *Nfkbiz*$^{-/-}$ mice. Strikingly, although the constitutive H3K4 trimethylation at three representative primary response promoters was comparable in wild-type and *Nfkbiz*$^{-/-}$ macrophages (*Cxcl2*, *Cxcl1*, Fig. 4*A*; *Tnf*, data not shown), the inducible H3K4 trimethylation observed in wild-type macrophages at the promoters of three IκBζ-dependent secondary response promoters was greatly reduced in *Nfkbiz*$^{-/-}$ cells (*Lcn2*, *Il12b*; Fig. 4*B*, *Il6*; data not shown). Furthermore, the recruitment of NF-κB p65, pol II, and TBP was greatly diminished at secondary response promoters in *Nfkbiz*$^{-/-}$ cells (*Lcn2*, *Il12b*; Fig. 4*D*, *Il6*; supplemental Fig. S3), whereas the recruitment of these proteins to promoters of primary response or IκBζ-independent secondary response genes was unaffected (*Cxcl2*, *Tnf*, or *iNOS* Fig. 4*C* and supplemental Fig. S4). These results are consistent with the gain-of-function results and support the view that IκBζ is a selective major regulator of chromatin structure and preinitiation complex assembly at IκBζ-dependent secondary response genes.

To determine whether IκBζ directly regulates IκBζ-dependent genes, ChIP experiments were performed. The results revealed that IκBζ associates with the *Il12b* and *Il6* promoters in LPS-stimulated macrophages (Fig. 5). The kinetics of binding was similar to that observed with two other factors previously shown to associate with these control regions, BRG1 and C/EBPβ (28). It is important to note that, in addition to its association with the promoters of IκBζ-dependent genes, inducible IκBζ association was observed in our hands at a

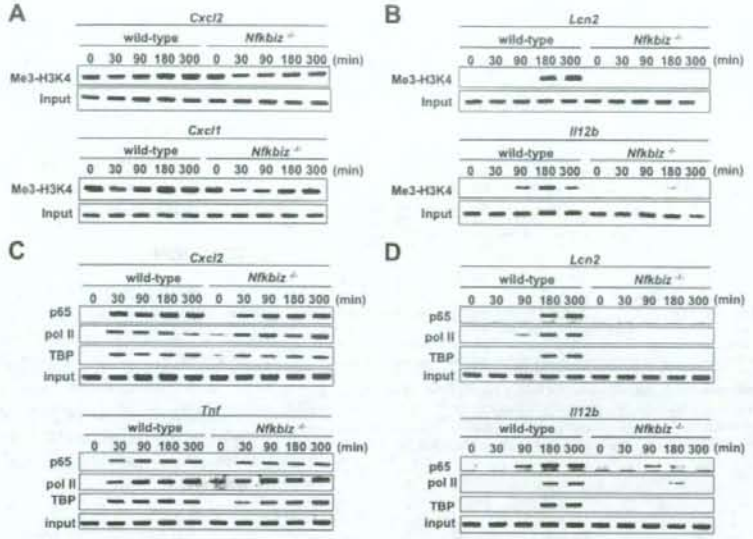

FIGURE 4. **Impaired trimethylation of histone H3 Lys-4 and preinitiation complex assembly at secondary response promoters in *Nfkbiz*$^{-/-}$ macrophages.** *A* and *B*, chromatin prepared from wild-type and *Nfkbiz*$^{-/-}$ bone marrow-derived macrophages treated with 100 ng/ml LPS for the indicated periods was immunoprecipitated with antibody against trimethyl histone H3 (Lys-4). Precipitated DNA was analyzed by PCR with promoter-specific primers for *Cxcl2*, *Cxcl1* (*A*), *Lcn2*, and *Il12b* (*B*). This is representative of three independent experiments. *C* and *D*, peritoneal macrophages from wild-type and *Nfkbiz*$^{-/-}$ mice were stimulated with 100 ng/ml LPS for the indicated periods and then chromatins were prepared and immunoprecipitated with antibody against NF-κBp65, pol II, or TBP. Precipitated DNA was analyzed by PCR with promoter-specific primers for *Cxcl2*, *Tnf* (*C*), *Lcn2*, and *Il12b* (*D*). The results are representative of three independent experiments.

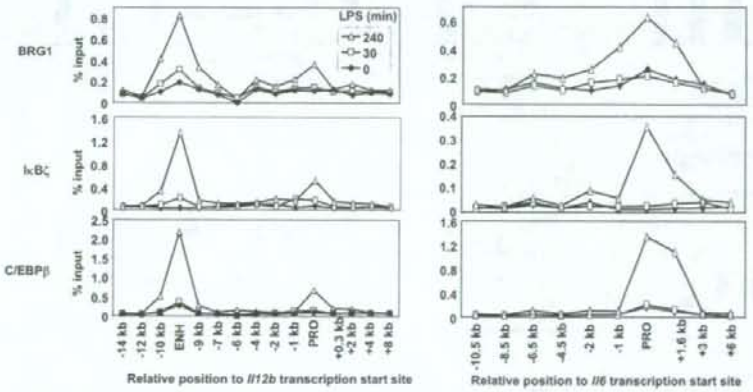

FIGURE 5. **Recruitment of IκBζ and BRG1 to the same control regions of the *Il12b* and *Il6* loci.** J774 cells were stimulated with LPS for 0, 30, and 240 min, and chromatins were prepared and precipitated with antibodies against BRG1, IκBζ, and C/EBPβ. Precipitated DNA samples were amplified using primer pairs specific to the indicated regions relative to the *Il12b* and *Il6* transcriptional start site.

recently described enhancer for the *Il12b* gene (Fig. 5), as well as at the promoters for a number of primary response genes that do not require IκBζ for expression (data not shown). Thus, although the analysis of *Nfkbiz*$^{-/-}$ macrophages provides strong evidence that IκBζ is selectively required for H3K4 trimethylation and for the recruitment of p65, TBP, and pol II to a subset of IκBζ-dependent secondary response genes, IκBζ associates with other LPS-induced genes with no apparent functional consequences.

IκBζ-independent Nucleosome Remodeling at Secondary Response Promoters—As shown previously (28) and in Fig. 2C, nucleosome remodeling by ATP-dependent remodeling complexes is generally required for the activation of secondary response genes following LPS stimulation. To determine whether IκBζ is required for nucleosome remodeling at IκBζ-dependent genes, we first used a ChIP assay to monitor recruitment of the BRG1 catalytic subunit of the SWI/SNF remodeling complexes. Following LPS stimulation, BRG1 was found to associate with representative IκBζ-dependent genes (*Il6* and *Lcn2* in Fig. 6*A* and *Il6* and *Il12b* in Fig. 6*B*). Interestingly, this inducible association was eliminated in *Myd88*$^{-/-}$ macrophages but was retained in *Nfkbiz*$^{-/-}$ macrophages (Fig. 6). These results suggest that IκBζ acts downstream of the remodeling event, with another MyD88 target required for remodeling. In contrast to the results obtained with secondary response genes, BRG1 associated constitutively with the *Cxcl2* primary response promoter (Fig. 6), as previously described, even though BRG1 is not important for the induction of this and other primary response genes (28). This constitutive association was retained in both *Myd88*$^{-/-}$ and *Nfkbiz*$^{-/-}$ cells (Fig. 6).

To further evaluate the role of IκBζ in nucleosome remodeling, restriction enzyme accessibility experiments were performed. Consistent with the BRG1 ChIP data, the LPS-induced increases in restriction enzyme cleavage observed at the *Il12b* enhancer, *Il12b* promoter, and *Il6* promoter were comparable in wild-type and *Nfkbiz*$^{-/-}$ macrophages stimulated with LPS (Fig. 7*A*). Finally, to determine whether IκBζ association with secondary response genes requires nucleosome remodeling, BRG1 and the closely related BRM catalytic subunits of the SWI/SNF remodeling complexes were depleted from J774 macrophages using a retrovirus that expresses a short interfering RNA targeted to a conserved region of BRG1 and BRM (28). In cells with reduced BRG1/BRM expression, LPS-induced recruitment of IκBζ to the *Il6* and *Lcn2* promoters and the *Il12b* enhancer was

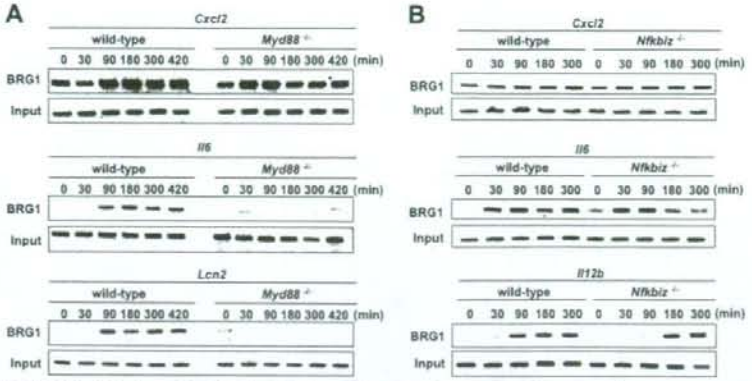

FIGURE 6. **MyD88-dependent, but IκBζ-independent, recruitment of BRG1 to secondary response promoters.** Bone marrow-derived macrophages from wild-type, *Myd88*$^{-/-}$ (*A*), and *Nfkbiz*$^{-/-}$ (*B*) mice were stimulated with 100 ng/ml LPS for the indicated periods. Then chromatins were prepared and precipitated with anti-BRG1 Ab. Precipitated DNA samples were amplified using promoter-specific primers for *Cxcl2* (*A* and *B*), *Il6* (*A* and *B*), *Lcn2* (*A*), and *Il12b* (*B*).

reduced compared with control cells, whereas recruitment to the constitutively open *Cxcl2* and *Tnf* promoters was unchanged (Fig. 7*B*). These findings indicate that nucleosome remodeling is required for the efficient recruitment of IκBζ to the transcriptional control regions of secondary response genes.

DISCUSSION

The results described in this study highlight the diverse mechanisms by which chromatin structure, signal transduction pathways, and transcription factors can control the activation of a large panel of inducible pro-inflammatory genes expressed by macrophages follow-

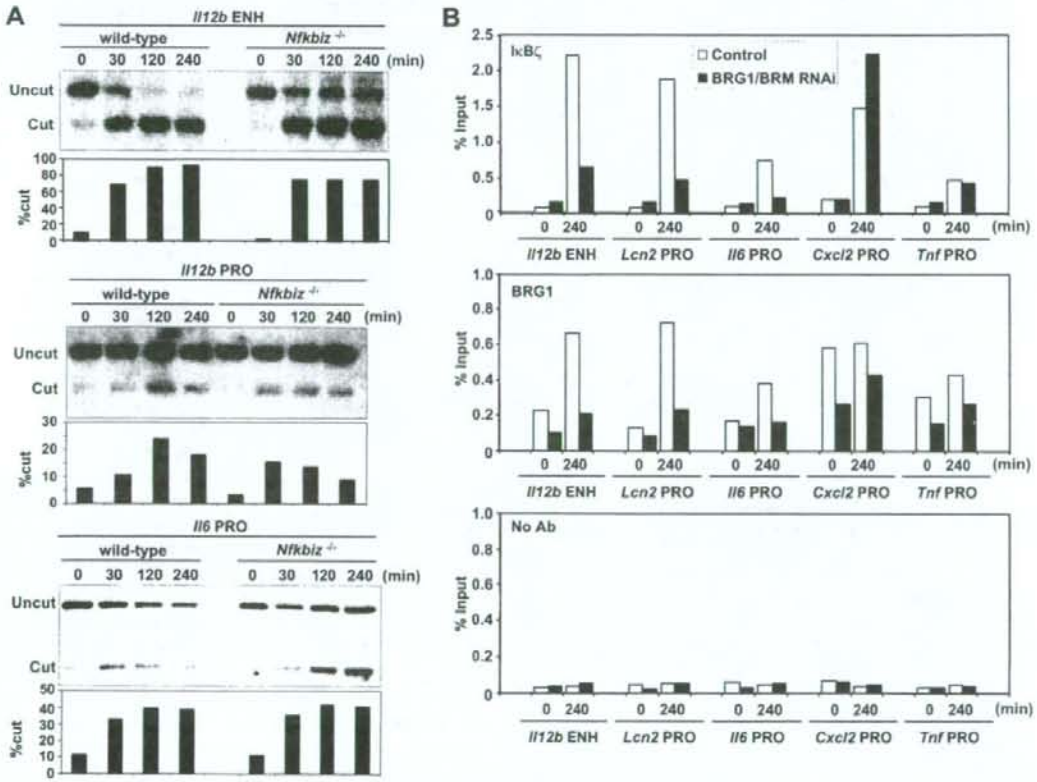

FIGURE 7. **Nucleosome remodeling at secondary response gene regulatory regions in the absence of IκBζ.** *A*, bone marrow-derived macrophages from wild-type and *Nfkbiz*$^{-/-}$ mice were stimulated with 10 μg/ml LPS for the indicated periods. Restriction enzyme accessibility assay at the *Il12b* enhancer (*upper*), *Il12b* promoter (*middle*), and *Il6* promoter (*bottom*) regions was performed. *B*, ChIP assay was performed with chromatin prepared from J774 cells infected with the empty RNA interference vector (*white bars*) and from BRG1/BRM short interfering RNA-depleted cells (*black bars*) treated with LPS for 0 and 240 min. Antibodies against IκBζ and BRG1 were used. Precipitated DNA was quantified by real time PCR using primers specific for the indicated control regions. Data were plotted relative to input DNA (% *Input*).

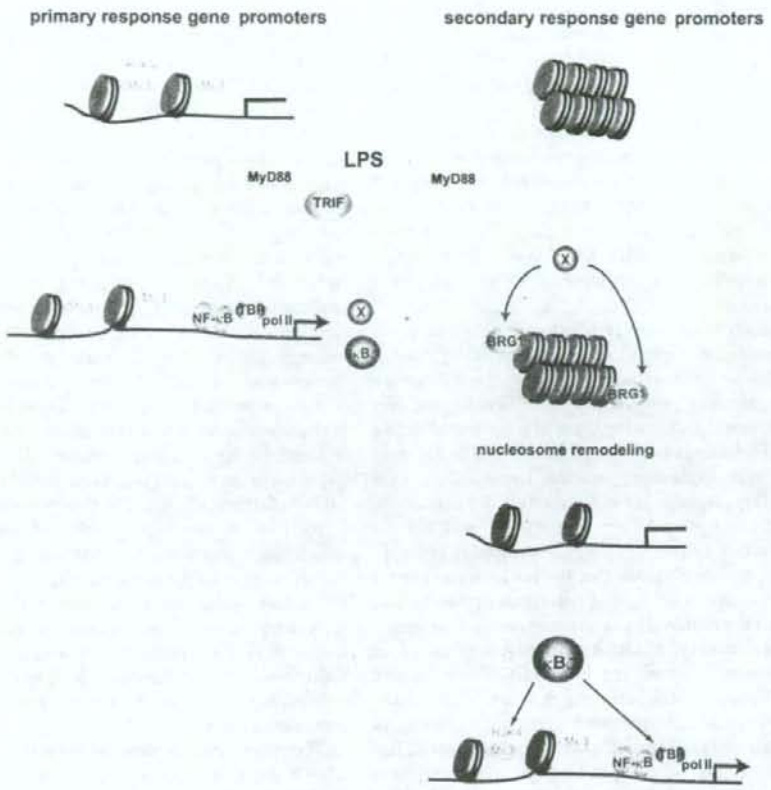

FIGURE 8. **Schematic model of primary and secondary response gene activation pathway.** Early primary response gene promoters have open nucleosome structures and are activated immediately after LPS stimulation. In contrast, secondary response gene promoters have closed nucleosome structures and are remodeled through an unknown primary response gene product (X)-dependent recruitment of the SWI/SNF complexes, including BRG1. Then another primary response gene product, IκBζ, mediates preinitiation complex assembly and histone H3K4 trimethylation, resulting in activation of the secondary response genes.

ing TLR4 stimulation. At one key class of genes that is induced rapidly in the absence of new protein synthesis, the early primary response class, the promoters appear to be assembled into chromatin structures that are poised for activation. In unstimulated cells, these chromatin structures consist of high histone acetylation and H3K4 trimethylation levels and high accessibility to nuclease cleavage (26, 28). After macrophage activation, the chromatin structure remains largely unchanged, but NF-κB rapidly associates with the promoters and presumably contributes to the rapid assembly of a preinitiation complex containing TBP and pol II. Preinitiation complex assembly in response to TLR4 signaling does not specifically require MyD88 because of its redundancy with TRIF. However, MyD88 is critical for the efficient induction of primary response gene transcription. It is possible that reduced expression of primary response genes in *Myd88*$^{-/-}$ cells is because of earlier shutdown of transcription. But there might be another unknown mechanism, because the MyD88 effect is just as strong at the 1-h time point.

Although secondary response genes can be induced quite rapidly after TLR4 stimulation, their mechanism of activation is dramatically different. In unstimulated macrophages, secondary response promoters are usually assembled into chromatin structures that are inaccessible to nuclease cleavage and exhibit low levels of histone acetylation and H3K4 trimethylation (26, 28). Substantial changes in chromatin structure are therefore required for transcriptional activation (27). One critical event appears to be the remodeling of nucleosomes by ATP-dependent nucleosome remodeling complexes of the SWI/SNF family. A previous study showed that nucleosome remodeling at the promoters of secondary response genes requires new protein synthesis (28). The results of this study show that MyD88 pathways are also required for nucleosome remodeling. Although IκBζ was an attractive candidate for an MyD88-dependent primary response gene product that might drive nucleosome remodeling at a subset of secondary response genes, our results strongly suggest that other MyD88-dependent primary response gene products carry out this critical function. IκBζ instead plays an important role downstream of the nucleosome remodeling step but prior to the binding of NF-κB p65, TBP, and pol II, and prior to histone H3K4 trimethylation (Fig. 8).