CD4⁺CD25⁺Foxp3⁺ regulatory T cells induce alternative activation of human monocytes/macrophages

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CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) are potent suppressors of the adaptive immune system, but their effects on innate immune cells are less well known. Here we demonstrate a previously uncharacterized function of Tregs, namely their ability to steer monocyte differentiation toward alternatively activated macrophages (AAM). AAM are cells with strong antiinflammatory potential involved in immune regulation, tissue remodeling, parasite killing, and tumor promotion. We show that, after coculture with Tregs, monocytes/macrophages display typical features of AAM, including up-regulated expression of CD206 (macrophage mannose receptor) and CD163 (hemoglobin scavenger receptor), an increased production of CCL18, and an enhanced phagocytic capacity. In addition, the monocytes/macrophages have reduced expression of HLA-DR and a strongly reduced capacity to respond to LPS in terms of proinflammatory mediator production (IL-1 β , IL-6, IL-8, MIP-1 α , TNF- α), NF κ B activation, and tyrosine phosphorylation. Mechanistic studies reveal that CD4⁺CD25⁺CD127^{low}Foxp3⁺ Tregs produce IL-10, IL-4, and IL-13 and that these cytokines are the critical factors involved in the suppression of the proinflammatory cytokine response. In contrast, the Treg-mediated induction of CD206 is entirely cytokine-independent, whereas the up-regulation of CD163, CCL18, and phagocytosis are (partly) dependent on IL-10 but not on IL-4/IL-13. Together these data demonstrate a previously unrecognized function of CD4⁺CD25⁺Foxp3⁺ Tregs, namely their ability to induce alternative activation of monocytes/macrophages. Moreover, the data suggest that the Treg-mediated induction of AAM partly involves a novel, cytokine-independent pathway.

alternatively activated macrophages | mannose receptor | phagocytosis | proinflammatory response | interleukin-10

The suppressive effects of CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) on the adaptive immune system and on CD4⁺ T cells in particular have been well documented (1, 2). CD4⁺CD25⁺ Tregs suppress T cell proliferation, down-regulate proinflammatory cytokine production (IFN- γ and TNF- α), and directly inhibit IL-2 mRNA transcription. *In vitro*, the mechanism behind T cell suppression appears to be IL-10- and TGF- β -independent and requires cell contact between Tregs and responder T cells (3–5) but does not require the presence of antigen-presenting cells (APC) *per se* (6). *In vivo*, however, IL-10 and TGF- β have been shown to play a prominent role (reviewed in refs. 7 and 8). Moreover, two recent *in vivo* studies showed that Tregs can form direct interactions with dendritic cells (DC) in lymph nodes, thereby preventing stable contacts between APC and responder CD4⁺ T cells, resulting in an inhibition of T cell activation (9, 10). Thus, the picture emerges that Treg-mediated suppression *in vivo* involves multicellular clusters consisting of responder T cells, APC, and regulatory T cells, and that during these cellular interactions, membrane-bound and/or soluble inhibitory molecules contribute to suppression.

In inflamed tissues, the interactions between CD4⁺CD25⁺ Tregs and APC are likely to involve not only DC but also monocytes/macrophages. Monocytes/macrophages play a critical

role in both innate and adaptive immunity through their ability to recognize pathogens and/or “danger signals” via TLRs and other pattern-recognition receptors; through their effector mechanisms, including phagocytosis, nitric oxide production, and killing of bacteria; and through their ability to produce a wide array of cytokines and chemokines. Furthermore, monocytes/macrophages can process antigen and present antigen-derived peptides via MHC class II molecules to CD4⁺ T cells. Importantly, monocytes/macrophages are involved in both the initiation and the resolution of an inflammatory response, and two corresponding activation states for macrophages have been described *in vitro* (11–13). The initial inflammatory response is carried out by macrophages that produce high amounts of proinflammatory cytokines and reactive oxygen species. These macrophages are also referred to as classically activated or M1 macrophages and can be generated *in vitro* by activation with IFN- γ /LPS. The resolution phase is associated with macrophages that produce mainly antiinflammatory cytokines and have a higher phagocytic capacity, and are characterized amongst others by an increased expression of the mannose receptor CD206 and/or the hemoglobin scavenger receptor CD163. These macrophages are often referred to as M2 or alternatively activated macrophages (AAM) and can be obtained *in vitro* after macrophage stimulation with IL-4/IL-13 (14, 15), a combination of immune complexes and LPS (15), or IL-10 or glucocorticoids (16). The three different induction methods lead to more or less distinct M2 macrophage subsets (also referred to as M2a, M2b, and M2c, respectively), each with a typical cytokine/chemokine and cell surface marker profile (17), although recent data indicate that a certain degree of versatility exists between the subsets (18–20).

The excessive presence or activity of either M1 or M2 subsets may cause damage to the host, because this could promote immune responses to healthy tissue resulting in inflammation, or, conversely, prevent an appropriate immune response leading to hampered tumor immunity (17, 21). Understanding the mechanisms behind the homeostatic control of monocyte/macrophage function is, therefore, of fundamental importance. Previously, indirect evidence for Treg-mediated suppressive effects on monocytes/macrophages was provided after adoptive transfer of CD4⁺CD25⁺ Tregs in a colitis model (22). Recent work by us (23) and others (24) demonstrated that in humans, CD4⁺CD25⁺ Tregs have direct inhibitory effects on the antigen-

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Fig. 1. Tregs induce an alternative activated phenotype in monocytes/macrophages. Monocytes were cultured without T cells (no T, black bars), with CD4⁺CD25⁻ T cells (CD25⁻, white bars), or with CD4⁺CD25⁺ T cells (CD25⁺, hatched bars) in the presence of anti-CD3 mAb (50 ng/ml). (A) The phenotype of monocytes was assessed after 40 h of culture by flow cytometry. The expression (average mean fluorescence intensity, MFI $\pm$ SEM) of CD206, CD163, and HLA-DR is shown for 15, 7, and 11 independent experiments, respectively. (B) The amount of CCL18 produced during the 40 h of coculture was analyzed in the supernatant by ELISA. (C) After 40 h of coculture, monocytes were stimulated with FITC-labeled latex beads or Zymosan, and phagocytosis was measured 18 h later ($n = 13$). The average MFI of phagocytosed particles (Zymosan or latex beads) per monocyte is shown for the three different monocyte cultures (no T vs. CD25⁻ vs. CD25⁺). Significant differences ($P < 0.05$; Θ , no T vs. CD25⁺; Δ , no T vs. CD25⁻; #, CD25⁻ vs. CD25⁺) are shown in the graphs.

presenting function of monocytes/macrophages, as shown by their reduced capacity to stimulate antigen or allo-specific T cell responses. Here we assessed a previously uncharacterized function of CD4⁺CD25⁺Foxp3⁺ Tregs, namely their ability to directly promote the alternative activation of monocytes/macrophages.

Results

CD4⁺CD25⁺ Tregs Induce Typical Characteristics of Alternative Activation in Monocytes/Macrophages. To investigate whether CD4⁺CD25⁺ Tregs can steer the differentiation of monocytes to AAM, CD14⁺ monocytes were cultured alone, with autologous CD4⁺CD25⁻ or CD4⁺CD25⁺ T cells. Anti-CD3 mAb was present in all conditions to stimulate the T cells. After 40 h, cells were collected for flow cytometry. Fig. 1A shows that, relative to control monocytes, Treg-treated monocytes displayed a strong up-regulation of the typical AAM markers CD206 ($n = 15$, $P < 0.05$) and CD163 ($n = 7$, $P < 0.02$). In addition, a reduced expression of HLA-DR ($n = 11$, $P < 0.04$) and CD86 ($n = 8$, $P < 0.02$, data not shown) was observed. We also determined the presence of CCL18, a chemokine that is specifically overexpressed by AAM (25), and found this to be significantly enhanced in monocyte/Treg cocultures (Fig. 1B, $n = 8$, $P < 0.008$). Furthermore, we assessed the phagocytic ability of Treg-modulated monocytes/macrophages by adding FITC-labeled latex beads or Zymosan after 40 h of coculture. Although only a slight increase in the percentage of phagocytosing monocytes was observed following Treg coculture [supporting information (SI) Fig. 6], on a per cell basis Treg-modulated monocytes/macrophages consistently phagocytosed more Zymosan particles

Fig. 2. Tregs suppress the LPS-induced proinflammatory response of monocytes/macrophages. Monocytes were cultured as described in the legend to Fig. 1. After 40 h of coculture, LPS (50 ng/ml) was added and 24 h later cytokine/chemokine production was measured by ELISA (IL-6, TNF- α , and IL-10) or Luminex (IL-1 β , IL-8, MIP-1 α , MCP-1, and IL-1Ra). The average production of eight independent experiments $\pm$ SEM of proinflammatory (A) and antiinflammatory (B) cytokines/chemokines are shown for the three culture conditions. Significant differences ($P < 0.03$; Θ , no T vs. CD25⁺; Δ , no T vs. CD25⁻; #, CD25⁻ vs. CD25⁺) are shown in the graphs. Neither GM-CSF nor IL-12p70 was not detected (data not shown). (C) After 40 h, the cocultures were depleted of T cells, and the purified monocytes were either unstimulated (control) or stimulated with LPS for 15 min before total cell lysates were generated. The mean NF- κ B p50 activation $\pm$ SEM of four independent experiments is shown. (D) Ten micrograms of total cell lysates from monocytes cultured in the absence (lanes 1 and 4) or presence of CD25⁻ (lanes 2 and 5) or CD25⁺ (lanes 3 and 6) T cells that were untreated (lanes 1–3) or treated with LPS (lanes 4–6) was separated on 10% SDS gels and immunoblotted with an antiphosphotyrosine antibody, pY (Upper) or a control anti- β -actin antibody (Lower). A representative example of four independent experiments is shown.

or latex beads compared with the control (no T or CD25⁻) monocytes ($P < 0.002$, Fig. 1C).

CD4⁺CD25⁺ Tregs Inhibit the Proinflammatory Response of Monocytes/Macrophages to LPS. Next we determined the ability of Treg-treated monocytes/macrophages to respond to LPS. Following coculture with Tregs, monocytes were significantly suppressed in their capacity to produce proinflammatory cytokines/chemokines (TNF- α , IL-6, IL-1 β , IL-8/CXCL8, and MIP-1 α /CCL3) compared with monocytes cultured alone (Fig. 2A). In contrast, the production of the antiinflammatory cytokines IL-1Ra and IL-10 was enhanced (Fig. 2B), indicating that rather than inducing a general cytokine down-regulation in monocytes/macrophages, Tregs shift the balance from a proinflammatory

toward an antiinflammatory cytokine profile. As a control, monocytes cultured in the presence of CD4⁺CD25⁻ effector T cells displayed increased production of both proinflammatory and antiinflammatory cytokines/chemokines. To exclude possible contributions of T cell-derived cytokines in these assays, we repeated these experiments with monocytes that were repurified after Treg coculture and obtained similar results (SI Fig. 7). Importantly, these latter experiments also demonstrate that the suppressive effects persist even once Tregs are removed from the assay. Moreover, in the presence of exogenous IFN- γ or LPS, we still observed intact Treg-mediated suppression of IL-6 and TNF- α production (SI Fig. 8). This demonstrates that the Treg effect on monocytes/macrophages is a dominant phenomenon and is not explained by a lack of IFN- γ or other inflammatory mediators in the Treg-monocyte cocultures. The decreased proinflammatory cytokine response to LPS of Treg-modulated monocytes was not explained by a decreased expression of TLR4 or CD14 after Treg contact (SI Fig. 9). However, the reduced proinflammatory response was reflected at the transcriptional level by a clear decrease in the basal activation levels of NF κ B p50 (and RelA/p65, data not shown) as well as by an impaired NF κ B up-regulation upon LPS stimulation (Fig. 2C). In addition to a lack of NF κ B activation, a profound block in tyrosine phosphorylation upon LPS stimulation was observed in CD25⁺ Treg-treated monocytes (Fig. 2D). In contrast, control monocytes or monocytes precultured with CD25⁻ T cells displayed an increase in both the number and intensity of tyrosine phosphorylated proteins and NF κ B activation upon LPS triggering (Fig. 2C and D).

Treg-Mediated Modulation of Monocyte/Macrophage Function Requires Cell Contact as Well as Soluble Factors. To investigate whether suppression of monocyte/macrophage function depended on cell contact or soluble factors, we cultured monocytes in the absence or presence of Tregs (or CD4⁺CD25⁻ T cells, data not shown) in either a coculture (CC) or a transwell (TW) system. After 40 h of culture, the top compartments (inserts) were removed, and the monocytes in the lower well were stimulated with LPS. By disrupting physical contact between monocytes and Tregs (TW) the suppression of both IL-6 and TNF- α production was significantly reduced compared with the coculture system, although the effect on TNF- α production was less marked than on IL-6 (Fig. 3A). This partial reversal of suppression could be due to the requirement of cell contact between Tregs and monocytes, or due to insufficient costimulation of Tregs in the absence of monocytes. We therefore restored cell contact in the upper well by adding monocytes to the Treg population in the insert (TW + mono). Interestingly, under these conditions suppression of cytokine production was completely restored to the levels seen in cocultures (Fig. 3A). This finding indicates that upon monocyte-Treg interaction soluble factors are produced that reduce the ability of monocytes in the lower well to respond to LPS, even once the inserts containing the Tregs are removed. Interestingly, only in the CC and TW + mono conditions, some IL-10 was detected in the supernatant, whereas IL-10 was completely absent from the TW conditions (data not shown), indicating that one of the soluble factors involved could be IL-10.

The Treg-Mediated Effect on Monocyte/Macrophage Function Is Partly Mediated via IL-10, IL-4, and IL-13. To determine the nature of the soluble factors involved in the modulation of monocyte/macrophage function, neutralizing antibodies against IL-10, IL-4/IL-13, or TGF- β were added to the cocultures. Treg-mediated inhibition of the LPS-induced TNF- α response (on average 70% suppression) was somewhat reduced after addition of neutralizing anti-IL-10, whereas anti-IL-4/IL-13 mAb had no effect (Fig. 3B). However suppression was significantly reversed (down to 24%, $P < 0.05$) when all three mAbs were added,

Fig. 3. Inhibition of proinflammatory cytokine production requires cell contact-dependent induction of soluble factors. (A) Monocytes were cocultured with Tregs in the same well (CC, black bars) or cultured separately in a transwell (TW, white bars), with Tregs in the insert and monocytes in the lower well. As a control, TW cultures were also set up with monocytes present in both compartments of the Transwell (TW + mono, hatched bars). After 40 h of culture, inserts were removed and cultures were stimulated with LPS (500 ng/ml). After 24 h, cytokine production was measured by ELISA. The mean percentage suppression of TNF- α (Left) and IL-6 (Right) production is shown for Treg-modulated monocytes compared with monocytes alone ($n = 5$). Significant differences ($P < 0.05$) are indicated for TW vs. CC (*) and TW vs. TW + mono (#). (B) Cocultures were set up as described in the legend to Fig. 1, in the absence or presence of neutralizing mAbs to IL-10 and/or IL-4/IL-13. The percentage suppression of TNF- α (Left, $n = 6$) and IL-6 (Right, $n = 4$) production in response to LPS is shown for Treg-modulated monocytes compared with monocytes alone.

indicating redundancy between these cytokines. Interestingly, suppression of IL-6 production was more readily reversed by using the neutralizing mAbs either alone or in concert (Fig. 3B). Blocking TGF- β had no effect (data not shown).

We next investigated the effects of neutralizing these three cytokines on AAM phenotype. Surprisingly, neutralizing IL-4/IL-13 had no effect on the Treg-induced up-regulation of CD206, nor did blocking of IL-10, or blocking all three cytokines in concert (Fig. 4). We did find that neutralizing IL-10 was sufficient to reverse the up-regulation of CD163 (Fig. 4) as well as the down-regulation of HLA-DR (data not shown). Although neutralizing IL-10 affected the overall production levels of the chemokine CCL18, the Treg-mediated increase in CCL18 production was still maintained in the presence of neutralizing IL-10 and/or IL-4/IL-13 mAb. Finally, the increased phagocytic capacity of Treg-treated monocytes/macrophages was only partially reversed by neutralizing IL-10, but not by IL-4/IL-13 blockade or the combination of mAbs (Fig. 4).

CD4⁺CD25⁺Foxp3⁺ Tregs Can Produce IL-4, IL-13, and IL-10 upon Stimulation. Although it has been shown that Tregs are able to produce some IL-4 and IL-10 upon stimulation (5, 26–29), we wanted to confirm that these cytokines were indeed present in our Treg cultures. CD4⁺CD25⁺ Tregs were isolated by magnetic cell separation as well as by stringent cell sorting for CD25^{hi}CD127^{low} cells, and intracellular stains were performed after stimulation with either CD3/CD28 beads or monocytes in combination with anti-CD3. Fig. 5A shows that the percentage of IL-10⁺, IL-13⁺, and

Tregs on T cell proliferation, which have been amply demonstrated *in vitro* to occur in a cell contact rather than cytokine-dependent manner. These data thus demonstrate that CD4⁺CD25⁺ Tregs can employ distinct modes of suppression when targeting different immune cells, and this may explain some of the contrasting findings in literature regarding the role of cytokines in Treg-mediated suppression *in vitro* and *in vivo*. The Treg-mediated suppression of the monocyte response to LPS is accompanied by a reduction in the up-regulation of NF- κ B DNA binding activity, as well as in the basal level of NF- κ B activity. NF- κ B activation is required for proinflammatory cytokine gene expression and involved in the regulation of surface markers such as CD40 and CD86. The decreased NF- κ B activation may well be due to the increased levels of IL-10 and IL-13 in the Treg-monocyte culture, because it has been shown that both IL-10 and IL-13 can suppress NF- κ B activation (32). Moreover, IL-10-induced suppression of NF- κ B activation is also found in tumor-associated macrophages, which have an alternatively activated phenotype (33). Furthermore, blocking NF- κ B activation in classically activated macrophages results in a decreased proinflammatory response and reduced expression of costimulatory markers, whereas the phagocytic capacity remains intact and bacterial killing is enhanced (34). The latter indicates that suppressed NF- κ B activation does not affect the phagocytic capacity of monocytes/macrophages, which is supported by our data. Of note, although some IL-10 and IL-4 production in human Treg culture supernatants was described before (26–29), to our knowledge this is the first report to describe IL-13 production by this cell population.

Recent *in vitro* and *in vivo* studies have shown that macrophages in tissues can undergo phenotypic switches from M1 to M2 macrophages and vice versa (18–20). Our data strongly suggest that, when entering tissues, monocytes/macrophages will not only be affected by the local microenvironment (cytokines, chemokines, growth factors, and tissue cells) but also by the presence of activated effector T cells and Tregs. For example, on the basis of the data presented here, it can be envisaged that the increased presence of Tregs at tumor sites as described previously (35, 36) together with the tolerogenic milieu skews newly recruited monocytes toward an AAM phenotype. Indeed the presence of M2-like tumor-associated macrophages with low proinflammatory and high phagocytic capacity have been described in various human and mouse tumors (17, 21). Thus Tregs may hamper effective tumor immunity not only by inhibiting CD4⁺ and CD8⁺ T cell responses but also by steering monocytes/macrophages toward an alternatively activated phenotype and function.

In conclusion our study shows that Tregs promote the induction of alternatively activated monocytes/macrophages, which will help to maintain tissue homeostasis and prevent local tissue damage but may also contribute to hampered antitumor immunity. This newly discovered ability of Tregs may help us to understand many disease processes and may also provide a novel tool to manipulate local immune responses.

Materials and Methods

Cell Isolation and Purification. Peripheral blood was obtained from healthy individuals with informed consent. Ethical approval for this study was obtained from the College Research Ethics Committee (02/03/67). Peripheral blood mononuclear cells (PBMC) were isolated by using density gradient centrifugation (Lymphocyte separation media, PAA). Monocytes (>95% purity) were isolated by using anti-CD14 microbeads or by depleting nonmonocytes (Miltenyi Biotec). CD4⁺ T cells (>95% purity) were purified by using a T cell isolation kit, and CD4⁺CD25⁺ T cells were enriched with anti-CD25 microbeads (Miltenyi Biotec) resulting in >90% CD4⁺CD25⁺ T cells with >80% expressing Foxp3⁺ and 90% CD127^{low} cells. In addition,

separation of CD4⁺CD25⁺CD127^{low} and CD4⁺CD25⁺CD127⁺ T cells from total CD4⁺ T cells was performed by using MoFlo (purities shown in SI Fig. 10). For flow cytometry, the following antibodies were used: anti-CD3-FITC, anti-CD4-PE-Cy5, anti-CD8-PE-Cy5, anti-CD14-PE-Cy5, anti-CD19-PE, anti-HLA-DR-FITC, CD86-PE, anti-CD25-FITC (all from Beckman-Coulter), anti-CD206-PE, anti-CD127-PE (BD Biosciences), anti-CD25-PE (Miltenyi Biotec), CD163-FITC (Santa Cruz Biotechnology), and Foxp3-APC (eBiosciences).

Cocultures and Transwell Experiments. Monocytes and T cells (2:1 ratio) were cocultured in RPMI medium 1640, supplemented with 1% penicillin/streptomycin, 1% glutamin, and 10% heat-inactivated FCS. Monocytes (5×10^5 /ml) were cultured in either 200- or 500- μ l cultures without T cells (no T), with CD4⁺CD25[−] T cells (CD25[−]) or CD4⁺CD25⁺ T cells (CD25⁺) for 40 h in the presence of 50 ng/ml anti-CD3 mAb (OKT3, Ortho Biotech), after which the different cultures were stimulated for 24 h with LPS (50 ng/ml, Sigma; this was found to be the optimal concentration out of a dose range (0–100 ng/ml) to stimulate monocytes for IL-6 and TNF- α production). Transwell experiments were performed in 24-well plates (0.4 μ m pore size, Corning Costar) by culturing monocytes in the lower well and the T cells with anti-CD3 mAb in the inserts (with or without monocytes). After 40 h of culture, the inserts were removed, and the monocytes in the lower well were stimulated with LPS (50 ng/ml). For neutralization experiments, neutralizing antibodies against IL-10 (5 μ g/ml, mIgG2b, clone 23738), IL-4 (5 μ g/ml, mIgG2b, clone 34019.111), IL-13 (5 μ g/ml, mIgG1, clone 31606), isotype control (mIgG2b, clone 20116 or mIgG1, clone MOPC 21, Sigma), or TGF- β (5 μ g/ml, mIgG1, clone 1D11) all from R & D Systems were added at the start of the coculture.

Detection of Cytokines, Chemokines, and Phagocytosis. IL-6, IL-10, TNF- α (Invitrogen), CCL18 (R & D Systems), and GM-CSF (Amersham Biosciences) were measured by ELISA, and IL-1 β , IL-1Ra, IL-8, IL-12-p70, MIP-1 α , and MCP-1 were measured by Luminex (Upstate) by using a Luminex 100 system, according to the manufacturer's instructions. Intracellular cytokine staining using anti-IL-10-PE (Miltenyi Biotec), anti-IFN- γ -FITC (eBiosciences), anti-IL-4-APC (eBiosciences) and anti-IL-13-PE (a kind gift of Catherine Hawrylowicz, King's College London, London) was performed 18 h after T cell stimulation with either anti-CD3 mAb and monocytes or CD3/CD28 beads (Invitrogen, 1 bead/cell). To measure phagocytosis, FITC-coupled carboxylate-modified latex beads (Sigma; 1:25 cell/bead ratio) or FITC-coupled Zymosan particles (25 μ g/ml) were added to the cocultures in the absence or presence of Cytochalasin D, an inhibitor of actin polymerization and particle internalization (10 μ M, Sigma). After stimulation, the cells were stained with CD14-PE-Cy5 and analyzed for uptake of FITC-coupled beads or Zymosan on a FACSCalibur.

Preparation of Cell Extracts, SDS/PAGE, and Western Blot Analysis. Cells were cocultured as described, and after 40 h, T cells were depleted by using CD2-microbeads (Miltenyi Biotec), and the purified monocytes (average purity 90%) were stimulated with medium or LPS (50 ng/ml) for 15 min. Total cell lysates were generated in lysis buffer containing 1 mM sodium orthovanadate (Upstate) supplemented with protease inhibitor mixture (Promega), for 10 min on ice, and supernatants were clarified by centrifugation at $16,100 \times g$ for 15 min at 4°C. Protein levels were quantified by using BCA protein assay kit (Pierce), and 10 μ g of each extract was separated on 10% SDS gels, transferred to Immobilon PVDF membranes (Millipore), and incubated with p-Tyr (PY99; Santa Cruz Biotechnology) or control anti-beta-actin monoclonal (Abcam) antibodies and developed by using the ECL Plus chemiluminescence kit (Amersham Biosciences).

NF- κ B Binding Activity. NF- κ B DNA-binding activity was assayed by using nonradioactive ELISA-based TransAM NF- κ B p50 and p65 specific chemiluminescence kits (Active Motif) according to the manufacturer's instructions. Each assay was performed by using 1 μ g of extract. Chemiluminescence was measured at 450 nm on a VICTORLight-1420 Luminescence counter (Perkin-Elmer).

Statistical Analysis. Statistical analysis was performed with GraphPad Prism 4.03 software by using Wilcoxon matched

pairs tests or paired *t* tests. *P* values <0.05 were considered significant.

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