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Pregnancy-Specific Down-Regulation of NF-κB Expression in T Cells in Humans Is Essential for the Maintenance of the Cytokine Profile Required for Pregnancy Success[1] FREE

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Pregnancy-Specific Down-Regulation of NF-κB Expression in T Cells in Humans Is Essential for the Maintenance of the Cytokine Profile Required for Pregnancy Success[1]

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It is accepted that human pregnancy is associated with a shift away from Th1 type and a bias toward Th2-type immune responses. The molecular mechanisms that regulate this shift are as yet unknown. We assessed the expression and activity of NF-κB, a transcription factor that plays a central role in regulating immune responses. We isolated T cells from PBMCs from nonpregnant and pregnant females and demonstrated that the NF-κB/IκB signaling pathway is down-regulated in T cells in pregnancy. Using Western blotting, high levels of NF-κB (p65) were detected in all nuclear fractions of T cells from nonpregnant females. In contrast, low levels of p65 were detected in nuclear fractions from T cells from pregnant females. Levels of IκBα and -β were also higher in cytoplasmic fractions from T cells from nonpregnant than from pregnant females. The reduction in p65 levels in pregnancy was reflected in the activity of NF-κB in EMSA; T cells from pregnant females contain less active NF-κB than from nonpregnant females. Stimulation of T cells from nonpregnant females with PMA/ionomycin resulted in IκBα degradation, p65 translocation, and subsequent production of the Th1 cytokines IFN-γ and IL-2. In contrast, PMA stimulation had no effect on NF-κB activity in T cells from pregnant females, and this was reflected in reduced Th1 cytokine production. Using the inhibitor of NF-κB activity, SN50, we were able to show that NF-κB activity was essential for the production of Th1 cytokines, suggesting that specific down-regulation of NF-κB in T cells throughout gestation is paramount to pregnancy success through specific regulation of cytokine production. ***The Journal of Immunology*****, 2004, 172: 4583–4591.**

The survival of the fetal allograft during normal human pregnancy remains poorly understood. The placenta has a paternal genetic contribution and is antigenically distinct from the mother. T cell immune responses similar to those seen in tissue rejection would therefore be expected to occur throughout pregnancy. T cells can differentiate into effectors whose cytokine profile is limited to type 1 (Th1; IFN-γ-dominant) or type 2 (Th2; IL-4, IL-5 dominant) (1). A Th1 cytokine profile is responsible for acute allograft rejection (2). It is proposed that T cell function is altered in pregnancy to facilitate a developing fetus by switching to a Th2 bias, which helps ameliorate potentially lethal Th1 and cytotoxic T cell responses (1).

The switch in the cytokine profile away from Th1-type reactivity to Th2 reactivity in normal pregnancy is well accepted. Th1 cytokines have been shown to induce fetal resorption in mice (3), and conversely, spontaneous fetal resorptions in certain mouse mating combinations can be reversed by Th2 cytokines (4). In the human, Th2 cytokines have been shown to be produced by T cells from normal pregnant females at levels higher than those in nonpregnant controls, whereas Th1 cytokines are produced in pregnant females at levels lower than those in nonpregnant controls (5). In addition, Th1 cytokines are present at higher levels in T cells from females who suffer from recurrent pregnancy losses and implantation failure (6–8). This is consistent with the observation that PBMCs from normal pregnant females produce Th2 cytokines, whereas PBMCs from patients with recurrent spontaneous abortions produce Th1 cytokines in response to placental/trophoblast Ags (9). Thus, there is a distinct suppression of Th1 responses and subsequent Th2 bias associated with normal pregnancy.

Despite the acceptance that pregnancy is associated with suppression of a Th1 immune response, the mechanism(s) responsible for regulation of this suppression is unknown. The NF-κB family of transcription factors is a critical regulator of the development and maintenance of the immune system. Five NF-κB subunits exist, the most abundant and active form is a heterodimer composed of p50 and p65. Most commonly, p50:p65 is retained in the cytoplasm of resting cells in a latent form bound to inhibitors of κB (IκB-α and IκB-β). Activation of cells results in degradation of IκBs and subsequent translocation of NF-κB to the nucleus, followed by gene transcription (10). NF-κB plays a key regulatory role in controlling Th1 and Th2 immune responses. Mice with a disrupted p50:p65 signaling pathway, and thus with inhibited NF-κB activity, develop a Th2 type response to infection (11). Conversely, activation and nuclear translocation of NF-κB are necessary for the development of a Th1 response (12). As Th1 responses culminate in allograft rejection, this is consistent with the observation that abrogation of the NF-κB signaling pathway is associated with markedly enhanced allograft survival in an animal transplantation model (13). NF-κB also plays a significant role in the survival of memory T cells by regulating the transcription of genes that protect cells against apoptosis (14–16).

We have attempted to delineate the molecular mechanisms involved in controlling the down-regulation of Th1 immune responses in pregnancy by performing studies to determine the regulation of NF-κB in circulating white cells in pregnancy. We have previously shown that NF-κB and IκBα are reduced in PBMCs

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from third-trimester pregnant women compared with those in normal nonpregnant control women (17) in a novel pregnancy-specific manner.

In this study we test the hypothesis that the cell type responsible for the down-regulation of NF-κB in PBMCs is the T lymphocyte in pregnancy. We have examined the expression and activity of NF-κB and some of its target genes in T cell from nonpregnant and pregnant females. We have demonstrated that NF-κB is down-regulated in T cells from pregnant females relative to that in nonpregnant females and that this reduction correlates with reduced T cell production of Th1 cytokines in pregnancy. This may reflect one mechanism that is an essential component for controlling the cytokine profile necessary for pregnancy success.

Materials and Methods

Sample cohort

For separation of T cells and PBMCs, blood was collected with informed consent from nonpregnant ($n = 10$) and pregnant ($n = 10$) females. Analysis of intracellular cytokine production was performed on PBMCs from nonpregnant ($n = 9$) and pregnant ($n = 9$) females. Nonpregnant females were fit and healthy, of reproductive age, nonmenstruating, and not taking oral contraception at the time of blood collection. Pregnant females were normotensive and in their third trimester of pregnancy. Patients who had any flu- or cold-like symptoms <7 days before blood collection were excluded from the study. The mean age, parity, and gestation of pregnancy of the study subjects are outlined in Table I. Ethical approval for this study was given by the Royal North Shore Hospital human research ethics committee.

Preparation of T cells

T cells were isolated from nonpregnant and pregnant females using the T Cell Negative Isolation Kit according to the manufacturer's instructions (Dynal, Victoria, Australia). Briefly, PBMCs were isolated using Ficoll-Paque (Amersham Pharmacia Biotech, Sydney, Australia) as described in the T cell isolation kit. PBMCs were incubated with the mixture of mAbs raised against non-T cells supplied with the kit on ice for 30 min. Ab-coated cells were identified using Depletion Dynabeads coated with human IgG4 Ab directed against mouse IgG and were removed from suspension using a magnetic particle concentrator. Isolated T cells were collected, and purity was determined by flow cytometry using a BD FACSVantage SE flow cytometer and CD3 FITC-conjugated Ab.

Cell culture

Isolated T cells and PBMCs were cultured in RPMI 1640 (Life Technologies, Melbourne, Australia), penicillin/streptomycin (Sigma-Aldrich, Castle Hill, Australia), 20 mM L-glutamine (Sigma-Aldrich), and 10% FCS at a concentration of 1×10^6 cells/ml. For T cell stimulation, cells were incubated with/without 10 ng/ml PMA (Sigma-Aldrich) and 500 ng/ml ionomycin (Sigma-Aldrich) for 1 h at 37°C in the presence of 5% CO_2. For analysis of intracellular cytokine production, PBMCs were incubated in culture medium alone or in the presence of SN50 (37.5 μg/ml; Biomol, Plymouth Meeting, PA) or SN50M (37.5 μg/ml; Biomol) for 30 min and then stimulated with/without 10 ng/ml PMA (Sigma-Aldrich) and 500 ng/ml ionomycin (Sigma-Aldrich) for 4 h at 37°C in the presence of 5% CO_2.

Sample preparation

Whole-cell lysates of T cells were prepared by resuspending 1×10^7 cells in 1 ml of double-detergent lysis buffer (50 mM Tris-HCl (pH 8), 150 mM NaCl, 0.02% sodium azide, 100 μg/ml PMSF, 1 μg/ml aprotinin, 1% IGEPAL CA-630 (Sigma-Aldrich), and 0.1% SDS) and storing at −70°C until use. Nuclear and cytoplasmic extracts were prepared from cells using a modified version of the method described by Shreiber et al. (18) as previously reported (17).

Table I. *Gestation, age, and parity of pregnant and nonpregnant females recruited for the study*[a]

	Nonpregnant ($n = 19$)	Pregnant ($n = 19$)
Age (years)	34.3 ± 8.4	30.14 ± 5.57
Gestation (wk)	N/A	32.14 ± 4.7
Parity (*n*)	0.83 ± 1.03	0.4 ± 0.7

[a] Values are given as means ± SD. N/A = not applicable.

Western blotting

Protein samples (10 μg) were mixed with an equal volume of 2× Laemmli sample buffer, boiled, and resolved through 8% SDS-PAGE. Proteins were transferred to nitrocellulose membranes and blocked in blocking buffer as previously described (17). Filters were washed three times for 5 min each time in PBS/0.1% Tween 20 (v/v) and incubated in primary Ab (rabbit anti-human p65 at 1/1000 (Serotec, Oxford, U.K.), rabbit anti-human IκBα at 1/1000 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-human IκBβ at 1/500 (Santa Cruz Biotechnology), or mouse anti-human actin at 0.1 mg/ml (Sigma-Aldrich) in 5% milk powder in PBS/0.05% BSA (w/v)/0.2% Tween 20 (v/v) with 0.01% azide (w/v) overnight at 4°C for the IκBs and for 2 h at room temperature for p65. Membranes were then incubated with HRP-conjugated goat anti-rabbit IgG or rabbit anti-mouse IgG for 60 min at room temperature. Bound Ab was visualized using enhanced chemiluminescence reagents (ECL Plus Western Blotting Kit; Amersham Pharmacia Biotech). Densitometric analysis was performed on a Macintosh computer (Apple Computer, Cupertino, CA) using the public domain National Institutes of Health Image program (developed at U.S. National Institute of Science). An arbitrary value for the intensity of the p65 and IκB bands was determined using the intensity of the corresponding actin band as a control for protein loading. Statistical analyses were performed using the Mann-Whitney *U* test for nonparametric variables, and $p < 0.05$ was considered statistically significant.

EMSAs

EMSAs were performed using a radiolabeled ([γ-^{32}P]dATP) NF-κB consensus oligonucleotide (AGT TGA GGG GAC TTT CCC AGG C) and the Gel Shift Assay System (Promega, Annandale, Australia). Briefly, nuclear proteins (6–10 μg) were preincubated for 10 min in a 9-μl reaction volume containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol, and 0.5 μg of poly(dI-dC)·(dI-dC). For controls and supershift analysis the reaction mixture was preincubated with a specific or nonspecific unlabeled probe and anti-p50 mAb (Serotec), respectively. The reaction mixture was then incubated with 1 μl of radiolabeled NF-κB for 50 min at room temperature. Samples were subject to nondenaturing 5% PAGE at room temperature in Tris/glycine running buffer. Gels were dried and exposed to x-ray film.

Intracellular cytokine detection

Intracellular cytokine production by T cells was determined after incubation of cells with PMA/ionomycin in the presence of 10 μg/ml brefeldin A (Sigma-Aldrich). Cells were harvested after 4 h of culture, washed in PBS/0.5% BSA (w/v), and incubated in the dark with FITC-conjugated CD3 mAb (DAKO, Botany, Australia) with gentle agitation for 40 min at room temperature. Unbound Ab was removed by washing in PBS/0.5% BSA (w/v), and cells were fixed in 1% paraformaldehyde in PBS overnight at 4°C. Fixed cells were washed and permeabilized in 0.1% saponin in PBS/0.5% BSA (w/v) for 20 min at room temperature. Cells were washed and resuspended in 0.1% saponin in PBS/0.5% BSA (w/v) and incubated with PE-conjugated mAbs to cytokines IFN-γ, IL-2, IL-4, and IL-10 (BD PharMingen, Ryde, Australia) for 40 min at room temperature. Unbound Ab was removed by washing, and cells were resuspended in PBS/0.5% BSA for analysis by flow cytometry.

Flow cytometric analysis of intracellular cytokine production

Cells were analyzed on a BD FACSVantage SE flow cytometer with an argon ion (488 nm) laser for FITC and PE fluorescence. Twenty thousand events were collected from the lymphocyte population of cells, and data were stored electronically for analysis using CellQuest software. Intracellular cytokine expression was determined in $CD3^+$ lymphocytes and expressed as the percentage of T cells expressing cytokine. Statistical analysis of cytokine expression was performed using the Mann-Whitney *U* test for nonparametric variables, and $p < 0.05$ was considered statistically significant.

Results

NF-κB expression is reduced in isolated T cells from pregnant females relative to nonpregnant controls

Fetal Ag presentation throughout pregnancy is thought to result in a maternal Th2 immune response, thus ameliorating potentially lethal Th1 and cytotoxic T cell responses to the fetus. The molecular mechanisms that direct this alteration in immune responses

during pregnancy are not fully understood. We recently demonstrated that NF-κB expression is down-regulated in PBMCs in normal pregnancies compared with nonpregnant controls (17). As NF-κB is known to play a significant role in the regulation of T cell function and immune regulation, we tested whether T cells were the cell type responsible for this specific down-regulation. We isolated T cells (>95% $CD3^+$ purity) from nonpregnant and pregnant females by negative bead depletion and determined the expression of NF-κB protein in nuclear extracts by Western blotting (Fig. 1*A*). One immunoreactive band of 65 kDa representing the p65 NF-κB molecule was detected in nuclear fractions from all ($n = 10$) nonpregnant samples analyzed. In contrast, p65 was undetectable in the majority (seven of 10) of nuclear samples from pregnant females, and in the pregnant samples in which p65 was detectable (three of 10) levels were consistently lower than those detected in nonpregnant females (Fig. 1*A*). The intensity of the band representing p65 in nuclear extracts was determined by densitometry, and the results were expressed as the p65/actin band intensity (Fig. 1*C*). Despite the detection of p65 in some pregnant samples, overall the expression of p65 was significantly reduced ($p < 0.002$) in pregnant females relative to nonpregnant controls (Fig. 1*C*). We assessed whether a reduced level of nuclear NF-κB was due to reduced translocation of active NF-κB or to a reduction in the expression of NF-κB. Levels of p65 were determined in whole cell lysates of isolated T cells from both nonpregnant and pregnant females. NF-κB (p65) was detected in all nonpregnant ($n = 10$) samples analyzed, but levels were either low in pregnant (three of 10) relative to nonpregnant samples or were not present at all (Fig. 1*B*). The intensity of the p65 band in whole cell lysates was again determined by densitometry (Fig. 1*C*). The expression of p65 in whole cell lysates was consistent with results from nuclear extracts; p65 was significantly reduced ($p < 0.002$) in T cells from pregnant females relative to nonpregnant controls.

Both IκBα and IκBβ are reduced in T cells from pregnant females relative to nonpregnant females

The regulation of NF-κB activation is well documented. Like other members of the family, p65 resides in the cytoplasm in an inactive form bound to inhibitory IκB proteins. Cellular activation results in the degradation of IκBs, translocation of p50:p65, and subsequent DNA binding of this complex, which initiates gene transcription. We have shown that p65 resides in the nuclear fraction of unstimulated T cells from nonpregnant females. This may reflect IκB degradation in these cells or a basal level of NF-κB activity. The observation that p65 is reduced in nuclear and whole cell lysates from T cells in pregnancy may reflect the down-regulation of the entire p50:p65 signaling pathway we previously described in PBMCs during pregnancy. To assess these possibilities, we determined the level of IκB proteins in cytoplasmic and whole cell lysates of T cells from nonpregnant and pregnant females (Fig. 2). All ($n = 10$) T cell cytoplasmic fractions from nonpregnant females contained detectable amounts of both IκBα and IκBβ (Fig. 2*A*). In contrast, the level of IκBs in T cell cytoplasmic fractions from pregnant females was low (three of 10) or undetectable using these assays. The intensity of the IκB bands detected in cytoplasmic fractions was determined by densitometry and was found to be significantly reduced in pregnant females relative to nonpregnant controls for both IκBα and IκBβ (Fig. 2*B*). Similar results were obtained from analysis of whole cell lysates (Fig. 2*C*). In all ($n = 10$) nonpregnant samples analyzed, both IκBα and IκBβ proteins were detectable; however, in T cells from pregnant females there were either very low levels (three of 10) of detectable IκBα and IκBβ protein or none at all. The differences in band intensities of IκBα and -β between nonpregnant and pregnant females were determined by densitometry, and the expression in whole cell lysates was shown to be significantly ($p < 0.02$) reduced in T cells from pregnant females relative to nonpregnant controls (Fig. 2*C*).

Reduced NF-κB expression in T cells from pregnant females correlates with reduced basal levels of NF-κB activity

Despite the detection of the inhibitory molecules, IκBs in the cytoplasmic fraction of T cells from nonpregnant females, p65 is clearly present in the nuclear fraction of these cells. The detection of NF-κB in the nucleus of isolated T cells in all nonpregnant and some pregnant females suggests a basal level of NF-κB activity in

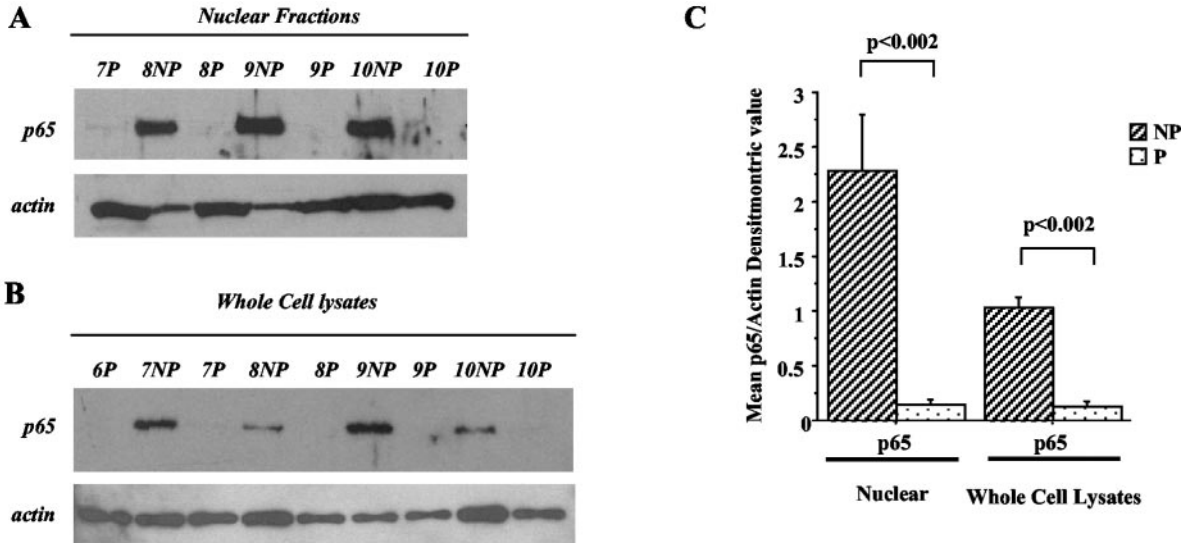

FIGURE 1. Analysis of p65 and actin protein levels in T cells from nonpregnant (NP) and pregnant (P) females. *A*, Western blot analysis of nuclear protein extracts (10 μg) from isolated T cells. *B*, Western blot analysis of whole-cell protein lysates (20 μl) from isolated T cells. The detection of actin in each sample acts as an internal loading control. Blots are representative of the results obtained for all NP and P females analyzed. The sample number is indicated to allow comparison between nuclear and whole cell lysates for the expression of p65. *C*, Bar graph representing mean p65 protein expression in nuclear extracts and whole-cell lysates of T cells from NP ($n = 10$) and P ($n = 10$) females obtained by densitometric analysis (see *Materials and Methods*).

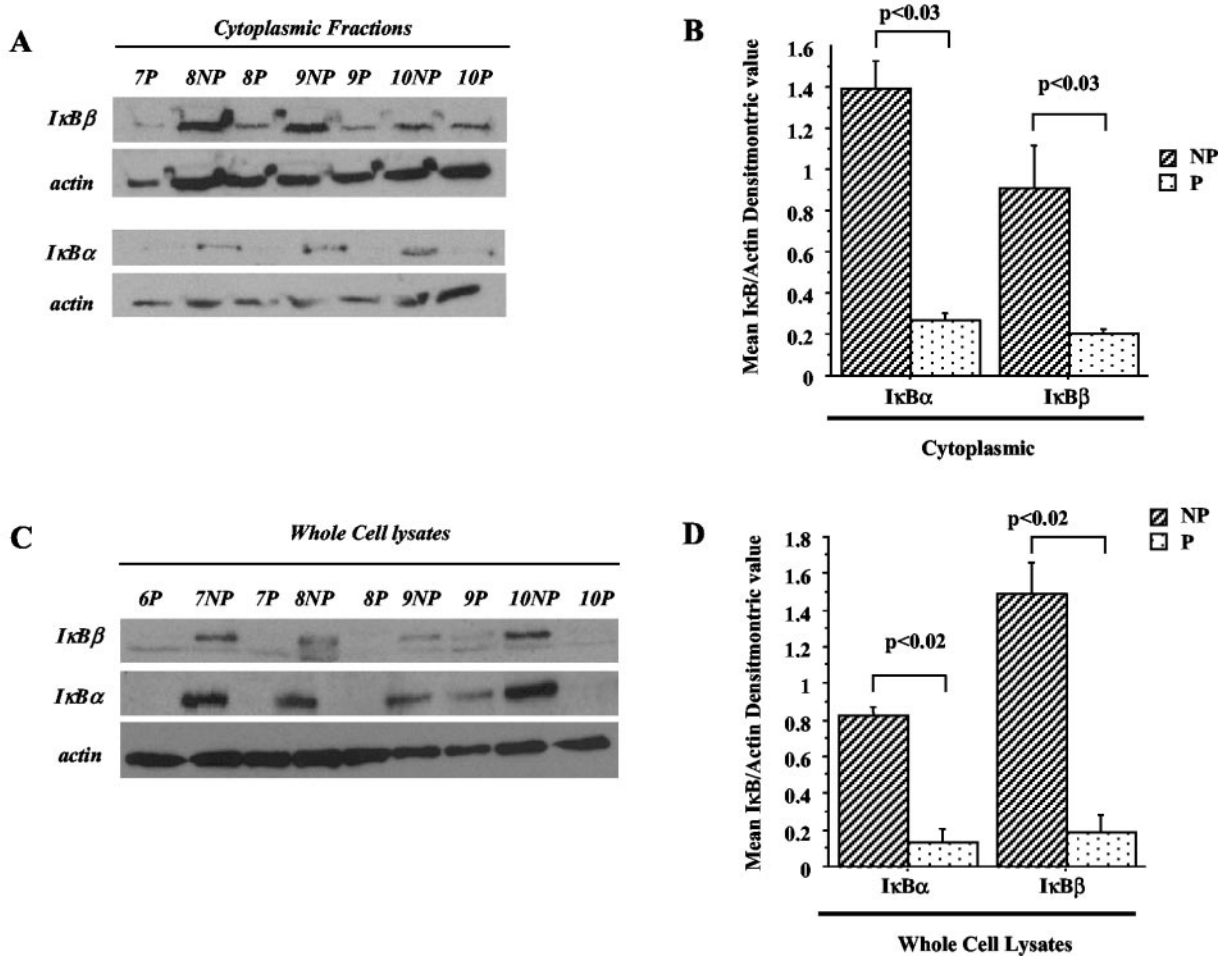

FIGURE 2. Analysis of IκBα/β and actin protein levels in T cells from nonpregnant (NP) and pregnant (P) females. *A*, Western blot analysis of cytoplasmic protein extracts (10 μg) from isolated T cells. The detection of actin in each sample acted as a loading control. *B*, Bar graph representing mean IκBα and IκBβ protein expression in cytoplasmic extracts of T cells from NP ($n = 10$) and P ($n = 10$) females obtained by densitometric analysis (see *Materials and Methods*). *C*, Western blot analysis of whole-cell protein lysates (20 μl) from isolated T cells. The detection of actin in each sample served as a loading control. *D*, Bar graph representing mean IκBα and IκBβ protein expression in whole cell lysates of T cells from NP ($n = 10$) and P ($n = 10$) females obtained by densitometric analysis (see *Materials and Methods*). Blots are representative of the results obtained for all NP and P females analyzed. The sample number is indicated to allow comparison between whole-cell and nuclear extracts for the expression of IκBα and IκBβ.

unstimulated T cells. Indeed, constitutive NF-κB activity has been shown to protect quiescent mature immune cells, including T cells, from apoptosis (19). Nuclear extracts were subjected to EMSA to elucidate whether NF-κB activity was detectable in unstimulated T cells and to determine whether the level of activity was reduced in T cells from pregnant females relative to those from nonpregnant controls. Using the NF-κB consensus sequence, we were able to demonstrated low levels of NF-κB activity in both nonpregnant and pregnant females (Fig. 3). Four bands that bound NF-κB were detectable by EMSA, but only three of these were shown to be specific to NF-κB (Fig. 3*A*). Using Abs raised against p50 and p65, we were able to show that the two slower migrating bands detectable in nuclear extracts contained both the p50 and p65 molecules, and as such represented the *trans*-activating p50:65 complexes of NF-κB (Fig. 3*B*). In contrast, the faster migrating band was only shifted using the p50 Ab, thus representing the p50 homodimer. Both bands representing the p50:p65 complex were detectable in all nonpregnant T cell extracts analyzed (Fig. 3*B*). In contrast, only the faster migrating p50:p65 complex was detectable in T cells from pregnant females, suggesting that the basal activity of NF-κB in T cells from pregnant females was reduced relative to that in T cells from nonpregnant females.

PMA stimulation of NF-κB activation is attenuated in T cells from pregnant females

The phorbol ester PMA is a strong and almost ubiquitous activator of NF-κB. In T cells, PMA has been shown to induce cell proliferation and cell survival (20), specifically through NF-κB activation (19, 21). As we have shown that the NF-κB/IκB expression and activation pathway is down-regulated in pregnancy, this would imply that T cell responses to stimuli known to activate NF-κB would be altered. To study the implication of NF-κB down-regulation in pregnancy, we stimulated T cells from nonpregnant and pregnant females with 10 ng/ml PMA and 500 ng/ml ionomycin for 1 h, and NF-κB activity was followed by Western blotting. As shown in Fig. 4, PMA induced NF-κB activity in T cells from nonpregnant females. PMA stimulation of T cells from nonpregnant females resulted in IκBα degradation, as indicated by the reduction of cytoplasmic levels of IκBα. The degradation of IκBα in the cytoplasmic fraction resulted in NF-κB release and subsequent translocation to the nucleus, as demonstrated by the increased level of p65 in the nuclear component of these cells. In contrast, PMA had no effect on NF-κB activation in T cells from pregnant females (Fig. 4). IκBα protein levels in the cytoplasmic

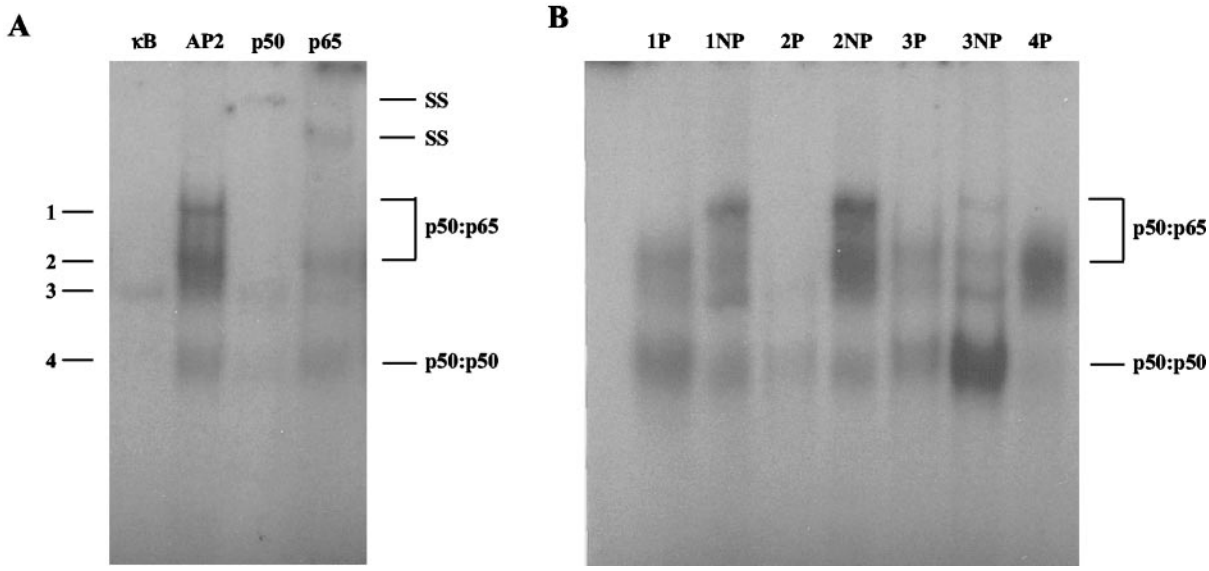

FIGURE 3. NF-κB activation in nuclear extracts (10 μg) of isolated T cells from nonpregnant (NP) and pregnant (P) females. *A*, Specificity for NF-κB binding was assessed by incubating nuclear extracts from NP T cells with unlabeled specific probe (NF-κB), nonspecific probe (AP2), and anti-p50 and -p65 mAbs before addition of labeled NF-κB. SS, supershift of NF-κB complex. *B*, Binding assay showing NF-κB-specific dimers in nuclear extracts from isolated T cells.

fraction of T cells from pregnant females did not change in response to PMA (Fig. 4). We were able to detect p65 in the cytoplasmic fraction of unstimulated T cells from pregnant females, and levels were not altered in response to PMA stimulation. In addition, there was no detectable p65 in the nuclear fraction of unstimulated T cells from pregnant females, and levels were unchanged in response to PMA stimulation.

Th1 cytokine production in response to PMA is defective in T cells from pregnant females

It is has been shown that the p50:p65 signaling pathway plays a pivotal role in the development of Th1 immune responses. Transgenic mice that fail to activate the p50:p65 pathway only mount Th2 cell-dependent responses to infection, but not Th1 responses (11). As we have demonstrated that NF-κB activation in response to PMA is abrogated in pregnancy, and normal pregnancy is associated with a bias toward a Th2 cytokine profile, we assessed the effect of PMA stimulation of the production of both Th1 and Th2 cytokines from T cells from pregnant females. PBMCs from nonpregnant and pregnant females were incubated with/without PMA/ionomycin for 4 h in the presence of the protein transport inhibitor brefeldin A. We analyzed intracellular cytokine expression (IFN-γ, IL-2, IL-4, and IL-10) in $CD3^+$ T cells from the lymphocyte population of PBMCs after 4 h of culture. We were unable to detect cytokine expression in unstimulated cells from either nonpregnant or pregnant females even after 4 h of culture. All subsequent results presented are from PMA/ionomycin-stimulated cells. The lymphocyte population gated on for analysis contain >80% $CD3^+$ T cells (data not shown). A dot plot of the expression of the intracellular cytokines IFN-γ and IL-2 is shown in Fig. 5. $CD3^+$ T cells from all nonpregnant and pregnant samples analyzed contained detectable levels of both cytokines. Significantly fewer

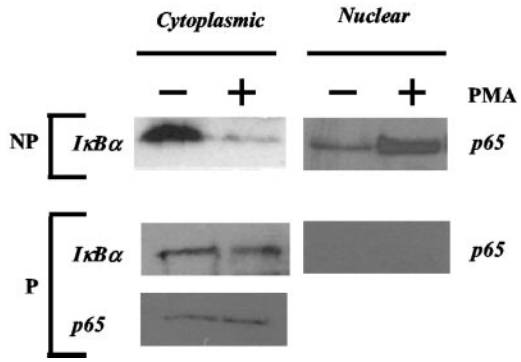

FIGURE 4. Effect of PMA stimulation of activation of isolated T cells from nonpregnant (NP) and pregnant females. PMA induces IκBα degradation and NF-κB translocation in T cells from NP females, but not from P females.

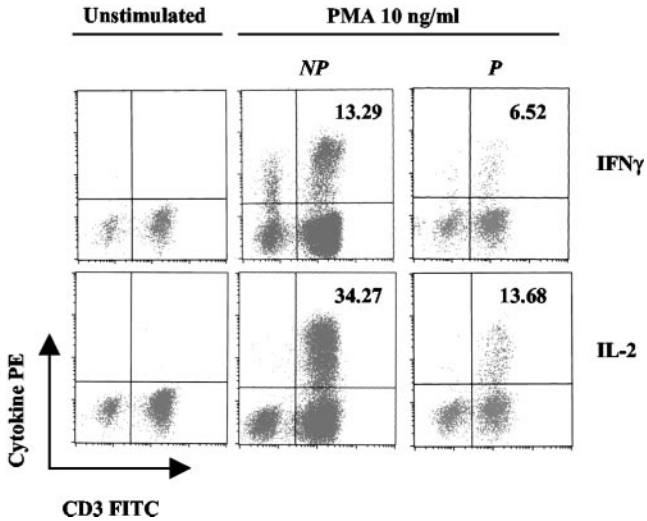

FIGURE 5. Activation of PBMCs with PMA stimulates Th1 cytokine production from $CD3^+$ T cells from nonpregnant (NP) and pregnant (P) females. PBMCs were cultured in the presence of brefeldin A with and without stimulation. Intracellular cytokine production was determined as described in *Materials and Methods*. The top three histograms are representative of staining for IFN-γ in NP and P samples, and the bottom three histograms are representative of staining for IL-2 in NP and P samples. Patients analyzed for intracellular cytokine production were 7–15NP and 7–15P. The samples shown are from patients 8NP and 8P. The percentage of cells doubly positive for CD3-FITC and cytokine-PE is indicated in the *top right* quadrant of each histogram.

Table II. *Intracellular cytokine expression in CD3$^+$ T cells*

Cytokine[a]	Nonpregnant (n = 9) Mean ± SD	Pregnant (n = 9) Mean ± SD	P Value
IFN-γ	17.36 ± 2.3	6.13 ± 3.11	0.0019
IL-2	33.36 ± 7.99	19.27 ± 4.78	0.0098
IL-4	0.398 ± 0.168	0.265 ± 0.134	NS
IL-10	0.206 ± 0.13	0.139 ± 0.105	NS

[a] Intracellular cytokines were detected in CD3$^+$ T cells after 4 h of stimulation with 10 ng/ml PMA and 500 ng/ml ionomycin as described in *Materials and Methods.*

CD3$^+$ T cells stained positively for IFN-γ ($p < 0.01$) and IL-2 ($p < 0.01$) in pregnant females relative to nonpregnant controls (Table II). In contrast, levels of the Th2 cytokines IL-4 and IL-10, although present in CD3$^+$ T cells, were detected at very low levels, and there was no significant difference in the levels of expression in nonpregnant and pregnant females.

Defective Th1 cytokine production from T cells in response to PMA from pregnant females can be induced in T cells from nonpregnant females using the NF-κB inhibitor SN50

The expression of both IFN-γ and IL-2 is dependent on NF-κB activation. In pregnant females, T cells express less p65 and IκBα and -β. In addition, the levels of both IFN-γ and IL-2 cytokine production are significantly reduced in T cells from pregnant females relative to nonpregnant controls. We tested whether the reduction in NF-κB expression in T cells in pregnancy could account for the lack of Th1 cytokine production in response to PMA. T cells from nonpregnant females were cultured in the presence of increasing concentrations of PMA (Fig. 6). A dose-dependent increase in nuclear translocation of NF-κB and a concomitant decrease in levels of cytoplasmic IκBα were detected by Western blotting (Fig. 6*A*). The stimulation of NF-κB correlated with a dose-dependent increase in both IFN-γ and IL-2 cytokine production (Fig. 6*B*). To assess the effect of blocking NF-κB translocation after stimulation on Th1 cytokine production, we used the synthetic peptide SN50. SN50 contains a signal sequence of the p50 subunit of NF-κB, which has been shown to block nuclear translocation of activated NF-κB (22, 23). PBMCs were preincubated with SN50 at a concentration of 37.5 μg/ml, the concentration predetermined to selectively inhibit NF-κB translocation (24), before stimulation with PMA. Cells were stimulated with the concentration of PMA that induced half-maximal expression of both IFN-γ and IL-2 (0.5 ng/ml; Fig. 6*B*). Nuclear extracts were prepared after 4 h of stimulation, and NF-κB activity was monitored by Western blotting (Fig. 7). Unstimulated cells contained low levels of nuclear p65 (Fig. 7*A*). In response to PMA, the level of p65 protein increased in the nuclear fraction of these cells. Treatment of cells with SN50 greatly reduced the accumulation of p65 in the nucleus (Fig. 7*A*). A control peptide, SN50M, was unable to block accumulation of p65 in the nuclear component (Fig. 7*A*). SN50M contains a mutation in the nuclear localization sequence residues and, as such, has a less inhibitory effect on NF-κB translocation (22, 23). Accumulation of p65 in the nucleus of stimulated

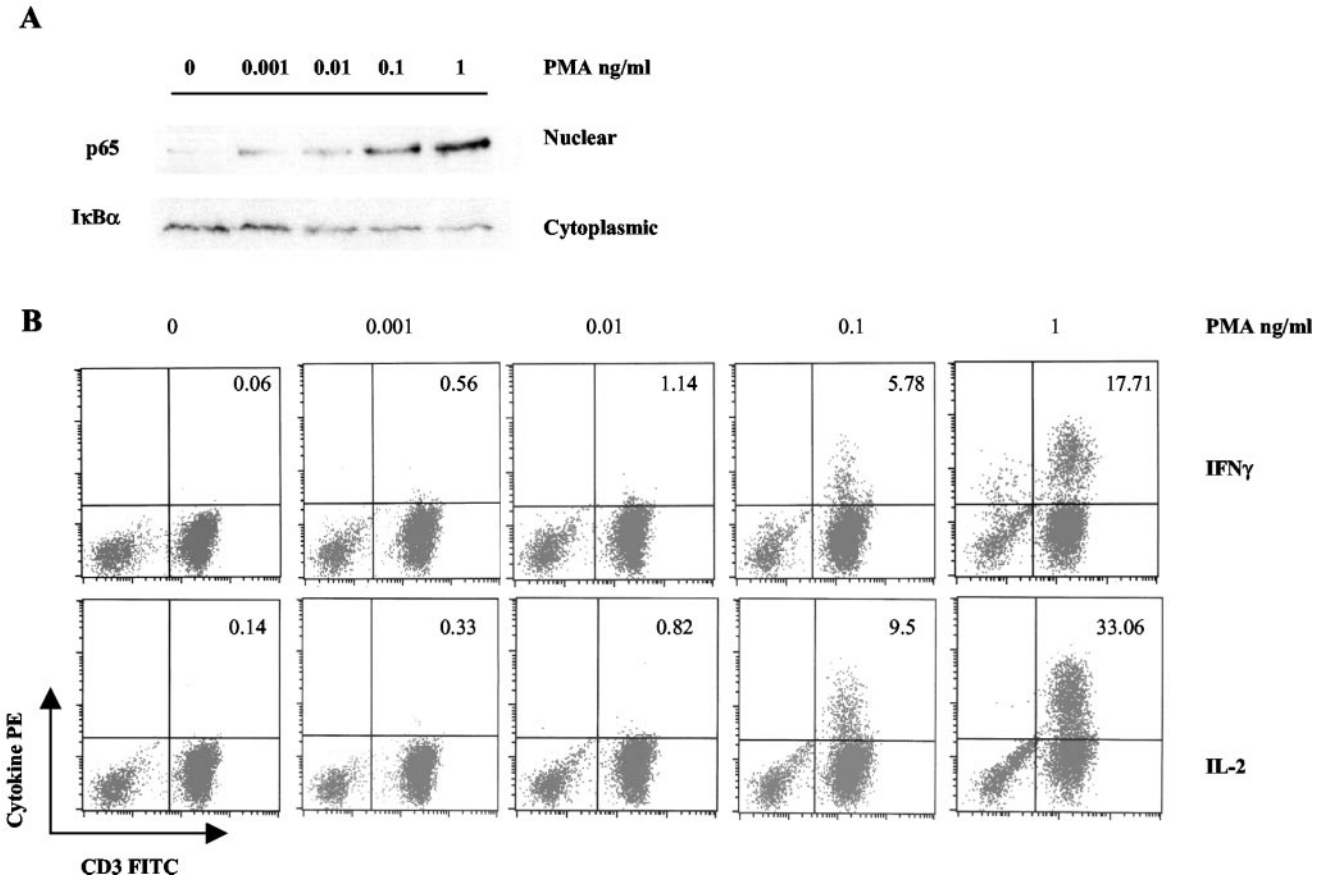

FIGURE 6. Effect of increasing concentrations of PMA/ionomycin on NF-κB activity and Th1 cytokine production. *A*, Western blotting analysis of IκBα degradation and NF-κB translocation in cytoplasmic (10 μg) and nuclear (10 μg) fractions of PBMCs, respectively. *B*, Flow cytometric analysis of Th1 cytokine production in CD3$^+$ T cells. The *top* five histograms are representative of staining for IFN-γ, and the *bottom* five histograms are representative of staining for IL-2. The percentage of cells doubly positive for CD3-FITC and cytokine-PE is indicated in the *top right* quadrant of each histogram.

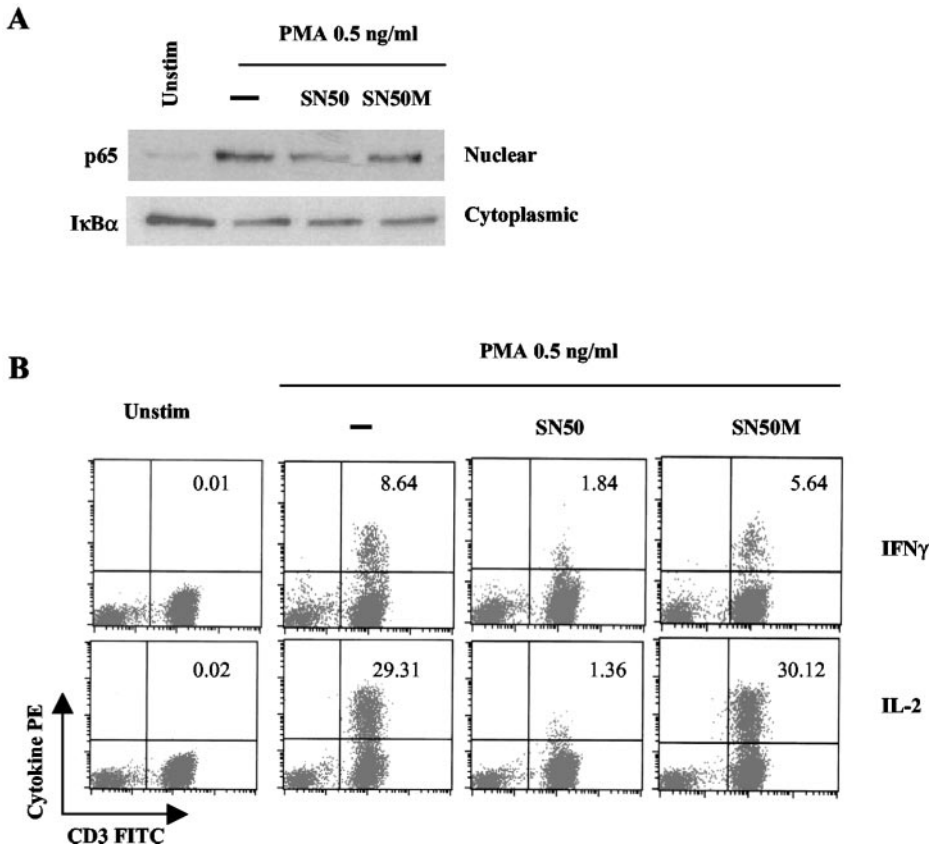

FIGURE 7. Effect of the NF-κB inhibitor SN50 on NF-κB activity and cytokine production in PBMCs cultured in the presence of PMA/ionomycin preincubated with or without SN50 or SN50M. *A*, Western blot analysis of nuclear and cytoplasmic extracts (10 μg) from a nonpregnant female. *B*, Flow cytometric analysis of Th1 cytokine production in $CD3^+$ T cells. The percentage of cells doubly positive for CD3-FITC and cytokine-PE is indicated in the *top right* corner. The experiment presented is representative of four different experiments.

cells was due to degradation of IκBα in the cytoplasmic fraction. The presence of SN50 in culture medium had no effect on the degradation of IκBα, merely on the translocation of p65 (Fig. 7*A*). Consistent with reduced NF-κB translocation, the expression of IFN-γ and IL-2 was attenuated in cells preincubated with SN50, but not SN50M (Fig. 7*B*).

Discussion

The molecular mechanisms that govern the changes necessary for the maintenance of normal pregnancy are not fully understood. We have shown that NF-κB, a molecule that plays a central role in the control of immunity, and its inhibitors, IκBα and -β, are down-regulated in T cells from pregnant females. In addition, we have shown that as a consequence of NF-κB/IκB down-regulation, T cell responses to known stimulators of this pathway are altered, which subsequently abrogates Th1 cytokine production.

We previously demonstrated with a different cohort of females that the NF-κB/IκB signaling pathway was down-regulated in PBMCs from pregnant females relative to nonpregnant controls (17). As alterations of T cell immune responses during pregnancy have been suggested to play a crucial role in pregnancy success (8), we assessed the possibility that T cells constituted the cell type responsible for the suppression of NF-κB in pregnancy. The results from this study demonstrating down-regulation of the entire NF-κB/IκB signaling pathway in T cells in pregnancy is concordant with the results we obtained from analysis of PBMCs, suggesting that T cell activity is specifically regulated in pregnancy

The majority of maternal immune effector cell interactions with fetal Ags occur at the maternal-fetal interface; however, it is imperative that the significance of immune cell regulation in the maternal circulation is not ignored. Indeed, fetal loss can be induced in pregnant mice by injecting them with the Th1 cytokine IL-2, which promotes Th1-type T cells responses at the expense of Th2-type responses (25). Definitive proof of the role of maternal T cells in the recognition of paternally inherited alloantigens that results in fetal loss came from studies using mice carrying either syngeneic or allogenic fetuses exposed to a pharmacological inhibitor of an enzyme called indoleamine 2,3-dioxygenase (IDO)[3] (26). IDO is expressed in the placental syncytiotrophoblast (27) and catabolizes tryptophan, an essential amino acid crucial for T cell activation (28). Treatment with the inhibitor of IDO resulted in uniform loss of allogenic pregnancies early in gestation, but had no effect on the development of syngenic pregnancies through to term (26). Our data demonstrating T cell-specific down-regulation of a molecular pathway that plays a central role in immune responses merely adds to the overall significance of delineating the regulatory mechanisms involved in controlling immune cells in the peripheral circulation during pregnancy.

Cellular immunity plays a crucial role in acute allograft rejection, whereas the generation of Th2 immunity is responsible for allograft tolerance (2). It is generally accepted that pregnancy is associated with a Th2 cytokine bias (1). Clinically, this bias is apparent from the observations that the symptoms of rheumatoid arthritis, an autoimmune inflammatory disease characterized by aggressive Th1 responses, improve during pregnancy (29, 30). In addition, systemic lupus erythematosus, a disease characterized by autoantibody production due to an increase in Th2 and a loss of Th1 cytokine production, is more severe during pregnancy (29). It has previously been shown that $CD4^+$ T cells from pregnant females produce significantly more IL-4 and IL-10 in response to PMA relative to nonpregnant controls (5). However, this study also used PMA to stimulate T cells, but was unable to show significant differences in the production of the Th2 cytokines IL-4 and

[3] Abbreviation used in this paper: IDO, indoleamine 2,3-dioxygenase.

IL-10 in $CD3^+$ T cells from pregnant females relative to nonpregnant controls. Much of the evidence that supports the proposal that pregnancy is associated with a Th2 cytokine bias has come from analysis of cytokine production from placental cells (1) and from the analysis of Th1 responses in T cells. In one study in which T cell function was assessed, Raghupathy et al. (8) demonstrated increased production of Th2 cytokines from PBMCs from normal pregnant females relative to females suffering from recurrent spontaneous abortion using placental Ags as a stimulus. PMA is a pharmacological stimulator of T cell responses, with a more physiological stimulation it is possible that a more pronounced Th2 immune response may predominate in T cells in pregnancy. However, what is more important with regard to the maintenance of pregnancy is the observation that the overproduction of Th1 cytokines is associated with recurrent pregnancy losses and implantation failure (6, 7, 9). Our results showing reduced IFN-γ and IL-2 production in activated $CD3^+$ T cells from uncomplicated pregnancies relative to nonpregnant controls are consistent with a previous report demonstrating reduced Th1 cytokine production in activated $CD4^+$ and $CD8^+$ T cells during pregnancy (5). Together these data confirm that Th1 cytokine production is down-regulated in pregnancy. The novel finding in our study is that the reduction in Th1 cytokine production from T cells in pregnancy is due specifically to the loss of NF-κB activity. Inhibition of NF-κB translocation by SN50 resulted in reduced production of Th1 cytokines from stimulated nonpregnant T cells to levels consistent with those of stimulated pregnant T cells. A direct link between NF-κB activity and the regulation of Th1 cytokines has been highlighted in many studies. Mice unable to activate the p50:p65 signaling pathway can only mount Th2 immune responses to infections (11). By manipulating the dietary intake of lupus-prone mice, Jolly et al. (31) were able to demonstrate that disease-associated losses in Th1 cytokine production were due to diminished NF-κB activation. In addition, the changes in T cell-mediated immunity after severe injury that result in suppressed Th1 responses and increased or persistent Th2 cytokine production are due to diminished NF-κB activity (32).

Consistent with Th1 responses playing a crucial role in acute allograft rejection, members of the NF-κB family of transcription factors have been shown to have an essential role in allograft rejection (33). Given that the fetus is generally likened to a fetal allograft, our results demonstrating down-regulation of NF-κB activity and concurrent down-regulation of Th1 cytokine production from T cells during pregnancy may reflect one mechanism used to prolong allograft survival.

Pregnancy-specific down-regulation of NF-κB may occur specifically to alter the cytokine milieu produced during pregnancy. However, there is growing evidence that NF-κB regulates the susceptibility of certain cell types to apoptosis through the transcriptional control of protective genes (15, 16). This is apparent because RelA (p65) knockout mice display embryonic lethality due to massive degeneration of the liver by apoptosis (34). In addition, constitutive NF-κB activity in T cells has been shown to protect T cells from apoptosis (19), and activation of T cells by the tumor promoter factor PMA results in T cell apoptosis when NF-κB translocation is inhibited (21). Immune responses to foreign Ags involve generating a pool of effector cells through activation, proliferation, and differentiation, followed by lymphocyte death to maintain T cell homeostasis (14). In pregnancy, maternal T cells are exposed to fetal Ags thoughout gestation and therefore have the potential to mount immune responses that culminate in fetal loss. Fas Ag is expressed in activated T cells to initiate apoptosis to avoid autoimmunity and maintain T cell homeostasis (35–37). Reinhard et al. (5) demonstrated the Fas Ag expression is up-regulated in T cells in pregnancy and suggest that this may represent a mechanism to eliminate possible autoreactive T cells directed against fetal tissues by apoptosis. We have shown that the antiapoptotic molecule NF-κB is down-regulated in T cells in pregnancy, which, together with an increase in Fas expression, may indeed represent an increased susceptibility of T cells to apoptosis, which would eliminate them from the circulation, and, as such, prevents immune responses that could result in fetal rejection.

How NF-κB activity is regulated in pregnancy is unknown. Steroids are known to have immunosuppressive functions in lymphocytes (38), specifically through the repression of NF-κB-dependent gene expression (39, 40). As steroids are present in the circulation at high concentrations throughout gestation, this makes them potential candidate molecules for regulating NF-κB activity in T cells. Steroid-induced repression of NF-κB occurs either through physiological interactions between steroid receptors and NF-κB (41, 42), which leads to inhibition of DNA binding, or through up-regulation of IκBα, which results in retention of NF-κB in the cytosol (42, 43). In reproduction, NF-κB is thought to play an active role in human labor by regulating the production of prolabor genes, including inflammatory cytokines that are necessary for the remodeling of fetal membranes and cervix and to stimulate myometrial contractions. In amnion cells, this is thought to occur due to the functional progesterone withdrawal that is normally associated with human pregnancies, resulting in increased NF-κB activity (44). In the peripheral circulation, T cells have been shown to express the progesterone receptor (45), but there are no reports to date that progesterone specifically regulates NF-κB activity in T cells in pregnancy. Our data suggest that down-regulation of NF-κB and IκB during pregnancy is transcriptionally regulated, because both NF-κB and IκBs are reduced at the protein level. If progesterone plays a role in regulating NF-κB activity in T cells throughout gestation, which would presumably be transcriptional, the mechanism for this would have to be either a receptor-mediated interaction that has not yet been described or some indirect mechanism.

In addition to the presence of steroids throughout pregnancy, human trophoblast express a nonclassical MHC molecule, HLA-G (reviewed by Le Bouteiller et al. (46)). HLA-G expression on trophoblast cells functions to protect the fetus from immune attack by down-regulating cytotoxic T cell responses to fetal trophoblast Ags (47). Soluble HLA-G down-modulates $CD4^+$ T cell alloproliferation in mixed lymphocyte reactions (48) and induces Fas/Fas ligand-mediated apoptosis in activated $CD8^+$ T cells (49). The molecular mechanisms involved in down-regulating these T cell responses and eliminating T cells by apoptosis have not been determined; however, given that NF-κB plays a significant role in protecting T cells against apoptosis (34), the apoptotic effect of HLA-G may be a consequence of NF-κB down-regulation in pregnancy. Alternatively, HLA-G may contribute to the control of NF-κB expression in pregnancy, thus indirectly affecting T cell responses.

In conclusion, we have shown that the NF-κB/IκB signaling pathway is down-regulated at what appears to be a transcriptional level in the third trimester of human pregnancy, and this suppression correlates with reduced expression of Th1 cytokines from activated T cells. These findings support the view that pregnancy is associated with the suppression of Th1 immune responses that results in a Th2-biased status and that the function of maternal immune effector cells is strictly regulated during pregnancy. In addition, our results suggest that the transcription factor NF-κB plays a central role in circumventing the changes necessary to control maternal immune responses throughout gestation.

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