Systemic Increase in the Ratio between Foxp3+ and IL-17-Producing CD4+ T Cells in Healthy Pregnancy but Not in Preeclampsia

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Systemic Increase in the Ratio between Foxp3⁺ and IL-17-Producing CD4⁺ T Cells in Healthy Pregnancy but Not in Preeclampsia

Brigitte Santner-Nanan, Michael John Peek, Roma Khanam, Luise Richarts, Erhua Zhu, Barbara Fazeekas de St Groth, and Ralph Nanan

Preeclampsia is the leading cause of morbidity and mortality in pregnancy. Although the etiology of preeclampsia is still unclear, it is believed to involve rejection of the fetus, possibly due to an imbalance between regulatory (Treg) and effector T cells. To test this, we compared the frequencies of circulating CD4⁺ T cells expressing Foxp3, IFN-γ, IL-10, or IL-17 at the end of the third trimester of healthy and preeclamptic pregnancies. The size of the Treg cell compartment, defined by the frequency of CD4⁺CD25high, CD4⁺CD127lowCD25⁺, and CD4⁺Foxp3⁺ cells was significantly higher in normal compared with preeclamptic pregnancies. CD4⁺CD25high and CD4⁺CD127lowCD25⁺ populations in preeclampsia were not significantly different from those in nonpregnant controls, whereas CD4⁺Foxp3⁺ cells numbers are slightly lower in preeclampsia. The suppressive activity of ex vivo-sorted CD4⁺ CD127lowCD25⁺ Treg cells was not significantly different between the three study groups. The percentage of CD4⁺IL-17-producing T cells decreased significantly in healthy compared with preeclamptic pregnancies and nonpregnant controls, whereas CD4⁺IL-10- and CD4⁺IFN-γ-producing cells remained unchanged. Consequently, the ratio of Foxp3⁺ Treg to IL-17-expressing CD4⁺ T cells was significantly increased in healthy but not in preeclamptic pregnancies. Thus, preeclampsia is associated with the absence of normal systemic skewing away from IL-17 production toward Foxp3⁺ expression. Finally, preeclamptic women had significantly higher levels of soluble endoglin, an inhibitor of TGF-β receptor signaling, which may bias toward IL-17 production. These results suggest that homeostasis between regulatory and proinflammatory CD4⁺ T cells might be pivotal for the semiallogeneic fetus to be tolerated within the maternal environment. The Journal of Immunology, 2009, 183: 7023–7030.
peripheral blood samples were obtained from healthy, nonpregnant, female volunteers (n = 28); third-trimester, healthy, pregnant patients (n = 47); and preeclamptic patients (n = 43). The characteristics of participants are summarized in Table I. Preeclampsia was defined as the onset of new hypertension in the second half of pregnancy (blood pressure > 140/90 mm Hg) and new proteinuria (>300 mg in a 24-h urine collection in the proven absence of a urinary tract infection), with a return to normal postnatally (19). Exclusion criteria for all pregnant participants were diabetes mellitus, gestational diabetes mellitus, preexisting renal disease, and/or chronic hypertension and/or infectious diseases diagnosed in the course of pregnancy. In addition, pregnancies in the control group were excluded if delivered before 37 wk gestation, as this indicated an abnormality inconsistent with inclusion as controls. The severity of preeclampsia was defined by the number of organ systems involved. These included impaired liver function (elevated liver enzymes), low platelet count (<150,000/μl), and/or cerebral or visual disturbances. As an additional measure of severity of preeclampsia, gestational age and in utero growth restriction (IUGR) were also analyzed. The nonpregnant group consisted of premenopausal age-matched female donors. These were not synchronized in terms of their menstrual cycle.

Blood samples from pregnant and preeclamptic patients were taken 2–5 h before delivery. Mononuclear cells were isolated by Ficoll-Hypaque (Amersham Biosciences) gradient centrifugation. The Ethics Committee of the Western Sydney Area Health Services approved this project according to the Declaration of Helsinki and all donors gave informed consent before sample collection.

**Cell staining and flow cytometry**

Mononuclear cells were suspended in cell staining buffer (PBS containing 0.1% FCS and 0.02% sodium azide). Surface Ab staining was performed as described previously (20). The following Abs were used: FITC-PE-, or biotin-conjugated anti-CD4 (BD Biosciences and eBioscience), anti-CD127 FITC or PE, anti-CD25-allophycocyanin, anti-IFN-γ-PE, anti-IL-10-PE (all from BD Pharmingen), and anti-IL-17-AlexaFluor647 (eBioscience). Biotin-conjugated anti-CD4 was developed with streptavidin conjugated with peridinin chlorophyll protein (BD Pharmingen). Intracellular staining for Foxp3 was performed using the anti-human staining set and protocol from BioLegend.

For intracellular staining with Abs against cytokines, cells were first stimulated as described below and then fixed in 4% paraformaldehyde (Sigma-Aldrich) in PBS, permeabilized in 0.1% saponin (Sigma-Aldrich), and stained with the indicated anti-cytokine Abs, followed by biotin-conjugated anti-CD4 Ab and streptavidin-peridinin chlorophyll protein. Data collection was performed on a FACSCalibur (BD Biosciences) and data files analyzed using CellQuest (BD Biosciences) and FlowJo software (Tree Star). Statistical analysis was based on at least 50,000 gated CD4+ cells.

**Cell enrichment and sorting**

For flow cytometric sorting, PBMCs were stained using a combination of anti-CD4-FITC, anti-CD127-PE and anti-CD25-allophycocyanin. Cells were sorted on a FACSARia cell sorter using the gates indicated in Fig. 2. Purity of the sorted populations was greater than 98%.

**Cell culture for intracellular cytokine measurement**

For analysis of cytokine production by CD4+ T cells, PBMCs were cultured for 5 h in the presence of 50 ng/ml phorbol myristate acetate (Sigma-Aldrich), 750 ng/ml ionomycin (Sigma-Aldrich), and 0.74 μl/ml Golgi stop protein transport inhibitor (BD Pharmingen) in RPMI 1640 medium containing 2 mM 1-glutamine supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin before analysis by intracellular cytokine staining and flow cytometry, as described above.

**In vitro suppression assay**

All suppression assays were performed in 96-well round-bottom plates (BD Biosciences) in a final volume of 200 μl/well. The culture medium used in all experiments was RPMI 1640 medium containing 2 mM l-glutamine supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Responder (sorted CD4+ CD127highCD25−) and suppressor (sorted CD4+ CD127highCD25+) cells were cultured in the presence of 5 × 10^4 irradiated allogeneic T cell-depleted PBMCs as APCs and anti-CD3 (H1α; BD Pharmingen) at 0.25 μg/ml. The responder cells were plated at 2.5 × 10^4/well and cocultured with suppressor cells at the ratio 1:1 in two to three replicate wells per patient. In control cultures, responders were added instead of suppressor cells at the indicated ratios. [3H]TdR incorporation was added at 72 h for the final 24 h of culture. The cells were then harvested onto glass fiber filters and assessed for uptake of the labeled thymidine by liquid scintillation counting. [3H]TdR incorporation in the suppressor:responder cultures was expressed as a percentage of the responder:responder controls.

**ELISA**

Serum samples of subjects were extracted from blood samples and then stored at −80 until analyzed. Levels of soluble endoglin in serum were measured by standard sandwich ELISA kits (R&D Systems) according to manufacturer’s instructions.

**Statistical analysis**

Statistical analysis was performed using Prism 4.0 software (GraphPad) and SPSS version 17.0 (SPSS). Data are presented as means ± SE. A one-way ANOVA followed by a Newman-Keuls multiple comparison test.

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**Table I. Characterization of study participants**

<table>
<thead>
<tr>
<th></th>
<th>Nonpregnant</th>
<th>Normal Pregnancy</th>
<th>Preeclampsia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample numbers</td>
<td>28</td>
<td>47</td>
<td>43</td>
</tr>
<tr>
<td>Age (year)</td>
<td>27 (± 1.0)</td>
<td>29 (± 0.8)</td>
<td>30 (± 1.0)</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>3449 (± 73)</td>
<td>2632 (± 152)</td>
<td></td>
</tr>
<tr>
<td>IUGR, n (%)</td>
<td>0</td>
<td>7 (16)</td>
<td></td>
</tr>
<tr>
<td>Gestational age at delivery (weeks)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mode of delivery, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaginal, not induced</td>
<td>16 (34)</td>
<td>9 (21)</td>
<td></td>
</tr>
<tr>
<td>Vaginal, induced</td>
<td>3 (6)</td>
<td>10 (23)</td>
<td></td>
</tr>
<tr>
<td>Cesarean section, not induced</td>
<td>26 (56)</td>
<td>20 (47)</td>
<td></td>
</tr>
<tr>
<td>Cesarean section, induced</td>
<td>2 (4)</td>
<td>4 (9)</td>
<td></td>
</tr>
<tr>
<td>Hypertension, proteinuria, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>One additional organ system involved</td>
<td>19 (44)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Two additional organ systems involved</td>
<td>12 (28)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Three additional organ systems involved</td>
<td>2 (5)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Data presented as means ± SE.*
was used to estimate the significance of differences in T cell subsets between nonpregnant controls, healthy pregnancies, and preeclamptic pregnancies. Levine’s test indicated that variances did not differ significantly between nonpregnant controls, healthy pregnancies, and preeclamptic pregnancies (2.11% ± 0.15) or nonpregnant controls (2.14% ± 0.10). CD4+CD25 T cells were also significantly higher (p < 0.001) in healthy pregnant women (6.26% ± 0.27) than in preeclamptic women (5.38% ± 0.37) and nonpregnant controls (5.88 ± 0.27%). Finally, CD4+Foxp3+ cells were significantly lower in patients with preeclampsia (4.42% ± 0.30) compared with healthy pregnant women (6.26% ± 0.32) and nonpregnant controls (5.56% ± 0.27). Thus, peripheral blood Treg cells were present at a significantly higher frequency in the blood of healthy pregnant women than in preeclamptic women, irrespective of the method used to identify Treg cells, whereas in preeclampsia the levels were at or below those of nonpregnant controls.

The healthy pregnant and preeclampsia groups differed in both gestational age and mode of delivery, with a bias toward lower gestational age and induced delivery in the preeclampsia group (Table I). We therefore tested whether either of these factors could explain the difference in Treg cell numbers between the two groups. There was no association between gestational age and CD4+Foxp3+ Treg cell numbers using Pearson’s correlation (r = −0.040, p = 0.815 for healthy pregnant; r = −0.034, p = 0.85 for preeclamptic; Fig. 1C). A two-way ANOVA analysis was performed to test the effect of mode of delivery on CD4+Foxp3+ Treg cell numbers in healthy pregnant vs preeclamptic groups (Fig. 1D). The analysis excluded induced vaginal delivery because only a single subject in the healthy pregnant group was delivered in this manner. Although the mode of delivery influenced CD4+Foxp3+ Treg cell numbers, with higher numbers in cesarean sections than vaginal delivery subjects (p = 0.040), the effect was relatively small when compared with the statistically highly significant (p = 0.001) difference in CD4+Foxp3+ Treg numbers between healthy

Results

Increased frequency of peripheral blood Treg cells in healthy vs preeclamptic pregnancy

The use of different methods for identifying Treg cells may have contributed to the controversy regarding Treg cell numbers in healthy vs preeclamptic pregnancy (10–13). We therefore compared the frequency of circulating Treg cells in healthy and preeclamptic pregnant women vs nonpregnant controls using the three marker combinations in current use (CD4+CD25hi (21), CD4−CD127loCD25hi (22, 23), and CD4−Foxp3hi (24) (Fig. 1). The percentage of CD4+CD25hi cells was significantly higher (p < 0.001) in healthy pregnancies (3.12% ± 0.26) than in preeclamptic pregnancies (2.11% ± 0.15) or nonpregnant controls (2.14% ± 0.10). CD4+CD127loCD25hi cells were also significantly higher in healthy pregnant women (6.98 ± 0.42%) than in women with preeclampsia (5.38% ± 0.37) and nonpregnant controls (5.88 ± 0.27%). Finally, CD4+Foxp3+ cells were significantly lower in patients with preeclampsia (4.42% ± 0.30) compared with healthy pregnant women (6.26% ± 0.32) and nonpregnant controls (5.56% ± 0.27). Thus, peripheral blood Treg cells were present at a significantly higher frequency in the blood of healthy pregnant women than in preeclamptic women, irrespective of the method used to identify Treg cells, whereas in preeclampsia the levels were at or below those of nonpregnant controls.
There was no significant correlation between gestational age and CD4+ IL-17+ T cell numbers ($r = -0.016$, $p = 0.94$ for healthy pregnancy; $r = 0.26$, $p = 0.30$ for preeclampsia) (Fig. 3C). In a two-way ANOVA comparing the effects of mode of delivery vs preeclampsia (excluding the induced vaginal and cesarean section groups, for which too few data points were available; Fig. 3D), the effect of mode of delivery was not significant ($p = 0.27$), whereas the effect of preeclampsia remained significant ($p = 0.017$). There was no effect of severity of preeclampsia on CD4+ IL-17+ T cell numbers ($p = 0.86$).

The decrease in CD4+ IL-17+ T cells in healthy pregnancy (Fig. 3), combined with the increase in CD4+ Foxp3+ T cells (Fig. 1), suggested that the ratio of Foxp3+ Treg to IL-17-expressing CD4+ T cells would be significantly increased compared with either preeclampsia or nonpregnant controls. Indeed, the increase in the ratio of Foxp3+IL-17-expressing CD4+ T cells was highly significant ($p < 0.01$) in healthy pregnancy (Fig. 4). The decrease in CD4+ IL-17-producing cells also led to a significant decrease ($p < 0.01$) in the IL-17:IL-10 ratio and an increase ($p < 0.001$) in the IFN-γ:IL-17 ratio, whereas the IFN-γ:IL-10 ratio did not change significantly (Fig. 4).

**Soluble endoglin is increased in preeclampsia**

It has recently been reported that soluble endoglin, an inhibitor of TGF-β signaling, is selectively increased in preeclampsia (15, 16). We tested whether the preeclamptic subjects in our study also showed evidence of increased serum-soluble endoglin (Fig. 5). The increase in preeclampsia vs normal pregnancy and healthy nonpregnant controls was highly significant ($p < 0.001$). However, the correlation between soluble endoglin levels and the ratio of Foxp3+IL-17-expressing CD4+ T cells in individual patients did not reach statistical significance (data not shown). Using a Pearson’s correlation test, we did not find significant correlations between levels of circulating endoglin and any of the T cell parameters (Treg, IL-17-, IFN-γ-, or IL-10-producing CD4+ T cells).

**Discussion**

In this study, we compared ex vivo-derived T cell subsets in nonpregnant women vs healthy and preeclamptic pregnant women. We have shown for the first time that the ratio of Foxp3+ Treg to IL-17-expressing CD4+ T cells is increased in normal pregnancy but not in preeclamptic patients at the time of delivery. An imbalance between Treg and Th17 cells has been proposed as a pathogenetic mechanism in several human immunopathologies, including autoimmune disease and acute transplant rejection (25–27). In this context, the anti-inflammatory properties of Treg cells are believed to antagonize the proinflammatory properties of IL-17-producing CD4+ T cells. These findings suggest that treatment aimed at changing the balance of Foxp3+ Treg cells and IL-17-producing CD4+ T cells may provide therapeutic benefit in preeclampsia.

Because the published data regarding changes in Treg cell populations in healthy and preeclamptic pregnancies have used different combinations of markers to define Treg cells, we compared Treg numbers obtained with the three commonly used marker combinations: CD4+CD25+Foxp3+, CD4+CD127lowCD25+, and CD4+CD127lowCD25- (Fig. 1). CD4+CD25high T cells have been reported to increase in pregnant mice (6, 28, 29) and throughout human pregnancy (9, 10, 30), although others found no significant difference between nonpregnant controls, healthy pregnancy, and preeclampsia (12, 13). However, human Treg cells are poorly defined by this marker combination. Not only does it exclude the majority of human CD4+Foxp3+ cells, but it does not clearly differentiate between Treg cells and activated conventional T cells, with the degree of cross-contamination depending on the placement of the
CD25<sup>high</sup> gate in individual data sets (21, 23, 31). Foxp3 has also been used to define Treg cells in human pregnancy (11). However, the use of Foxp3 as the sole marker to define human Treg cells has also been questioned since the discovery that conventional CD4<sup>+</sup> T cells also express Foxp3 after activation (31). The extent to which conventional CD4<sup>+</sup> T cells are present within the Foxp3<sup>+</sup> population in vivo remains unknown. To date, no studies have determined Treg cell numbers in human pregnancy using the recently defined CD4<sup>+</sup>CD127<sup>low</sup>CD25<sup>+</sup> phenotype (22, 23).

In the current study, the proportion of CD4<sup>+</sup>CD25<sup>high</sup> and CD4<sup>+</sup>CD127<sup>low</sup>CD25<sup>+</sup> cells was significantly increased in healthy pregnancy compared with nonpregnant controls, whereas CD4<sup>+</sup>Foxp3<sup>+</sup> cells increased but the difference failed to reach statistical significance. It has previously been noted, using the CD4<sup>+</sup>CD25<sup>high</sup> marker combination, that Treg cell numbers fall precipitously toward the end of normal pregnancy (9). It was therefore highly significant that Treg cell frequencies, as determined by all three marker combinations, were decreased in preeclamptic compared with healthy pregnancies, even though the period of gestation in the preeclamptic pregnancies was on average 3 wk shorter than in the normal pregnancy group (Table I and Fig. 1C). This difference in mean gestational age between the preeclampsia and the healthy pregnancy group can be considered as a possible confounder in this study. We therefore performed a direct comparison of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg frequencies between the healthy pregnancy group (by definition ≥37 wk gestational age) and the subset of preeclamptic pregnancies delivered at 37 wk of gestation and above. The decrease in the preeclampsia group was highly significant (t test, p < 0.001). In addition, there was no effect of possible confounding factors, such as mode of delivery (Fig. 1D), IUGR, or severity of preeclampsia on the CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell frequency, suggesting that a factor underlying preeclampsia itself, rather than any functional consequences of preeclampsia, was associated with the reduction in Treg cell numbers.

For both CD4<sup>+</sup>CD25<sup>high</sup> and CD4<sup>+</sup>CD127<sup>low</sup>CD25<sup>+</sup> cells, the preeclampsia group did not differ from nonpregnant controls,
whereas CD4+/Foxp3+ cells were significantly decreased compared with nonpregnant controls. These data confirm that healthy pregnancy is associated with an increase in the proportion of Treg cells, whereas in preeclampsia the levels are at or below those of nonpregnant controls. Given the importance of TGF-β in the differentiation and function of human Treg cells (17), these results are consistent with a crucial role for TGF-β in setting the immune balance in normal human pregnancy, in addition to its known roles in implantation and normal placental development and function (32).

It has been reported that the functional activity of CD4+CD25high cells is significantly reduced in patients with preeclampsia in comparison to those of healthy pregnancies (11). However, Treg cells purified using this method may be contaminated with activated T cells that interfere with the suppression assay (33). By using the surface CD4+CD127low/CD25high phenotype to isolate the Treg cell population, we were able to assess the functional capacity of pure Treg cells (23). We found no significant differences in Treg cell activities in samples derived from preeclamptic patients compared with healthy pregnant and nonpregnant controls (Fig. 2).

We found that healthy pregnancies were associated with a decrease in IL-17-producing cells, whereas preeclamptic pregnancies were not (Fig. 3B). Once again, gestational age, mode of delivery, IUGR, and severity of preeclampsia had no on the number of IL-17-expressing CD4+ T cells (Fig. 3, C and D). In vitro studies have indicated that differentiation of human IL-17-producing cells is inhibited by high TGF-β concentrations but requires IL-1β and IL-6 and/or IL-21 (18, 34). The relative increase in IL-17-producing cells in preeclampsia is consistent with previous data showing that monocytes from preeclamptic women secrete more IL-1β and IL-6 than do those from healthy pregnant women (35). In addition, the inhibitory effect of TGF-β on differentiation of IL-17-producing cells during pregnancy may be reduced in preeclampsia due to the abnormally high levels of soluble endoglin (Fig. 5). Endoglin potently blocks the action of TGF-β (14). Soluble endoglin has been described as a characteristic marker of preeclampsia, with markedly increased levels detectable before the onset of preeclampsia at the beginning of the second trimester of pregnancy (15). These high serum levels reflect a local overproduction from endothelial cells and syncytiotrophoblasts (16). The effects of increased endoglin levels on TGF-β signaling and consequently T cell differentiation are likely to occur throughout the second and third trimesters. Together with the fact that endoglin levels could be influenced by several other confounders around the time of birth, this might explain why the percentages of Treg and IL-17-expressing CD4+ T cells did not correlate with serum endoglin levels in individual patients in our study.

There is some evidence that normal third trimester pregnancy is associated with a bias toward the Th2 lineage (36, 37). However, these findings are mainly based on serum cytokine levels. In this study, we found similar frequencies of IL-10- and IFN-γ-producing cells in all three cohorts (Fig. 3B).
The reciprocal changes in Foxp3+ and IL-17-producing CD4+ T cells in healthy vs preeclamptic pregnancies led to a highly significant (p < 0.01) drop in the final ratio of Foxp3+IL-17-expressing CD4+ T cells at the end of preeclamptic pregnancy (Fig. 4). How skewing of responses toward IL-17-producing CD4+ T cells and away from Foxp3+ Treg cells is controlled during the course of normal and preeclamptic pregnancy remains to be determined. The processes that lead to the differentiation of naïve CD4 T cells into Th17 effector cells involve multiple factors including TGF-β, IL-6, IL-21, and IL-23 (reviewed in Ref. 34). Although the sources of Th17 differentiation factors have been partially revealed in vivo in murine models, investigation of this differentiation process in humans is difficult to analyze in vivo and cannot easily be recapitulated by ex vivo studies that do not take into account both the long timeframe involved in pregnancy and the possibility that distinct processes are occurring in the placenta as compared with maternal lymphoid tissues.

Our results suggest a number of future research directions, including the degree to which human peripheral blood CD4+ T cells producing IL-17 after direct ex vivo stimulation express RORγt, IL-23R, IL-21, and/or IL-22, characteristic of murine Th17 cells. Recent reports of ex vivo coexpression of IL-17 and either IL-21 or IFN-γ in human inflammatory diseases such as rheumatoid arthritis and ankylosing spondylitis suggest that human Th17 cells may not be as clearly characterized by production of IL-22 as are murine Th17 cells (38, 39). After prolonged in vitro culture, IL-21 is produced by a minority of both IFN-γ-producing (~6.1%) and IL-17-producing human cells (~10.8%) (40). Although 42% of in vitro-polarized IL-17-producing human cells coexpress IL-22 (41), the vast majority (~80%) of human IL-22-producing CD4+ T cells appear to be distinct from IL-17-producing cells (42).

In summary, our results suggest that homeostasis between Foxp3+ and IL-17-producing CD4+ T cells might be essential for the semil agaroid fetus to be tolerated within the maternal environment. A similar shift from Treg cells to IL-17-producing CD4+ T cells has been postulated to contribute to the development and/or progression of autoimmune disease and acute transplant rejection in humans (25, 27). The mechanism underlying the shift in cytokine profiles in preeclampsia remains undefined, but as the changes described here are likely to have evolved in vivo over time, they reflect the influence of a number of factors, including hormones, soluble endoglin, and cytokines, both in the serum and locally in the placenta. Whether these immune changes are a primary or secondary cause of preeclampsia remains to be determined.

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Disclosures
The authors have no financial conflict of interest.

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