Published in final edited form as:

J Autoimmun. 2012 May ; 38(2-3): J88–J96. doi:10.1016/j.jaut.2011.07.001.

Placenta Suppresses Experimental Autoimmune Hypophysitis through Soluble TNF Receptor 1

Melissa A. Landek-Salgado¹, Noel R. Rose^{1,2}, and Patrizio Caturegli^{1,2}

¹Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, MD, USA

²Feinstein Department of Molecular Microbiology and Immunology, The Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA

Abstract

Pregnancy modulates autoimmune diseases through diverse and still incompletely defined mechanisms, in part operating at the decidua-placenta interface. To assess the immunological contribution of placenta, we administered mouse placental proteins to a mouse model of autoimmune hypophysitis, a disease known to be strongly associated with pregnancy. Emulsified placental proteins suppressed both the cellular and humoral aspects of hypophysitis. Suppression was specific to self antigens and not seen when two foreign antigens, tetanus toxoid or tuberculin purified protein derivative, were used. Proteomic analysis revealed high levels of soluble TNF receptor 1 in placenta, suggesting that blockade of the TNF- α pathway was a mechanism of disease suppression. Placentas derived from mice deficient in TNF receptor 1 lost the ability to suppress hypophysitis. Notably, hypophysitis suppression was seen only when the TNF- α pathway was blocked locally, at the site of immunization, and not systemically. These findings provide evidence that placenta contributes to the immune tolerance of pregnancy by locally inhibiting the TNF- α pathway.

Keywords

Hypophysitis; pituitary antibodies; pregnancy; placenta; TNF- α

1. Introduction

Pregnancy is intimately connected with autoimmune diseases [1]. It can significantly alter their course but at the same time be altered by them through severe obstetric and perinatal complications. Pregnancy modulates the course of autoimmune diseases in numerous ways. Some diseases, like multiple sclerosis, improve during pregnancy [2]. Others, like rheumatoid arthritis and lupus, can improve or exacerbate mainly depending upon the level of disease activity before conception [3]. A few more diseases, like hypophysitis and thyroiditis, are actually brought to light by pregnancy and diagnosed during the third

© 2011 Elsevier Ltd. All rights reserved.

Corresponding author: Patrizio Caturegli, Johns Hopkins Pathology – Ross 656, 720 Rutland Avenue, Baltimore, MD 21205, pcat@jhmi.edu, Phone: 443-287-8911, Fax: 410-614-3548.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

trimester or early post-partum [4]. The mechanisms responsible for these modulatory effects of pregnancy on autoimmune diseases are diverse, complex, and still incompletely defined.

Several investigators have used rodent models to gain mechanistic insights. The Morton group discovered an early pregnancy serum factor, later identified as a homologue of chaperonin 10 [5], that appears within 24 hours of fertilization and disappears by day 14 to 16 of pregnancy [6] and is capable of delaying the onset and suppressing the severity of experimental autoimmune encephalomyelitis [7, 8]. The Steinman group reported that immunization during late pregnancy improved disability and protected against relapses, possibly through a late pregnancy serum factor [9]. These findings were confirmed by the Whitacre group who also showed that immunization during late pregnancy improved disease severity by reducing TNF- α production, increasing the frequency of IL-10 secreting cells [10], and, more recently, by releasing placental exosomes [11]. A more recent study in genetically susceptible rats refined the importance of the timing of antigenic exposure by showing that immunization during the third week of pregnancy aggravated disease whereas it improved it during the second week [12]. A few additional studies have examined the effect of pregnancy in animal models other than encephalomyelitis. In thyroiditis, thyroglobulin immunization led to pregnancy loss and disease exacerbation in the post-partum period [13, 14]. In systemic lupus erythematosus, repeated pregnancies improved skin lesions and decreased plasma IFN- γ levels but exacerbated nephritis [15]. In arthritis, pregnancy improved a proteoglycan-induced model of the disease [16], and placental culture supernatants ameliorated a collagen-induced model possibly via inhibition of TNF- α and IFN- γ [17].

Given the strong temporal association between pregnancy and hypophysitis, we used a mouse model of experimental autoimmune hypophysitis (EAH) [18] to expand the mechanisms through which pregnancy modulates the course of autoimmune diseases. We report that placenta derived soluble TNF receptor 1 (sTNF-R1) ameliorates disease through blockade of TNF- α .

2. Materials and methods

2.1 Mice

The study used a total of 260 female mice. SJL (N= 192), CBA (N= 48), and C57BL6 (N= 10) mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and used between 7 and 27 weeks of age. All mice were wild type except for 10 TNF receptor 1 knockout mice on the C57BL6 background (a gift from Dr. Robert Anders, Johns Hopkins School of Medicine). Mice were maintained in pathogen-free conditions and received water and food ad libitum, and used in accordance with the standards established by the United States Animal Welfare Acts, set forth in the National Institutes of Health guidelines, and approved by the Johns Hopkins University Animal Care and Use Committee.

2.2 Immunogen types and preparations

The following tissues from mice of mixed strains and sexes were obtained from the Johns Hopkins animal care facility: pituitary gland, skeletal muscle, liver, and thyroid gland. Placentas were collected at term (around day 18) from a pool of SJL and FVB/N mice, or from wild type C57BL6 or TNF-R1 knockout mice on the C57BL6 background. Placentas, pituitary glands, skeletal muscle, and liver were homogenized as described in detail in [19]. For the thyroid gland, a purified preparation of thyroglobulin was obtained by gel filtration chromatography, as previously described [20].

2.3 Induction of experimental autoimmune hypophysitis and thyroiditis

For hypophysitis induction, SJL mice were immunized on days 0 and 7 with 1 mg of mouse pituitary proteins and sacrificed on day 28, as detailed in [19, 21], with or without the addition of mouse placental proteins. Placental proteins were diluted in PBS to a final concentration of 8, 16 or 20 mg/ml and emulsified 1:1 in CFA, supplemented with 5 mg/ml *Mycobacterium tuberculosis*. As control, an emulsion was made by mixing 1:1 PBS in CFA. These two emulsions were combined 1:1 with the pituitary emulsion before injection, except for some experiments where placental and pituitary emulsions were injected at separate anatomical sites. In addition, placental proteins were also administered systemically, rather than locally, by injecting subcutaneously every other day a soluble (non-emulsified) placental extract for a total of 14 injections, spanning from day 0 to day 26 of immunization.

For thyroiditis induction, CBA mice were injected on days 0 and 7 with 0.1 mg of mouse thyroglobulin emulsified in CFA, with or without the addition of placental proteins, and sacrificed on day 21 [20].

2.4 Measurement of autoantibodies to pituitary proteins and thyroglobulin, and foreign antibodies to purified protein derivative and tetanus toxoid by ELISA

Autoantibodies to pituitary proteins were measured in the sera of immunized SJL mice on day 28 post-immunization by ELISA, assessing both the total IgG and IgM content and the individual IgG1 and IgG2c isotypes, using a home-made standard curve and the reagents described in [18]. In the SJL strain the Igh1-b allele, which produces IgG2a, is deleted and replaced by the IgG2c-producing Igh1-a allele [22]. Autoantibodies to thyroglobulin were measured in CBA mice on day 21 post-immunization, assessing total IgG and IgM content, IgG1, and IgG2a isotypes and following the approach detailed in [20].

Foreign antibodies to the purified protein derivative that is part of the *Mycobacterium tuberculosis* contained in CFA were measured in SJL mice 28 days after immunization. ELISA plates (Immunolon2 from ISC BioExpress, Kaysville, UT) were coated with *M. tuberculosis* H37Rv PPD (1 µg/well, Mycos Research, LLC, Loveland, CO), incubated overnight at 4 °C with sera diluted 1:100 in TBS, and then washed with TBS-Tween 20. A secondary antibody conjugated to alkaline phosphatase recognizing mouse IgG and IgM (from Jackson Laboratories, West Grove, PA) was used to detect specific binding. After addition of the p-nitrophenylphosphate substrate (Bio-Rad Laboratories, Hercules, CA), color development was assessed and results expressed as optical density. Sera were tested in triplicate and excluded if the coefficient of variation was >20%.

Tetanus toxoid was obtained from the Statens Serum Institut (Copenhagen, Denmark) and diluted in PBS at a concentration of 50 limit of flocculation (Lf) units per ml (corresponding to 125 mg/ml). Mice were injected on days 0 and 7 with 2.5 Lf units, emulsified 1:1 in the same CFA adjuvant preparation used for the other immunization experiments, and bled on days 0 and 28 after the first immunization. Immunolon2 ELISA plates were coated with tetanus toxoid (0.084 Lf units per well) and incubated overnight at 4 °C with mouse sera diluted in TBS (1:40 for day 0 or 1:6400 for day 28). Antibody binding and detection were carried out as described for purified protein derivative antibodies.

2.5 TNF-α and sTNF-R1 levels in serum and tissue extracts by ELISA

Sera from immunized and control mice were tested for TNF-α and sTNF-R1 levels using a commercial kit (R&D Systems, Inc.), following the manufacturer's recommendations. Tissue extracts were also tested for sTNF-R1 levels using a commercial kit (R&D Systems, Inc.).

2.6 Pituitary and thyroid histopathology

After euthanasia, pituitary or thyroid glands were removed, fixed overnight in the zinc-based Beckstead's solution, processed, and embedded in paraffin. Several non-sequential sections (5 microns thick) were cut, stained with hematoxylin and eosin. The section with the most severe mononuclear cell infiltration was scored from 0 to 5 based on the extent of tissue damage. Grade 1 indicated a less than 20% involvement; grade 2, 20%–30%; grade 3, 30%–50%; grade 4, 50%–90%; grade 5, greater than 90%. All sections were scored blindly by MALS and PC.

2.7 Measurement of cytokines and chemokines in placental and muscle extracts

Placental and muscle protein extracts were adjusted to 500 µg/ml and incubated overnight at 4 °C on a mouse cytokine array (Array 2 by RayBiotech, Norcross, GA), which allows the simultaneous detection of 31 molecules. After addition of a biotin-conjugated cytokine antibody cocktail and streptavidin conjugated to horseradish peroxidase, a colorimetric signal was induced by the addition of detection buffer and quantified by exposure to radiographic films (X-Omat AR; Kodak, Rochester, NY). Signals were analyzed for gray-scale intensity using Image J software (<http://rsb.info.nih.gov/ij>).

2.8 Biological activity of sTNF-R1 after emulsification

Placental proteins were emulsified as described in section 2.3. The placenta emulsion was then placed in a microcentrifuge tube containing an equal volume of PBS and incubated at 37 °C for 48 hours. After centrifugation, the PBS, which contained proteins that diffused out of the emulsion, was removed and subsequently incubated with 2.5 µg of recombinant mouse TNF-α (Biolegend, San Diego CA) at 4 °C with gentle rotation. Twenty-four hours later, 5 µg of a hamster anti-mouse TNF-R1 antibody (Biolegend) or a hamster anti-TNP isotype control antibody (Pharmingen, San Diego, CA) was added to the tube and rotated for 24 hours at 4 °C. Another tube containing non-emulsified placental proteins served as a positive control and was incubated in an identical fashion with TNF-α and then the anti-mouse TNF-R1 antibody. Protein A/G agarose beads (Pierce Biotechnology, Rockford, IL) were added to each tube and incubated for another 24 hours with gentle rotation at 4 °C. Following this incubation, the tubes were centrifuged and the supernatant removed. The protein A/G was extensively washed and incubated for 5 minutes at 95 °C with electrophoresis loading buffer and then fractionated on a 12% Tris-HCl polyacrylamide gel, under denaturing and reducing conditions. Proteins were subsequently transferred to a nitrocellulose membrane. Membranes were blocked for 1 hour at room temperature in 3% BSA 0.1% Tween 20, incubated with a biotinylated anti-mouse TNF-α antibody (R&D systems, Inc.) and then streptavidin conjugated to horseradish peroxidase (Dako, Carpinteria, CA). After incubating with a chemiluminescent substrate (GE Healthcare, Piscataway, NJ), membranes were exposed to radiographic film.

2.9 Injections of anti-TNF-α and anti-horseradish peroxidase antibodies

Monoclonal rat IgG1 antibodies directed against mouse TNF-α or horseradish peroxidase (both from Bio X Cell, West Lebanon, NH) were diluted in PBS and injected intraperitoneally into SJL mice immunized with pituitary proteins. The antibodies were administered at a dose of 0.39 mg/mouse on day -1 and day 0 and then every 3 days until day 27 post-immunization. As control, immunized mice received PBS with the same injection schedule.

2.10 Statistical analysis

The main outcome measure of the study was the histopathological score, expressed on a discrete scale from 0 to 5. Differences in the scores among groups were analyzed using the

non-parametric Wilcoxon's rank sum test. Additional outcomes were serum antibodies and expression levels of chemokines and cytokines. Differences in antibody units among groups were assessed by linear regression. Expression level ratios greater than 2 fold were considered significant. All analyses were performed using Stata statistical software, release 10 (Stata Corp., College Station, TX). A p value ≤ 0.05 was considered statistically significant.

3. Results

3.1 Placental proteins combined with the immunogen decrease the severity and incidence of EAH and experimental autoimmune thyroiditis

To determine whether placental proteins influence EAH, we co-immunized SJL mice with mouse pituitary and placental proteins such that each injection site contained both immunogens. This co-immunization decreased the severity of EAH from a median of 3 to a median of 0 (Figure 1A, $p = 0.004$, comparing the third to the fourth group), as well as its incidence (risk ratio 0.44, 95% CI, 0.25–0.79; $p = 0.009$). Most of the mice immunized with pituitary alone developed a diffuse mononuclear cell infiltration in the anterior pituitary (Figure 1B, representing the mouse indicated by the open arrow in Figure 1A), whereas mice receiving also placental proteins showed a milder disease (Figure 1C, representing the mouse indicated by the closed arrow in Figure 1A). No pituitary infiltration was seen in CFA-only control mice or in mice receiving only placental proteins (Figure 1A, first and second groups, respectively). The suppressive effect on EAH was unique to placental proteins and not observed in co-immunizations using equal amounts of muscle or liver proteins (data not shown).

To assess whether the suppressive effect of placental proteins was restricted to EAH or rather applicable to other autoimmune diseases affected by pregnancy, we tested the same placental co-immunization procedure in a mouse model of experimental autoimmune thyroiditis. Placental proteins decreased the severity of thyroiditis from a median of 2 to 1, although this decrease did not reach statistical significance ($p = 0.069$, Figure 1D). Overall, thyroiditis severity was low in both groups, but the mononuclear cell infiltration was milder in the group receiving placental proteins (compare Figure 1E, open arrow in panel D, to Figure 1F, closed arrow in panel D).

3.2 Autoantibodies to pituitary proteins and thyroglobulin are decreased by placenta co-immunization

In EAH, placental co-immunization significantly decreased the total serum IgG and IgM levels of pituitary-specific autoantibodies (Figure 2A, $p = 0.014$). The same effect was observed for the IgG1 subclass (Figure 2B, $p = 0.005$) and for IgG2c, although in this latter case the decrease did not reach statistical significance (Figure 2C, $p = 0.058$). Pituitary-specific IgG and IgM (Figure 2A) and IgG2c (Figure 2C) antibodies were significantly increased by immunization with placenta in CFA as compared to the CFA-only controls, suggesting the existence of antigens shared by both the placenta and pituitary. Pituitary antibodies did not significantly change when mice were co-immunized with pituitary extract and either muscle or liver extract (data not shown).

In experimental autoimmune thyroiditis, placental proteins significantly lowered the total serum levels of IgG and IgM thyroglobulin-specific antibodies (Figure 2D, $p = 0.026$). The effect was not seen in the IgG1 subclass (Figure 2E) but was strongly replicated for IgG2a (Figure 2F, $p < 0.001$), in keeping with the notion that disease in this mouse model is chiefly mediated by Th1 CD4 T cells [23].

3.3 Foreign antibodies differ from autoantibodies in their response to placental proteins

To determine whether placental proteins globally suppress the antibody response or if instead the suppression is restricted to self-antigens, we immunized SJL mice with tetanus toxoid with or without placental proteins and measured the titers of tetanus toxoid antibodies 28 days later. The immunization significantly increased antibody titers in both groups, but the increase was significantly higher in the group co-immunized with placenta (Figure 3A, $p = 0.003$).

We then evaluated the antibody response to a second foreign antigen, purified protein derivative from *M. tuberculosis*, in mice where EAH was induced in the presence of increasing concentrations of placental proteins. Purified protein derivative antibodies increased in a dose-dependent manner from a median OD of 0.67 in the group that did not receive placenta to a median of 1.4 in the one that received 0.5 mg of placental proteins (Figure 3B, $p = 0.017$). Overall these results are in keeping with the observation that foreign antigens induce in B cells a stronger calcium response and a broader nuclear signaling activation than self antigens [24].

3.4 The suppressive effect of placenta requires direct contact with the immunogen

To assess whether placental proteins exert the same immunosuppressive effect if given systemically, we prepared a soluble placenta extract and administered it subcutaneously throughout the duration of EAH. Soluble (i.e., non-emulsified) placental proteins did not alter EAH, which showed a median severity of 3 comparable to that observed in the control group receiving systemic PBS (Figure 4A, compare groups 2 and 3). The incidence and severity of EAH in these two groups were significantly greater than those observed when placenta was given locally in an emulsion mixed with pituitary proteins (median histopathology score of 0, $p = 0.029$ versus group 2 and $p = 0.008$ versus group 3, Figure 4A).

Furthermore, when the CFA-emulsified placental proteins were injected at a different anatomical location than the CFA-emulsified pituitary proteins, they lost the ability to suppress EAH (compare group three to group one in Figure 4B, $p = 0.004$), inducing a disease that was similar to that observed in the PBS controls (compare group 3 to group 2 in Figure 4B, $p = 0.971$).

3.5 Soluble TNF receptor 1 (sTNF-R1) is specifically enriched in placenta

To determine the factor(s) in the placental extract responsible for EAH suppression, we compared placental and muscle proteins by cytokine arrays. The molecule with the highest differential between the two tissues was sTNF-R1, showing 7-fold higher levels in placenta than muscle (Figure 5A). The elevation in sTNF-R1 was confirmed by ELISA, which yielded 9-fold higher sTNF-R1 levels in placenta than in muscle (906 versus 108 pg/mg of protein). Several other proteins showed a >2-fold ratio, however, they were all preferentially expressed in muscle: IL-5, IFN- γ , IL-12 p70, CCL21, and CCL11 (Figure 5A).

In non-pregnant mice, injection of emulsified placental proteins increased the concentration of sTNF-R1 locally (i.e., at the injection site) but did not significantly change its serum levels: 806 ± 186 pg/ml in mice immunized with pituitary proteins versus 793 ± 199 pg/ml in mice immunized with pituitary and placental proteins (Figure 5B, $p = 0.826$). In non-immunized pregnant mice, the high placental content of sTNF-R1 did not seem to result in higher systemic serum levels: serum sTNF-R1 levels: 865 ± 320 pg/ml before pregnancy and 931 ± 243 pg/ml during pregnancy, Figure 5B.

3.6 sTNF-R1 retains its biological activity after emulsification

We verified that emulsification does not destroy the biological activity of sTNF-R1 by performing a co-immunoprecipitation experiment. sTNF-R1 was eluted from the emulsion and retained its biological activity. In fact, when the emulsified placental proteins were incubated with recombinant TNF- α and then pulled down by an anti-TNF-R1 antibody, sTNF-R1 was still capable of binding TNF- α (Figure 6, lane 3). On the contrary, when an isotype control was used instead of the anti-TNF-R1 antibody, no significant amount of TNF- α was detected by immunoblotting after co-immunoprecipitation (Figure 6, lane 2). The low level of recognition by the anti-TNF- α antibody most likely represents non-specific binding of the recombinant TNF- α to the protein A/G agarose beads. As expected, non-emulsified placental proteins contained sTNF-R1 that was capable of binding TNF- α , which could be pulled down by an anti-TNF-R1 antibody and detected by an anti-TNF- α antibody on immunoblotting (Figure 6, lane 1).

3.7 Lack of sTNF-R1 in placenta abolishes its ability to suppress EAH

To delineate the role of sTNF-R1 in disease suppression, we compared EAH in mice co-immunized with wild type C57BL/6 placenta (which contains a sTNF-R1 level of 1298 pg/mg of protein) or placenta derived from TNF-R1 knockout C57BL/6 mice (which contains a sTNF-R1 level less than 9 pg/mg of protein). The lack of sTNF-R1 in placenta abolished its ability to suppress EAH, which showed a median disease severity of 3, significantly higher than the score found when using wild type placenta (median score of 1, $p = 0.026$, Figure 7A). These results strongly suggest that the high sTNF-R1 content in placenta is a critical mediator of disease suppression.

The increased sTNF-R1 levels could act as a sink that locally depletes its ligands, the major one of which is TNF- α . We therefore assessed whether systemic depletion of TNF- α could induce suppression similar to that observed when co-immunizing with placenta. To this end, EAH was induced in mice that were systemically injected with a monoclonal antibody directed against TNF- α , with an isotype control recognizing horseradish peroxidase, or with PBS. No difference in EAH severity was observed in mice systemically depleted of TNF- α (Figure 7B), reinforcing the notion that the suppressive effect of placenta occurs locally at the site of immunization. In addition, serum levels of TNF- α were similar in mice immunized with pituitary alone (23.4 ± 14 pg/ml) or pituitary plus placenta (24.6 ± 16 pg/ml).

4. Discussion

We report that placenta suppresses a mouse model of hypophysitis through its high content of sTNF-R1. Notably, the suppression occurred only when placenta was administered locally (i.e., at the site of immunization), expanding the array of mechanisms used by the immune system to achieve the selective immune tolerance typical of pregnancy.

sTNF-R1, formed by proteolytic cleavage of the membrane receptor [25, 26], binds and neutralizes TNF- α [27], thus acting as a counter-regulatory molecule that attenuates the pro-inflammatory responses induced by TNF- α [28]. Serum levels of sTNF-R1 increase slightly during the course of normal human pregnancy [29], but markedly in pregnant women with malaria [30]. In these women the presence of sTNF-R1 in the peripheral blood positively correlates with parasitemia, thus signaling the presence of an inflammatory response somewhere in the body [30]. Our findings demonstrate that placenta is an important source of sTNF-R1 and implicates sTNF-R1 in the immunosuppressive mechanisms operating at the placenta-decidua interface. However, it is likely that sTNF-R1 is not the only placental factor that contributes to the suppression of EAH. Nevertheless, our findings are in line with other studies that looked at the effect of placenta on inflammation. Cortes *et al.* reported that

supernatants of placental cultures diminish disease severity in a rat model of collagen-induced arthritis [17] while Jash *et al.* found that porcine placental extract decreased the severity of a contact hypersensitivity reaction [31]. Interestingly, both of these groups noted that TNF- α , along with several other cytokines, was decreased in the placenta treated group.

The demonstration that TNF- α is critically involved in the pathogenesis of some autoimmune diseases like arthritis [17] and Crohn-like ileitis [32] has paved the way for the development of TNF- α blocking therapies that have revolutionized the management of patients affected by rheumatoid arthritis [33, 34], Crohn disease [35], or psoriasis [36]. The same anti-TNF- α therapy, however, has proved deleterious in other autoimmune diseases like multiple sclerosis where it exacerbates [37, 38] or even induces demyelination-dependent manifestations [39, 40]. Our results suggest for the first time a contribution of the TNF- α pathway to the pathogenesis of autoimmune hypophysitis. This observation is clinically relevant since some hypophysitis patients do not respond to conventional glucocorticoid immunosuppression [41], and require the introduction of more aggressive medications like methotrexate [42] and azathioprine [43].

Another interesting finding from our studies concerns the locality of the suppressive effect. We surmise that the high placental levels of sTNF-R1 neutralize TNF- α locally (at the site of immunization) based on the findings that they did not influence the systemic levels of TNF- α and that systemic blockade of TNF- α did not suppress EAH. We cannot exclude the possibility that sTNF-R1 not only neutralizes TNF- α at the site of immunization but also lymphotoxin (LT), specifically LT α_3 or LT $\alpha_2\beta_1$, additional ligands of sTNF-R1 [44]. Studies assessing the role of LT α_3 in autoimmunity are complicated by the fact that LT α deficient mice lack peripheral lymphoid organs [45]. However, two studies that specifically blocked LT α_3 or used LT α deficient bone marrow cells found that LT α_3 did not contribute to disease in mouse models of autoimmunity. For instance, Chiang *et al.* showed that blockade of LT α_3 with an antibody did not improve disease in experimental autoimmune encephalomyelitis and collagen-induced arthritis [46]. Similarly, in an elegant set of experiments Riminton *et al.* reconstituted lethally irradiated mice with LT α deficient bone marrow cells and found that LT does not play a role in experimental autoimmune encephalomyelitis [47].

The suppressive effect of placenta was also specific for autoantigens and not seen with two foreign antigens (tetanus toxoid and tuberculin purified protein derivative). These findings are consistent with the notion that self antigens induce a smaller calcium response and a more limited nuclear signal activation profile than foreign antigens [24]. The findings are also consistent with the observation that pregnant women do make antibodies against paternal antigens [48] and mount a response against vaccines [49] but at the same time suppress some autoimmune diseases [2]. Overall, these results are in line with current views indicating that pregnancy induces a selective, rather than generalized, state of immune tolerance [50].

In conclusion, we report that an increased level of placenta derived sTNF-R1 locally suppresses the TNF- α pathway, offering an additional mechanism through which pregnancy modulates the activity of autoimmune diseases.

Acknowledgments

The authors are grateful to Drs. Hiroaki Kimura and Shey-Cherng Tzou for their insights into the project. The work was supported by NIH grant DK080351 to PC.

ABBREVIATIONS

CFA	complete Freund's adjuvant
EAH	experimental autoimmune hypophysitis
LT	lymphotoxin

References

1. Borchers AT, Naguwa SM, Keen CL, Gershwin ME. The implications of autoimmunity and pregnancy. *J Autoimmun.* 2010; 34:J287–99. [PubMed: 20031371]
2. Lee M, O'Brien P. Pregnancy and multiple sclerosis. *J Neurol Neurosurg Psychiatry.* 2008; 79:1308–11. [PubMed: 19010945]
3. Ostensen M, Brucato A, Carp H, Chambers C, Dolhain RJ, Doria A, et al. Pregnancy and reproduction in autoimmune rheumatic diseases. *Rheumatology (Oxford).* 2010
4. Landeck-Salgado MA, Gutenberg A, Lupi I, Kimura H, Mariotti S, Rose NR, et al. Pregnancy, postpartum autoimmune thyroiditis, and autoimmune hypophysitis: intimate relationships. *Autoimmun Rev.* 2010; 9:153–7. [PubMed: 19539059]
5. Cavanagh AC, Morton H. The purification of early-pregnancy factor to homogeneity from human platelets and identification as chaperonin 10. *Eur J Biochem.* 1994; 222:551–60. [PubMed: 7912672]
6. Morton H, Hegh V, Clunie GJA. Studies of the Rosette Inhibition Test in Pregnant Mice: Evidence of Immunosuppression? *Proc R Soc Lond B.* 1976; 193:413–9. [PubMed: 11471]
7. Zhang B, Harness J, Somodevilla-Torres MJ, Hillyard NC, Mould AW, Alewood D, et al. Early pregnancy factor suppresses experimental autoimmune encephalomyelitis induced in Lewis rats with myelin basic protein and in SJL/J mice with myelin proteolipid protein peptide 139–151. *J Neurol Sci.* 2000; 182:5–15. [PubMed: 11102634]
8. Zhang B, Walsh MD, Nguyen KB, Hillyard NC, Cavanagh AC, McCombe PA, et al. Early pregnancy factor treatment suppresses the inflammatory response and adhesion molecule expression in the spinal cord of SJL/J mice with experimental autoimmune encephalomyelitis and the delayed-type hypersensitivity reaction to trinitrochlorobenzene in normal BALB/c mice. *J Neurol Sci.* 2003; 212:37–46. [PubMed: 12809997]
9. Langer-Gould A, Garren H, Slansky A, Ruiz PJ, Steinman L. Late pregnancy suppresses relapses in experimental autoimmune encephalomyelitis: evidence for a suppressive pregnancy-related serum factor. *J Immunol.* 2002; 169:1084–91. [PubMed: 12097417]
10. McClain MA, Gatson NN, Powell ND, Papenfuss TL, Gienapp IE, Song F, et al. Pregnancy suppresses experimental autoimmune encephalomyelitis through immunoregulatory cytokine production. *J Immunol.* 2007; 179:8146–52. [PubMed: 18056357]
11. Gatson NN, Williams JL, Powell ND, McClain MA, Hennon TR, Robbins PD, et al. Induction of pregnancy during established EAE halts progression of CNS autoimmune injury via pregnancy-specific serum factors. *J Neuroimmunol.* 2010; 10.1016/j.jneuroim.2010.09.010
12. Barac-Latas V, Muhvic D, Radosevic-Stabic B. The influence of pregnancy on development and course of chronic relapsing experimental autoimmune encephalomyelitis in rats: implications for multiple sclerosis. *Coll Antropol.* 2010; 34 (Suppl 1):267–71. [PubMed: 20402331]
13. Imaizumi M, Pritsker A, Kita M, Ahmad L, Unger P, Davies T. Pregnancy and murine thyroiditis: thyroglobulin immunization leads to fetal loss in specific allogeneic pregnancies. *Endocrinology.* 2001; 142:823–9. [PubMed: 11159855]
14. Imaizumi M, Pritsker A, Kita M, Ahmad L, Unger P, Davies TF. Non-MHC driven exacerbation of experimental thyroiditis in the postpartum period. *Autoimmunity.* 2001; 34:95–105. [PubMed: 11905847]
15. Kokeny G, Godo M, Nagy E, Kardos M, Kotsch K, Casalis P, et al. Skin disease is prevented but nephritis is accelerated by multiple pregnancies in autoimmune MRL/LPR mice. *Lupus.* 2007; 16:465–77. [PubMed: 17670844]

16. Buzas EI, Hollo K, Rubliczky L, Garzo M, Nyirkos P, Glant TT. Effect of pregnancy on proteoglycan-induced progressive polyarthritis in BALB/c mice: remission of disease activity. *Clin Exp Immunol.* 1993; 94:252–60. [PubMed: 8222315]
17. Cortes M, Canellada A, Miranda S, Dokmetjian J, Gentile T. Placental secreted factors: their role in the regulation of anti-CII antibodies and amelioration of collagen induced arthritis in rats. *Immunol Lett.* 2008; 119:42–8. [PubMed: 18538417]
18. Tzou SC, Lupi I, Landek M, Gutenberg A, Tzou YM, Kimura H, et al. Autoimmune hypophysitis of SJL mice: clinical insights from a new animal model. *Endocrinology.* 2008; 149:3461–9. [PubMed: 18388197]
19. Tzou, SC.; Landek-Salgado, MA.; Kimura, H.; Caturegli, P. Preparation of mouse pituitary immunogen for the induction of experimental autoimmune hypophysitis; *J Vis Exp.* 2010. p. 46<http://www.jove.com/details.stp?id=2181>
20. Kimura H, Kimura M, Tzou SC, Chen YC, Suzuki K, Rose NR, et al. Expression of class II major histocompatibility complex molecules on thyrocytes does not cause spontaneous thyroiditis but mildly increases its severity after immunization. *Endocrinology.* 2005; 146:1154–62. [PubMed: 15591134]
21. Landek-Salgado, MA.; Tzou, SC.; Kimura, H.; Caturegli, P. Induction of experimental autoimmune hypophysitis in SJL mice; *J Vis Exp.* 2010. p. 46<https://www.jove.com/details.stp?id=2182>
22. Jouvin-Marche E, Morgado MG, Leguern C, Voegtli D, Bonhomme F, Cazenave PA. The mouse Igh-1a and Igh-1b H chain constant regions are derived from two distinct isotypic genes. *Immunogenetics.* 1989; 29:92–7. [PubMed: 2563358]
23. Flynn JC, Conaway DH, Cobbold S, Waldmann H, Kong YC. Depletion of L3T4+ and Lyt-2+ cells by rat monoclonal antibodies alters the development of adoptively transferred experimental autoimmune thyroiditis. *Cell Immunol.* 1989; 122:377–90. [PubMed: 2569935]
24. Healy JI, Dolmetsch RE, Timmerman LA, Cyster JG, Thomas ML, Crabtree GR, et al. Different nuclear signals are activated by the B cell receptor during positive versus negative signaling. *Immunity.* 1997; 6:419–28. [PubMed: 9133421]
25. Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, et al. A metalloproteinase disintegrin that releases tumour-necrosis factor- α from cells. *Nature.* 1997; 385:729–33. [PubMed: 9034190]
26. Reddy P, Slack JL, Davis R, Cerretti DP, Kozlosky CJ, Blanton RA, et al. Functional analysis of the domain structure of tumor necrosis factor- α converting enzyme. *J Biol Chem.* 2000; 275:14608–14. [PubMed: 10799547]
27. Van Zee KJ, Kohno T, Fischer E, Rock CS, Moldawer LL, Lowry SF. Tumor necrosis factor soluble receptors circulate during experimental and clinical inflammation and can protect against excessive tumor necrosis factor α in vitro and in vivo. *Proc Natl Acad Sci U S A.* 1992; 89:4845–9. [PubMed: 1317575]
28. Bradley JR. TNF-mediated inflammatory disease. *J Pathol.* 2008; 214:149–60. [PubMed: 18161752]
29. Arntzen KJ, Liabakk NB, Jacobsen G, Espevik T, Austgulen R. Soluble tumor necrosis factor receptor in serum and urine throughout normal pregnancy and at delivery. *Am J Reprod Immunol.* 1995; 34:163–9. [PubMed: 8561873]
30. Thevenon AD, Zhou JA, Megnekou R, Ako S, Leke RG, Taylor DW. Elevated levels of soluble TNF receptors 1 and 2 correlate with *Plasmodium falciparum* parasitemia in pregnant women: potential markers for malaria-associated inflammation. *J Immunol.* 2010; 185:7115–22. [PubMed: 20980627]
31. Jash A, Kwon HK, Sahoo A, Lee CG, So JS, Kim J, et al. Topical application of porcine placenta extract inhibits the progression of experimental contact hypersensitivity. *J Ethnopharmacol.* 2011; 133:654–62. [PubMed: 21040771]
32. Marini M, Bamias G, Rivera-Nieves J, Moskaluk CA, Hoang SB, Ross WG, et al. TNF- α neutralization ameliorates the severity of murine Crohn's-like ileitis by abrogation of intestinal epithelial cell apoptosis. *Proc Natl Acad Sci U S A.* 2003; 100:8366–71. [PubMed: 12832622]

33. Furst DE, Breedveld FC, Kalden JR, Smolen JS, Burmester GR, Bijlsma JW, et al. Updated consensus statement on biological agents, specifically tumour necrosis factor {alpha} (TNF{alpha}) blocking agents and interleukin-1 receptor antagonist (IL-1ra), for the treatment of rheumatic diseases, 2005. *Ann Rheum Dis.* 2005; 64(Suppl 4):iv2–14. [PubMed: 16239380]
34. Garrison L, McDonnell ND. Etanercept: therapeutic use in patients with rheumatoid arthritis. *Ann Rheum Dis.* 1999; 58 (Suppl 1):165–9. [PubMed: 10577976]
35. Targan SR, Hanauer SB, van Deventer SJ, Mayer L, Present DH, Braakman T, et al. A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. *N Engl J Med.* 1997; 337:1029–35. [PubMed: 9321530]
36. Reich K, Nestle FO, Papp K, Ortonne JP, Evans R, Guzzo C, et al. Infliximab induction and maintenance therapy for moderate-to-severe psoriasis: a phase III, multicentre, double-blind trial. *Lancet.* 2005; 366:1367–74. [PubMed: 16226614]
37. TNF neutralization in MS: results of a randomized, placebo-controlled multicenter study. The Lenercept Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group. *Neurology.* 1999; 53:457–65. [PubMed: 10449104]
38. van Oosten BW, Barkhof F, Truyen L, Boringa JB, Bertelsmann FW, von Blomberg BM, et al. Increased MRI activity and immune activation in two multiple sclerosis patients treated with the monoclonal anti-tumor necrosis factor antibody cA2. *Neurology.* 1996; 47:1531–4. [PubMed: 8960740]
39. Gomez-Gallego M, Meca-Lallana J, Fernandez-Barreiro A. Multiple sclerosis onset during etanercept treatment. *Eur Neurol.* 2008; 59:91–3. [PubMed: 17934280]
40. Pfueller CF, Seipelt E, Zipp F, Paul F. Multiple sclerosis following etanercept treatment for ankylosing spondylitis. *Scand J Rheumatol.* 2008; 37:397–9. [PubMed: 18720264]
41. Caturegli P, Newschaffer C, Olivi A, Pomper MG, Burger PC, Rose NR. Autoimmune Hypophysitis. *Endocr Rev.* 2005; 26:599–614. [PubMed: 15634713]
42. Tubridy N, Saunders D, Thom M, Asa SL, Powell M, Plant GT, et al. Infundibulohypophysitis in a man presenting with diabetes insipidus and cavernous sinus involvement. *J Neurol Neurosurg Psychiatry.* 2001; 71:798–801. [PubMed: 11723207]
43. Lecube A, Francisco G, Rodriguez D, Ortega A, Codina A, Hernandez C, et al. Lymphocytic hypophysitis successfully treated with azathioprine: first case report. *J Neurol Neurosurg Psychiatry.* 2003; 74:1581–3. [PubMed: 14617725]
44. Hehlhans T, Mannel DN. The TNF-TNF receptor system. *Biol Chem.* 2002; 383:1581–5. [PubMed: 12452434]
45. De Togni P, Goellner J, Ruddle NH, Streeter PR, Fick A, Mariathasan S, et al. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. *Science.* 1994; 264:703–7. [PubMed: 8171322]
46. Chiang EY, Kolumam GA, Yu X, Francesco M, Ivelja S, Peng I, et al. Targeted depletion of lymphotoxin-alpha-expressing TH1 and TH17 cells inhibits autoimmune disease. *Nat Med.* 2009; 15:766–73. [PubMed: 19561618]
47. Sean Riminton D, Korner H, Strickland DH, Lemckert FA, Pollard JD, Sedgwick JD. Challenging cytokine redundancy: inflammatory cell movement and clinical course of experimental autoimmune encephalomyelitis are normal in lymphotoxin-deficient, but not tumor necrosis factor-deficient, mice. *J Exp Med.* 1998; 187:1517–28. [PubMed: 9565643]
48. Van Rood JJ, Eernisse JG, Van Leeuwen A. Leucocyte antibodies in sera from pregnant women. *Nature.* 1958; 181:1735–6. [PubMed: 13566127]
49. Zuccotti G, Pogliani L, Pariani E, Amendola A, Zanetti A. Transplacental antibody transfer following maternal immunization with a pandemic 2009 influenza A(H1N1) MF59-adjuvanted vaccine. *Jama.* 2010; 304:2360–1. [PubMed: 21119083]
50. Erlebacher A. Immune surveillance of the maternal/fetal interface: controversies and implications. *Trends Endocrinol Metab.* 2010; 21:428–34. [PubMed: 20304670]

Figure 1. Placental proteins suppress experimental autoimmune hypophysitis (EAH) and thyroiditis

(A) EAH in female SJL mice 28 days after immunization with CFA only (none group), placental proteins only (placenta group), pituitary proteins only (pit. group), or pituitary and placental proteins (pit. & plac. group). (B and C) Representative H&E staining of the anterior pituitary in a mouse immunized with pituitary proteins (B) or with pituitary and placental proteins (C) (magnification, X40). Arrows in A identify the mice that are represented in B and C. (D) Experimental autoimmune thyroiditis in female CBA mice 21 days after immunization with CFA only (none group), thyroglobulin only (thyroglob. group), or thyroglobulin and placental proteins (thyroglob. & placenta group). (E and F) Representative H&E staining of the thyroid in a mouse immunized with thyroglobulin only (E) and a mouse immunized with thyroglobulin and placental proteins (F) (magnification, x20). Arrows in D identify the mice that are represented in E and F. The horizontal lines represent the median severity in each group.

Figure 2. Placental proteins decrease pituitary- and thyroglobulin-specific autoantibody titers IgG and IgM (A), IgG1 (B) and IgG2c (C) pituitary-specific antibody titers were measured by an ELISA in female SJL mice 28 days after immunization with CFA only (none group), placental proteins only (placenta group), pituitary proteins only (pit. group), or pituitary and placental proteins (pit. & plac. group). IgG and IgM (D), IgG1 (E) and IgG2a (F) thyroglobulin-specific antibody titers were measured by an ELISA in female CBA mice 21 days after immunization with CFA only (none group), thyroglobulin only (thyroglob. group), or thyroglobulin and placental proteins (thyroglob. & placenta group).

Figure 3. Placental proteins increase foreign antibody titers

(A) IgG and IgM tetanus toxoid-specific antibody titers were measured by an ELISA in female SJL mice on days 0 and 28 after immunization with tetanus toxoid only (black circles) or tetanus toxoid and placental proteins (open circles). (B) IgG and IgM purified protein derivative (PPD)-specific antibody titers were measured by an ELISA in female SJL mice 28 days after immunization with pituitary proteins emulsified in CFA (which contains PPD) or pituitary proteins in CFA supplemented with three doses of placental proteins. The horizontal lines represent the median severity in each group.

Figure 4. Placental proteins need to be at the same anatomic location as the immunogen to suppress EAH

(A) EAH severity in female SJL mice after immunization with pituitary and local placental proteins (box plot), pituitary proteins and subcutaneous administration of control buffer (pituitary & systemic PBS group) or non-emulsified placental proteins (pituitary & systemic placenta group). (B) EAH severity in female SJL mice 28 days after immunization with pituitary proteins in the left hind leg and either PBS emulsified in CFA (pituitary & PBS at different injection sites group) or placental proteins emulsified in CFA (pituitary & placenta at different injection sites group) in the right hind leg. The pituitary & placenta at same

injection site group (box plot) was co-immunized with pituitary proteins and placental proteins. The horizontal lines represent the median severity in each group.

Figure 5. sTNF-R1 is specifically enriched in the placenta

(A) Cytokines and chemokines in two placental and muscle extracts were measured by protein array. Results are expressed as placenta over muscle ratios, reporting only values where the difference was greater than two-fold. (B) sTNF-R1 levels in serum of immunized non-pregnant SJL mice and non-immunized virgin and pregnant SJL mice.

Figure 6.

sTNF-R1 retains its biological activity after emulsification. Western blot to detect TNF-α after co-immunoprecipitation. Non-emulsified placental proteins (lane 1) or emulsified placental proteins (lanes 2 and 3) were incubated with recombinant TNF-α and then an anti-TNF-R1 antibody (lanes 1 and 3) or an isotype control antibody (lane 2). After capturing the anti-TNF-R1 or isotype control antibody with protein A/G agarose, samples were run on a polyacrylamide gel and probed with an anti-TNF-α antibody.

Figure 7. A placental extract deficient in TNF-R1 does not suppress EAH

(A) EAH severity in female SJL mice 28 days after immunization with pituitary proteins and wild type placenta or pituitary proteins and TNF-R1^{-/-} placenta (B) EAH severity in female SJL mice 28 days after immunization with pituitary proteins and systemic PBS, anti-HRP, or anti-TNF- α antibody. The horizontal lines represent the median severity in each group.