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Contraceptive Effect of a Vaccine Targeting Leukemia Inhibitory Factor that is Vital for Implantation

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**Contraceptive Effect of a Vaccine Targeting
Leukemia Inhibitory Factor that is Vital for Implantation**

Angela Rae Lemons

**Thesis submitted to the
School of Medicine
At West Virginia University
In partial fulfillment of the requirements
for the degree of**

**Master of Science
In
Biomedical Science**

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ABSTRACT

Contraceptive Effect of a Vaccine Targeting Leukemia Inhibitory Factor that is Vital for Implantation

by Angela Rae Lemons

Immunomodulation at the fetal-maternal interface is essential for the establishment and maintenance of pregnancy. Several interleukins have been shown to be key in this process. Leukemia inhibitory factor (LIF), a member of the interleukin-6 family of proteins, is vital for blastocyst implantation in both humans and mice. LIF is expressed by the uterine epithelium and stromal cells surrounding the blastocyst prior to implantation and acts via binding to the LIF receptor expressed on the endometrium. This study investigates the contraceptive effect of a vaccine prepared with peptides derived from LIF and LIF receptor in the murine model. The vaccine was created by conjugating the LIF and LIF receptor peptides to various carrier proteins. Vaccines were administered to female mice by intramuscular, subcutaneous or intranasal immunization. The contraceptive effect of the vaccine was investigated by mating vaccinated animals and counting the number of pups born. Immunization of female mice with the LIF/LIF receptor peptide vaccine induced peptide-specific antibody titers (IgG and IgA) in the sera as well as locally in the genital tract. These antibodies provide significant reductions in fertility as compared to controls. Targeting mucosal surfaces with intranasal immunization appeared to further reduce litter size in vaccinated animals. Peptide-specific antibodies were shown to remain in immunized mice up to 11 months after their final immunization. LIF/LIFR peptide antibodies not only reduced LIF bioactivity *in vivo*, they also neutralized LIF biological function in an *in vitro* assay. These studies suggest that LIF/ LIF receptor is an attractive target for immunocontraception.

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LIST OF ABBREVIATIONS

CFA	complete Freund's adjuvant
CTB	cholera toxin B subunit
CV	contraceptive vaccine(s)
ELISA	enzyme-linked immunosorbent assay
IFA	incomplete Freund's adjuvant
i.m.	intramuscular
i.n.	intranasal
i.p.	intraperitoneally
i.vag.	intravaginally
IVF	<i>in vitro</i> fertilization
LA	antagonist of leukemia inhibitory factor
LIF	leukemia inhibitory factor
LIFR	leukemia inhibitory factor receptor
MBS	<i>m</i> -maleimidobenzoyl- <i>N</i> -hydroxysuccinimide ester
MTT	1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan
PBS	phosphate buffered saline
PEG	polyethylene glycol
s.c.	subcutaneous

INTRODUCTION

Introduction to Contraceptive Vaccines

Despite the many efforts to implement birth control methods around the world, there are still many unintended pregnancies that are only exacerbating the already increasing world population. The current methods of birth control, particularly steroidal contraceptives and intrauterine devices, have several serious side effects that limit their use in many women. Consequently, the need to develop a more targeted, less invasive approach becomes more and more relevant. A contraceptive vaccine (CV) provides a novel solution that would be easy to administer, less expensive, reversible, require less frequent administration and, most importantly, offer the specificity current methods lack. By targeting factors that are essential for establishment of pregnancy, a CV would block the action of a factor(s) and prevent the onset of pregnancy.

One of the essential factors that has been extensively studied is the chorionic gonadotropin (CG). Human chorionic gonadotropin (hCG) is a major systemic regulator of embryo development and implantation and is secreted by the implanting trophoblast [1], making it an ideal pregnancy-specific target for CV development. Several forms of hCG vaccines have undergone clinical trials in women, both phase I and phase II, displaying positive contraceptive effects [2, 3]. While the outlook of the hCG vaccine looks promising, research on additional potential targets continues with an ultimate goal of finding a vaccine that is more immunogenic and efficacious. This research focuses on finding additional factors that are involved in the development and implantation of the embryo, with a focus on those that have been shown to be essential for normal embryonic development and/or implantation, and pregnancy-specific. The goal is to target one or more of these molecules for the development of a highly specific, non-steroidal, and efficacious vaccine for birth control.

Pregnancy-Essential Factors

Research began with a database search to identify factors that are indispensable in the establishment and maintenance of pregnancy in mice and/or humans. A summary of cytokines, chemokines, growth factors, and other factors essential for successful pregnancy are described in Tables A1-A3. Those factors essential for embryo development, implantation, uterine receptivity and maternal tolerance are summarized in the following schematic (Figure 1). This figure highlights the molecules among these factors which are both essential and pregnancy-specific.

Figure 1. Schematic of the factors involved in the establishment of pregnancy from Lemons & Naz [4]. Factors that are essential and pregnancy-specific are represented in **RED**.

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Possible Immunocontraceptive Targets

The purpose of this database search was to review the factors that are involved in the establishment of pregnancy, and delineate which of those factors are essential and pregnancy-specific. Among the essential factors identified, three, namely chorionic gonadotropin (CG), leukemia inhibitory factor (LIF), and preimplantation factor (PIF), were found to be pregnancy-specific molecules that could be exploited for contraceptive vaccine development. By selecting the proteins that are essential and pregnancy-specific, it is highly likely that targeting these molecules will reduce fertility without affecting any other molecule and process.

Research in this area has been rapidly progressing over the past decade. The pregnancy-specific protein, hCG, was initially used for detection of pregnancy in women. Now, it is being investigated as a contraceptive target for development of a birth control vaccine. Several vaccines based on the β subunit of hCG incorporating various carriers and adjuvants have undergone phase I and phase II clinical trials in women. A study completed in 1994 by Talwar et al recorded that women administered an hCG vaccine developed antibody titers that prevented pregnancy. Only 1 pregnancy was recorded in over 1224 cycles observed in these vaccinated women [2]. Another trial demonstrated that a heterospecies dimer (HSD)-hCG vaccine (containing beta hCG and alpha ovine luteinizing hormone) was reversible and that titers below the protective threshold showed no teratogenic effect on pregnancy outcome [3].

A more recent protein of interest is LIF, a member of the interleukin-6 family of proteins involved in embryo development and uterine response to implantation [5]. It is known that the LIF molecule is required for successful blastocyst implantation. Hindering the ability of LIF to bind its receptor (LIFR) has been shown to block implantation *in vivo* [6, 7]. Another interesting molecule, PIF, is an embryo-derived peptide detected in the serum just before implantation [1]. It has recently been shown to be essential for implantation by promoting adhesion, regulating immunity, and apoptosis [8, 9].

These three factors (hCG/LIF/PIF) may provide viable targets for immunocontraception. The contraceptive vaccines targeting factors involved in pregnancy establishment have two potential concerns: 1) Although these factors are involved in the early events of embryonic development and preimplantation, the vaccines against them are not contraceptives in the true sense because they target the post-fertilization stages, and 2) They are “self” molecules and it may be a challenging proposition to induce enough antibodies to neutralize these factors. However, the findings of phase I and phase II of clinical trials of hCG vaccine in women indicate that by using appropriate carriers and adjuvants, one can modulate the “self” molecule to raise antibodies in humans. Also, hCG vaccine trials have indicated that there are no teratogenic effects of the low titer residual antibodies. The hCG vaccine trials in women have established the basis for developing a birth control vaccine targeting various factors involved in establishment of pregnancy. Research now continues to find a vaccine target that is more immunogenic and efficacious.

Leukemia Inhibitory Factor in Blastocyst Implantation

LIF is an IL-6 family cytokine shown to be required for successful blastocyst implantation in mice. Stewart et al developed LIF null mice which expressed a mutated *LIF* gene encoding a truncated, non-functional form of LIF [6]. Female mice expressing this mutant LIF demonstrate complete implantation failure. It appeared that maternal LIF expression was required as blastocysts from LIF null females were viable and implanted successfully when transferred to wild type recipient females. This was one of the first studies indicating the importance of cytokines in the reproductive process. Because LIF was so critical to the implantation process, it drew interest as a possible means to control pregnancy.

Fifteen years later, White et al addressed the possibility of targeting LIF to prevent implantation in mice [7]. They administered a high affinity LIF antagonist (LA) to female mice to

block LIF function. Because LIF is secreted in the uterine glandular epithelium on day 3.5 of pregnancy, the antagonist was administered from days 2.5-4.5. Successful blockage of implantation was achieved by the administration of 7 mg/kg per day by four intraperitoneal (i.p.) injections on top of continuous administration through a miniosmotic pump. They then conjugated the LA to polyethylene glycol (PEG-LA) to increase the serum stability of the antagonist. PEG-LA (37.5 mg/kg per day) completely blocked implantation in mice with three i.p. injections administered on days 2.5 and 3.5.

These were key studies indicating that LIF, due to its critical nature in the process of implantation, could be targeted for successful prevention of pregnancy in mice. White et al demonstrated that preventing LIF interaction with its receptor is sufficient to block implantation. While the LA is adequate for blocking implantation when administered at high doses over the course of the implantation window, it may not provide a practical means of contraception. In this sense, immune targeting could provide a more realistic approach for exploiting the requirement of LIF for contraceptive purposes.

Evidence for LIF Requirement in Human Implantation

Although the experiments mentioned here have been limited to the murine model, the requirement for LIF in successful pregnancy extends to many other species, most importantly, humans. It has been shown that LIF mRNA expression levels peak in the human endometrium at the time of implantation [10], indicating a possible role in implantation similar to that seen in mice. Although its requirement is difficult to study in women, low levels of LIF have been shown to be associated with infertility in some women. LIF secretion was significantly lower in endometrial explants from women suffering repeated implantation failures or unexplained infertility compared to fertile women [11]. LIF immunostaining of endometrial biopsies from fertile women was also much stronger than that of women with unexplained infertility [12].

These studies seem to suggest a requirement for increased LIF expression to achieve successful implantation in women, given that lower expression can be correlated to implantation failure and unexplained infertility.

Not only have low levels of LIF secretion been demonstrated in women with unexplained infertility, studies have also revealed *LIF* gene mutations that correlate to poor *in vitro* fertilization (IVF) outcome in women with unexplained infertility and endometriosis [13]. These women had a simple G to A substitution that led to a switch from valine to methionine at codon 64. When compared to other infertile women displaying endometriosis or unexplained fertility that do not carry this mutation, mutation-positive women had significantly decreased pregnancy rates after IVF. These reports suggest an important role of LIF in implantation in humans that is similar to that seen in mice and other species.

Aims of the Study

Because of its essential role and promising results as a contraceptive target, we chose to target LIF for immunocontraceptive vaccine studies. It is not known whether production of LIF and LIFR antibodies can block LIF biological function. The objective of this research is to determine if LIF/LIFR antibodies created as a result of LIF/LIFR peptide vaccination will prevent blastocyst implantation. We hypothesized that blocking the interaction of LIF with its receptor with antibodies will reduce the fertility of female mice. We also expect that higher antibody titers localized to the genital tract will produce higher contraceptive effects. In the present study, peptides for both the LIF protein and the LIF receptor were created from regions where LIF interacts with its receptor [14-16]. These peptides were then used to create a LIF/LIFR vaccine intended to produce peptide-specific antibodies that would reduce LIF biological function, in turn reducing the fertility of female mice. A CV targeting LIF/LIFR provides specificity and a possible alternative to hormonal contraception.

Specific Aim 1: To determine if a LIF/LIFR peptide vaccine has any effect on fertility.

The working hypothesis was that immunization with LIF peptides will reduce fertility in female mice. Female BALB/c mice were immunized via subcutaneous and intramuscular injection. Boosters were given until significant antibody titers are obtained. The mice were then mated and effects on fertility were determined by comparing the litter sizes to those of control mice given only vehicle injections. We expected that litter sizes in immunized mice would be significantly less than those of controls.

Specific Aim 2: To determine if targeting the mucosa increases the effect on fertility.

Previous research led to the hypothesis that intranasal immunization targeting the mucosa combined with intramuscular injection will provide higher antibody titers in the genital tract than intramuscular or subcutaneous immunization alone. We expected that because this method of immunization targets the mucosal surfaces, the effect on fertility will be even greater than systemic immunization alone.

Specific Aim 3: To determine if the vaccine delivery route produces antibody titers in the genital tract.

Most studies to this point simply looked at antibody titers in the vaginal washings as an indication that intranasal and other forms of mucosal immunization resulted in an antibody response in the genital tract. Since the goal of our studies was to reduce fertility by blocking LIF function in the uterus, we aimed to determine if our vaccination strategy produced antibodies in the different regions of the genital tract. Antibodies in the vagina, uterus and oviduct were analyzed by ELISA. We expected, since we saw high titers in the vagina in previous studies, that there would also be high titers in the uterus and oviduct.

Specific Aim 4: To determine if LIF/LIFR peptide antibodies reduce LIF biological function in vitro.

To verify the results seen in *in vivo* studies, we used an *in vitro* method to analyze the ability of each peptide antibody to neutralize the biological function of LIF. We expected that the antibodies would be able to reduce LIF biological function in this assay because of their ability to reduce fertility in mice. This assay also gave us the ability to determine which antibodies appeared to be most effective at neutralizing LIF biological function.

The aim of this study was to identify a peptide candidate(s) that may be combined with other efficacious peptides for further development of a multi-epitope contraceptive vaccine. Such a vaccine would provide a cheaper, more reliable method of contraception that would be available worldwide. This new approach to contraception would be completely non-hormonal. Given the many risks that go along with hormonal contraception methods, an alternative that is safer and more reliable is becoming more and more relevant.

CHAPTER 1:

Contraceptive potential of LIF/LIFR peptide vaccine

Materials and Methods

Animals

Balb/C 8-10 week old virgin female mice were used for immunization in this study. The Balb/C females were mated with 10-15 week old fertile Balb/C males in fertility testing. Animals were maintained by West Virginia University Office of Laboratory and Animal Resources personnel. All experimental protocols used in this study were approved by the West Virginia University Animal Care and Use Committee (WVU-ACUC).

Peptide design and synthesis

The LIF and LIFR peptides were selected based on regions and specific amino acids previously shown to be important in their interaction (Figure 2). Pro 51 and Phe 52 within the LIF amino acid sequence are essential for LIF/LIFR binding [14, 17]. The LIFp1 was chosen from amino acids 40-54 to span these important interactions. Also shown to be essential were Ile-103, Asn-105 and Ser-107 [14] leading us to choose LIFp2 to include amino acids 100-115. The selection of LIFp3 (156-180) was based upon the essential amino acids Phe-156 and Lys-159 [17] as well as a previous finding that 9 C-terminal amino acids (166-180) are required for LIF biological function [18].

LIF has been shown to interact with LIFR through the Ig-like domain (residues 260-263) with Ser-262 providing a hydrogen bond with Lys-159 of LIF [14]. This was the rationale for selection LIF-Rp1 (250-263). Other important interactions involve Val-314, Pro-316 and Glu-338, leading us to choose a second LIFR peptide (LIF-Rp2) to cover residues 314-339.

Figure 2. Essential interactions for LIF/LIFR binding. The interface of hLIF:mLIFR illustrated by Huyton et al [14] is shown, with hLIF residues shown in salmon and mLIFR residues shown in yellow. Residues targeted for peptide design and synthesis in the present study are indicated by the black boxes (LIF residues) and red boxes (LIFR residues).

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These peptides from both the murine LIF and murine LIF receptor proteins were analyzed using the Invitrogen Peptide Select™ Online program to determine the peptides which were hydrophilic and immunogenic. Five peptides were selected and synthesized by GenScript Corp. (Piscataway, NJ) (Table 1). Peptides from LIF were designated LIFp1, LIFp2 and LIFp 3 and peptides from the LIF receptor were LIF-Rp1 and LIF-Rp2. For conjugation purposes, the glycine-cysteine residues were added to the N-terminal ends of the peptides.

Table 1. Murine LIF and LIFR Peptide Sequences Used for Immunization

<i>Antigen</i>	<i>Designation</i>	<i>Sequence</i>
LIF (40-54)	LIFp1	GC-LFISYYTAQGEPFPN
LIF (100-115)	LIFp2	GC-DQKVLNPTAVSLQVK
LIF (156-180)	LIFp3	GC-FQRKKLGCQLLGRYKQVISVVVQAF
LIF receptor (250-263)	LIF-Rp1	GC-AIHILNIPVSENSG
LIF receptor (314-339)	LIF-Rp2	GC-GPRNTEYTLFESISGKSAVFHRIEG

Vaccine preparation

For i.m. and s.c. immunizations, the carrier proteins used were keyhole limpet hemocyanin (Calbiochem, San Diego, CA), chicken γ -globulin (Calbiochem), chicken ovalbumin (Calbiochem) and bovine serum albumin (Sigma-Aldrich, St. Louis, MO). Peptides were conjugated to these carrier proteins at a 1:1 ratio using the heterobifunctional reagent *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) (Pierce Biotechnology, Rockford, IL) using a previously described procedure [19]. Peptides were conjugated to CTB (List Biological Labs, Campbell, CA) for i.n. immunizations using a two step glutaraldehyde (Sigma-Aldrich) procedure described previously [20] or using MBS. Peptides were combined with the CTB at a 5:1 ratio. CTB-peptide conjugates were dialyzed in phosphate buffered saline (PBS) for 48 hours using the Slide-A-Lyzer dialysis cassette system (Pierce Biotechnology).

Immunization

Mature, virgin BALB/c female mice were immunized i.m., s.c., and/or i.n. against the LIF peptide-carrier vaccine, the LIF receptor peptide-carrier vaccine, a combination of LIF and LIFR peptide-carrier vaccines, or PBS. 10-12 mice were immunized in each experimental group. For i.m. and s.c. immunizations, each animal received 50 μ l injections containing 50 μ g of each peptide emulsified with Freund's adjuvant (Sigma-Aldrich). For i.n. administration, each animal received 10-15 μ l per nostril containing a total of 50 μ g of each peptide conjugated to CTB.

Fertility Testing

Following the last boost, the immunized females were mated with 10-15 week male BALB/c mice. Females were placed with the males in the evening and mating was confirmed the following morning by the presence of a vaginal plug. Mated females were separated until they delivered. The number of pups delivered by each female were counted at birth and then euthanized by decapitation. Three separate fertility trials were conducted.

Analysis of antibodies in the blood and genital tract

After being administered a series of immunizations, sera and vaginal washings were collected from each mouse prior to fertility testing. Blood was collected through the saphenous vein. Vaginal washings were collected by washing the vagina with 50 μ l of PBS.

The presence and titers of antibodies were analyzed in these samples using the enzyme-linked immunosorbent assay (ELISA), as described earlier [19, 21]. Each well was coated overnight at 4°C with 4 μ g peptide. Sera was diluted 1:40 and vaginal washings 1:20 in 0.05% Tween in PBS (PBS-T) containing 3% BSA. Alkaline phosphatase conjugated anti-mouse IgG (Sigma-Aldrich) and IgA α -chain (Sigma-Aldrich) were diluted 1:800 and 1:400, respectively, in PBS-T containing 3% BSA. The plates were developed with substrate solution (1 mg/ml *p*-nitrophenyl phosphate diluted in 0.05 M carbonate buffer, pH 9.6 with 1.0 mM MgCl₂) and the reaction was read at 405 nm. Absorbance readings were converted to standard deviation units by using the following formula: SD units = absorbance (test) - mean absorbance

(control group)/standard deviation of the control group. The test samples with SD units greater or equal to 3 were considered as having a positive reaction with a peptide.

Statistical analysis

In Table 2, which is comprised of multiple groups, ANOVA was used to determine if differences exist among means of various groups. Since the differences were significant, the follow-up Fisher's Significant Difference (FSD) methods were used to examine the pair-wise differences. The significance of difference between the mean pups born in the vaccinated and control groups was analyzed by using an unpaired Student's *t*-test in Table 3. A *P*-value of <0.05 was considered significant.

Antibody Response and Contraceptive Effect of Systemic Immunization

Female mice vaccinated systemically by intramuscular (i.m.) and/or subcutaneous (s.c.) administration with the LIF/LIFR peptide vaccines showed peptide-specific antibody titers (IgG) in both serum and vaginal washings (Fig. 3A-B). All animals immunized with LIF peptides developed peptide-specific IgG in the sera while 83% showed these same antibodies in the vaginal mucosa. Of the animals immunized with LIFR peptides 83% and 8% developed peptide-specific IgG in the serum and vaginal mucosa, respectively. When immunized with both LIF and LIFR peptides, all animals showed peptide-specific IgG in the serum and vaginal mucosa.

Corresponding to the development anti-LIF and anti-LIFR antibodies in these mice, an average reduction in litter size below that of the controls was seen in all groups (Table 2). Interestingly, the most significant reduction, 50.6% below controls, was seen in mice immunized with LIFR peptides, who showed the lowest number of animals with peptide-specific antibodies in the vaginal mucosa. These results indicate that systemic immunization with LIF and LIFR peptides can elicit an immune response that causes reductions in fertility. Given the high variability of antibodies in the vaginal mucosa, we believed targeting mucosal immunity in the genital tract could boost the contraceptive effect.

Table 2. Contraceptive Effect of LIF/LIFR Peptide Systemic Followed by Mucosal Immunization

Antigen	Systemic Immunization (i.m./s.c.)				Mucosal Immunization (i.n./i.m.)			
	Animals (n)	Pups born (n)	Pups born/animal (mean $\pm$ SEM)	Fertility reduction (%)	Animals (n)	Pups born (n)	Pups born/animal (mean $\pm$ SEM)	Fertility reduction (%)
PBS	12	92	7.7 $\pm$ 0.48 [†]	---	10	80	8.0 $\pm$ 0.52 [†]	---
LIF peptides	12	68	5.7 $\pm$ 1.25*	26.00%	6	31	5.2 $\pm$ 1.51 ^{†*}	32.6%
LIFR peptides	12	45	3.8 $\pm$ 1.27*	50.60%	10	39	3.9 $\pm$ 0.95*	49.2%
LIF + LIFR peptides	10	51	5.1 $\pm$ 1.05*	33.80%	1	3	3.0 $\pm$ 0.00	60.9%

[†] * Values with different superscripts are statistically significant ($P \leq 0.05$), and with the same superscript are statistically non-significant ($P > 0.05$).

Figure 3. Antibody titers in sera and vaginal washings of mice vaccinated systemically against LIF and LIF-R peptides. Female Balb/C mice were immunized i.m. or s.c. with LIF peptides (LIFp), LIF-R peptides (LIF-Rp) or a combination of both along with Freund's adjuvant (CFA for initial injections and IFA for subsequent boosts). IgG titers were analyzed in the serum (A) and vaginal washings (B) by ELISA and expressed as SD units. SD units ≥ 3 were considered positive. The horizontal dotted line (---) indicates 3 SD units.

Antibody Response and Contraceptive Effect of Mucosal Immunization

In an attempt to boost the antibody response in the vaginal mucosa, we targeted the mucosal immune system by immunizing intranasally (i.n.) along with i.m. The same female mice vaccinated previously were boosted twice with an i.n. vaccine of peptide conjugated to cholera toxin B subunit (CTB) along with the i.m. immunization. The vaginal washings of these mice showed positive antibody reactivity showing high IgG and relatively high IgA titers (Fig. 4A-B). All animals immunized with LIF peptides showed peptide-specific IgG in the vaginal mucosa while 67% had peptide-specific IgA. Of those animals immunized with LIFR peptides, 40% and 70% of animal showed peptide-specific IgG and IgA, respectively, in the vaginal mucosa. This result was much greater than that seen after systemic immunization alone, where only 8% of mice showed IgG in the vaginal mucosa. Animals immunized with LIF and LIFR peptides showed peptide-specific IgG, but not IgA, in the vagina mucosa.

Reductions in fertility were again observed by the reduction in litter size of the vaccinated groups (Table 2). Contraceptive effects were again decreased after mucosal immunization when compared to control mice. When comparing the initial fertility effect after systemic immunization to the effect after the addition of mucosal immunization, the contraceptive effect was similar. While significant data for some groups could not be obtained due to the unexpected mortality of several animals, the contraceptive effect did not appear to be any different when the vaccine was targeted to mucosal surfaces.

Figure 4. Antibody titers in vaginal washings of mice with the addition of intranasal vaccination against LIF and LIF-R peptides. Female Balb/C mice were boosted i.n. and i.m. with LIF peptides (LIFp), LIF-R peptides (LIF-Rp) or a combination of both. Vaginal washings were analyzed for peptide-specific IgG (A) and IgA (B) by ELISA and expressed as SD units. SD units ≥ 3 were considered positive. The horizontal dotted line (---) indicates 3 SD units.

A second trial was conducted to confirm if i.n. combined with i.m. immunization would be the best route for delivery of the LIF/LIFR peptide vaccine. In this trial, mice were given a series of immunizations i.n. and i.m. as described previously of either PBS or a combination of LIF and LIFR peptides. After the final boost, antibody titers in the serum and vaginal mucosa were analyzed for peptide-specific IgG and IgA. 93% and 27% of vaccinated animals showed IgG and IgA in the serum, respectively (Fig. 5A-B). IgG and IgA were detected in the vaginal mucosa of 53% and 67% of vaccinated mice, respectively (Fig. 5C-D). Antibody production in these animals was similar to that seen in the previous trials.

The effect of i.n./i.m. immunization on fertility was tested as described in the previous trial. While the vaccinated group showed a reduction in fertility of 29%, this reduction did not appear to be greater than the effect seen after systemic immunization alone (Table 3). These results suggest that systemic immunization is just as effective as mucosal immunization at eliciting a protective antibody response that reduces fertility in mice.

Table 3. Effect of LIF/LIF-R Peptide Vaccine Administered Intranasally and Intramuscularly

<i>Antigen</i>	<i>Animals (n)</i>	<i>Pups born (n)</i>	<i>Pups born/animal (mean ± SEM)</i>	<i>Fertility reduction (%)</i>
PBS	10	70	7.0 ± 0.86 [†]	---
LIF + LIFR peptides	11	55	5.0 ± 0.76*	28.6%

[†] * Values with different superscripts are statistically significant ($P \leq 0.05$)

Figure 5. Antibody titers in sera and vaginal washings of mice immunized mucosally against LIF and LIFR peptides. Female Balb/C mice were immunized i.n. and i.m. with a combination of both LIF and LIFR peptides. Serum was analyzed for peptide-specific IgG (A) and IgA (B). Antibodies in the vaginal washings, IgG (C) and IgA (D) were also analyzed. Antibodies were detected by ELISA and expressed as SD units. SD units ≥ 3 were considered positive. The horizontal dotted line (---) indicates 3 SD units.

CHAPTER 2:

Generation of antibodies in various regions of the genital tract

Materials and Methods

Animals

CD-1 8-10 week old virgin female mice were used for immunization in this study. Animals were maintained by West Virginia University Office of Laboratory and Animal Resources personnel. All experimental protocols used in this study were approved by the West Virginia University Animal Care and Use Committee (WVU-ACUC).

Vaccine preparation

For i.m. immunizations, the carrier proteins used were keyhole limpet hemocyanin (Calbiochem, San Diego, CA), chicken γ -globulin (Calbiochem), chicken ovalbumin (Calbiochem) and bovine serum albumin (Sigma-Aldrich, St. Louis, MO). Peptides were conjugated to these carrier proteins at a 1:1 ration using the heterobifunctional reagent MBS (Pierce Biotechnology) using a previously described procedure [19]. Peptides were conjugated to CTB (List Biological Labs) for i.n. immunizations using a two step glutaraldehyde (Sigma-Aldrich) procedure described previously [20] or using MBS. Peptides were combined with the CTB at a 5:1 ratio. CTB-peptide conjugates were dialyzed in phosphate buffered saline (PBS) for 48 hours using the Slide-A-Lyzer dialysis cassette system (Pierce Biotechnology).

Immunization

Mature, virgin CD-1 female mice were immunized i.m. and i.n. against the LIF peptide-carrier vaccine, the LIF receptor peptide-carrier vaccine, a combination of LIF and LIFR peptide-carrier vaccines, or PBS. 11-12 mice were immunized in each experimental group. For i.m. immunizations, each animal received 50 μ l injections containing 50 μ g of each peptide emulsified with Freund's adjuvant (Sigma-Aldrich). For i.n. administration, each animal received 10-15 μ l per nostril containing a total of 50 μ g of each peptide conjugated to CTB. The final

boost was given i.m., i.n. and also intravaginally (i.vag.). Each animal was administered 20 μ l i.vag. containing a total of 50 μ g of each peptide conjugated to CTB. Two weeks after the final immunization, mice were euthanized for sample collection and antibody analysis.

For passive transfer experiments, antibodies to all the LIF and LIFR peptides were developed in the rabbit model (Antagene, Inc, Sunnyvale, CA). For each peptide antibody, 2-3 female CD-1 mice were injected i.p. with 300 μ l of rabbit anti-sera. Mice were euthanized 6-8 days following immunization for antibody analysis.

Analysis of antibodies in the blood and genital tract

After a series of immunizations, mice were euthanized by CO₂ asphyxiation for sample collection and antibody analysis. Blood was collected through the saphenous vein prior to euthanization. Vaginal washings were collected by washing the vagina with 50 μ l of PBS. The uterus and oviduct from these mice were then removed. The uterus was flushed with 200 μ l of PBS while oviduct was macerated in 200 μ l of PBS.

The presence and titers of antibodies were analyzed in these samples using the enzyme-linked immunosorbent assay (ELISA), as described earlier [19, 21]. Each well was coated overnight at 4°C with 4 μ g peptide. Sera was diluted 1:40 and genital tract samples 1:20 in 0.05% Tween in PBS (PBS-T) containing 3% BSA. Alkaline phosphatase conjugated anti-mouse IgG (Sigma-Aldrich) was diluted 1:800 in PBS-T containing 3% BSA. The plates were developed with substrate solution (1 mg/ml *p*-nitrophenyl phosphate diluted in 0.05 M carbonate buffer, pH 9.6 with 1.0 mM MgCl₂) and the reaction was read at 405 nm. Absorbance readings were converted to standard deviation units by using the following formula: SD units = absorbance (test) - mean absorbance (control group)/standard deviation of the control group. The test samples with SD units greater or equal to 3 were considered as having a positive reaction with a peptide.

Statistical analysis

Correlations between the antibody titers (SD units) in different region of the genital tract as well as in the serum were analyzed by linear regression.

Antibody Generation in the Genital Tract After Active Immunization

Mice were immunized i.n./i.m., as described in the second trial. Rather than mating them to determine the effect of vaccination on fertility, the mice were euthanized to determine if peptide-specific antibodies were generated in different regions of the genital tract. Samples were analyzed for presence of antibodies to each individual peptide. Animals showed significant IgG titers in the genital tract specific to all peptides (Fig. 6). All immunized animals revealed significant amounts of peptide-specific IgG in the oviduct compared to controls. Over 90% of immunized animals had high IgG titers in the vaginal washings while uterine flushings had a significant, but much lower number of animals (~57%) positive for peptide-specific IgG. These results suggest that the i.n./i.m. immunization strategy is sufficient for eliciting a peptide-specific immune response in the genital tract. We also saw that some peptides may be better at eliciting a systemic response (higher titers in the serum), while others elicit a better mucosal response (higher titers in the genital mucosa).

Correlations among antibody titers were calculated to determine if antibodies in the different regions of the genital tract correlated to high titers in the sera. In general, higher titers in the genital tract appeared to be correlated to titers in the sera, especially in the oviduct (data not shown). This brings up the possibility that antibodies in the genital tract may not only be locally produced and may be a result of circulating antibodies passively transudating to the genital tract.

Figure 6. LIF/LIFR peptide-specific antibodies in different areas of the genital tract following immunization. Female CD-1 mice were immunized i.n. and i.m. with LIF and LIFR peptides ($n=12$ for LIF peptides and $n=11$ for LIFR peptides). Antibodies to each individual peptide in the oviduct (top panel), uterine flushings (second panel), vaginal washings (third panel) and serum (bottom panel) were analyzed by ELISA and converted to SD units. SD units ≥ 3 were considered positive results. The horizontal dotted line (---) indicates 3 SD units.

Dispersal of Antibodies into the Genital Tract After Passive Immunization

To determine if antibodies are capable of passive transudation into different regions of the genital tract from the circulation, mice were injected intraperitoneally (i.p.) with rabbit anti-LIF/LIFR peptides and antibodies in the oviduct, vaginal mucosa and serum were analyzed. One week after injection, about 57% of animals showed antibodies percolating to the oviduct and vaginal mucosa (Fig. 7). Over 85% of these animals showed that these antibodies remained in circulation. These results confirm that the presence of antibodies in the genital tract may be due to local production and/or passive transudation of circulating antibodies to these regions.

Figure 7. LIF/LIFR peptide-specific antibodies in different areas of the genital tract following passive transfer. Female CD-1 mice were injected i.p. with sera from rabbits immunized with LIF and LIFR peptide antibodies ($n=2-3$ mice per peptide antibody). Antibodies to each individual peptide in the oviduct (top panel), vaginal washings (middle panel) and serum (bottom panel) were analyzed by ELISA and converted to SD units. SD units ≥ 3 were considered positive results. The horizontal dotted line (---) indicates 3 SD units.

CHAPTER 3: Duration of Peptide-Specific Antibodies

Materials and Methods

Animals

Balb/C female mice from our first immunization trial were used in this study. The Balb/C females were kept for approximately 1 year after their final immunization and were maintained by West Virginia University Office of Laboratory and Animal Resources personnel. All experimental protocols used in this study were approved by the West Virginia University Animal Care and Use Committee (WVU-ACUC).

Analysis of antibodies in the blood and genital tract

Mice were euthanized 11 months after their final immunization by CO₂ asphyxiation for analysis of antibodies in the genital tract. Blood was collected through the saphenous vein prior to euthanization. Vaginal washings were collected by washing the vagina with 50 µl of PBS. The uterus and oviduct from these mice were then removed. The uterus was flushed with 200 µl of PBS while oviduct was macerated in 200 µl of PBS.

The presence and titers of antibodies were analyzed in these samples using the enzyme-linked immunosorbent assay (ELISA), as described earlier [19, 21]. Each well was coated overnight at 4°C with 4 µg peptide. Sera was diluted 1:40 and genital tract samples 1:20 in 0.05% Tween in PBS (PBS-T) containing 3% BSA. Alkaline phosphatase conjugated anti-mouse IgG (Sigma-Aldrich) and IgA α-chain (Sigma-Aldrich) were diluted 1:800 and 1:400, respectively, in PBS-T containing 3% BSA. The plates were developed with substrate solution (1 mg/ml *p*-nitrophenyl phosphate diluted in 0.05 M carbonate buffer, pH 9.6 with 1.0 mM MgCl₂) and the reaction was read at 405 nm. Absorbance readings were converted to standard deviation units by using the following formula: SD units = absorbance (test) - mean absorbance (control group)/standard deviation of the control group. The test samples with SD units greater or equal to 3 were considered as having a positive reaction with a peptide.

Antibodies in the Sera and Genital Tract 11 Months After Immunization

A few mice from each group of the first trial were kept for 11 months to examine the reversibility of the immune response. Eleven months after the last immunization, mice were euthanized and antibodies were analyzed in the serum, vagina, uterus and oviduct. All of the control mice showed no peptide-specific IgG or IgA in the serum or genital tract (SD units < 3). All vaccinated mice had peptide-specific IgG in the serum and oviduct (SD units > 3), while 4 of 5 showed IgG in the uterus (SD units >3). None of the mice had peptide-specific IgG in the vaginal washings. In contrast, peptide-specific IgA was not detected in the serum or any part of genital tract (SD units < 3). The fertility of these animals could not be examined as these mice were too old to mate. These findings indicate that the antibodies generated after immunization are long-lasting. It is highly unlikely that these residual antibodies will affect fertility.

CHAPTER 4:

***In vitro* analysis of peptide antibody neutralization of LIF/LIFR binding**

Materials and Methods

Cell culture and proliferation assays

BA/F3 mLIFR-mgp130 cells, pro-B-cells transfected with mLIFR and mgp130 cDNA [22], were maintained in RPMI 1640 (Invitrogen, Carlsbad, California) supplemented with 10% FBS (Invitrogen), 50 U/ml penicillin-50 µg/ml streptomycin (Invitrogen) and 40 ng/ml recombinant mouse LIF (eBioscience, San Diego, CA). These cells have been shown, in previous studies, to only proliferate when the culture medium is supplemented with LIF. Other IL-6 family cytokines can also mimic the function of LIF in this cell line, namely IL-11 and IL-6. Recombinant human LIF is capable of reacting with murine LIFR complex to cause proliferation of BA/F3 mLIFR-mgp130. A rabbit anti-human LIF antibody was able to neutralize the LIF activity in these conditions [23].

For antibody neutralization assays, 2 µl of each rabbit anti-LIF/LIFR peptide sera or pre-immune control sera (Antagene, Inc.) was incubated for 2h at 37°C with 40 ng recombinant mLIF. A LIF polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was also included as a positive control using the same volumes as the rabbit sera. This antibody was generated against human LIF residues 24-203 and is reactive with mouse, rat and human LIF. Cells were plated in 24 well culture plates, 500 µl per well. Peptide antibody-neutralized LIF was then added to each well. Positive controls were cultured in the presence of LIF with no antibody and negative controls were cultured without LIF or antibody. Proliferation was measured using 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) (Invitrogen) as described previously [23, 24]. After 72 h incubation at 37°C and 5% CO₂ with or without neutralizing antibody, 50 µl of MTT was added to each 500 µl culture and allowed to incubate for 4h. 250 µl of 10% SDS/0.01 M HCl was then added and the absorbance (A₅₇₀) was determined after overnight incubation at 37°C.

Statistical analysis

The significance of the difference in proliferation between each of the antibody treated groups and the control group (with the addition of LIF) was analyzed by an unpaired Student T-test as well as an ANOVA one-way analysis of variance. The follow-up Dunnett multiple comparisons test was used to examine the pair-wise differences. A *P*-value of <0.05 was considered significant.

Neutralization of LIF Bioactivity

The results of our *in vivo* studies in the mouse model indicate that LIF/LIFR peptide antibodies are capable of neutralizing the biological function of LIF resulting in reduced fertility of female mice. To verify this finding, *in vitro* studies were conducted to determine if these antibodies were capable of neutralizing LIF biological function in a cell culture model. BA/F3 mLIFR-mgp130 cells express the murine LIFR complex and require LIF supplementation in the culture media to proliferate (Fig. 8A). These cells were cultured in the presence of or without LIF and proliferation was measured over the course of 4 days by MTT assay. The concentration of cells at the start of growth was approximately 0.4 million cells/ml. This concentration increased 2.5 fold in the presence of LIF to a concentration of approximately 1 million cells/ml after 72 hours. By 88-93 hours, the concentration had increased to 5 times the starting concentration. This proliferation was less marked in cells cultured without LIF. Addition of LIF-neutralizing antibody has been shown to prevent the proliferation of these cells [23]. This system was used to determine if our peptide-antibodies were capable of preventing proliferation in the same manner.

Sera from LIF/LIFR peptide-immunized rabbits was used to neutralize LIF and then added to the BA/F3 mLIFR-mgp130 culture. Proliferation was assessed using MTT. The LIF positive controls demonstrated a 2-fold increase in proliferation compared to the LIF negative

controls. Addition of LIF/LIFR peptide antibodies significantly reduced the proliferation of the BA/F3 mLIFR-mgp130 cells (Fig. 8B). The LIF-Rp1 and LIF-Rp2 antibody treatments showed the most significant reductions compared to both cells cultured with LIF without antibody treatment, while the other peptide antibodies showed less marked reductions. Pre-immune sera was tested as a control to rule out the possibility of serum-toxicity. The control sera showed no significant reductions in cell proliferation, as expected. These results indicate that the LIF/LIFR peptide antibodies were capable of neutralizing the biological function of LIF *in vitro*.

Figure 8. Neutralization of LIF bioactivity *in vitro*. (A) BA/F3 mLIFR-mgp130 cells were cultured in the presence of LIF or without LIF. Cell proliferation was analyzed by MTT assay over the course of 96 hours. Results are expressed as the absorbance at 570nm. (B) Serum from rabbits immunized with LIF/LIFR peptides was used to neutralize recombinant mouse LIF (2h at 37°C). Each peptide antibody is indicated. A polyclonal LIF antibody was tested as a positive control (Poly-LIF) and pre-immune rabbit serum was included as an antibody control (Control). Antibody-neutralized LIF was then added to BA/F3 mLIFR-mgp130 cultures. Controls were cultured in the presence of LIF with no antibody and without LIF or antibody. After 72 h, proliferation was analyzed by MTT cell proliferation assay. Results are expressed as fold increase compared to the negative control. Results are representative of two experiments. The difference between the means of LIF + and LIF – cultures was statistically significant ($P < 0.01$). When compared to the LIF + culture, all the antibody-neutralized cultures were all found significant by an unpaired student's T test ($P < 0.05$). Using ANOVA one-way analysis of variance followed by a Dunnett multiple comparisons test, the LIF-Rp1 and LIF-Rp2 antibody-neutralized cultures were found to be statistically significant ($P < 0.05$).

DISCUSSION

The present study investigated the ability of peptide vaccines based upon LIF and its receptor to elicit an immune response targeting the LIF/LIFR interaction and reduce fertility in mice. LIF has been shown in previous studies to be required for successful blastocyst implantation in mice. A non-functional LIF mouse knockout showed no signs of implantation [6]. Blocking the binding of LIF to its receptor by injecting mice with a high affinity antagonist was able to block implantation completely [7]. Our results have provided further evidence of the requirement of the LIF/LIFR interaction for successful pregnancy in mice. Impairing the interaction with LIF and LIFR peptide-specific antibodies resulted in reduced fertility in vaccinated mice. Targeting LIF for immunocontraceptive purposes has not previously been attempted.

Immunization with the selected peptides allowed us to generate LIF- and LIFR-specific antibodies that targeted specific regions within each protein essential for binding. These antibodies were present in both the circulation as well as locally in the genital tract. Intranasal immunization has been shown to elicit both circulating IgG and mucosal IgA responses in the genital tract and appears to be the most effective route of mucosal immunization for providing genital tract immunity [25-27]. We chose to investigate the effect of i.n. administration of LIF/LIFR peptides vaccines composed of peptides conjugated to CTB. CTB has been shown to elicit high mucosal responses in the genital tract in many previous studies, [20, 21, 28-30]. Immunization of mice i.n. seemed to increase the antibody response in the genital tract, as shown by an increase in peptide-specific antibodies in the vaginal mucosa after i.n. administration. Antibody production in these areas could be due to local mucosal antibody production or due to percolation of these antibodies from the circulation to the genital tract. On the other hand, the i.n. immunizations did not seem to boost the contraceptive effect of the

vaccine. Although we expected the i.n./i.m. vaccination strategy to further enhance the contraceptive effect, reductions were similar to those seen after the systemic immunization.

Not only did the LIF/LIFR peptide antibodies block LIF function *in vivo* by reducing fertility, we demonstrated that the antibodies were able to neutralize biological function in an *in vitro* model. In this model, all LIF/LIFR peptide antibodies demonstrated marked reductions in LIF-dependent proliferation of BA/F3 mLIFR-mgp130 cells. LIFR peptide antibodies showed reduced bioactivity that appeared slightly greater than the LIF peptide antibodies. This *in vitro* effect correlated to the greater contraceptive effect seen in mice immunized with LIFR peptides in our first trial. We predict this is due to a lower abundance of cell-bound LIFR in respect to secreted/supplemented LIF in the system.

Although the experiments discussed here have been limited to studies in mice, the requirement for LIF in successful pregnancy extends to many other species, most importantly, humans. LIF mRNA has been shown to peak in the human endometrium at the time of implantation [10], indicating a possible role in implantation. Although its requirement is difficult to study in women, low levels of LIF have been shown to be associated with infertility in some women [11, 12]. Studies on women with unexplained infertility and endometriosis revealed LIF gene mutations that correlate to poor *in vitro* fertilization outcome [13]. These reports suggest an important role of LIF in humans that is similar to that seen in mice and other species.

A CV based upon LIF could meet many of the prerequisites of an ideal contraceptive. By developing a vaccination strategy that targets mucosal immunity, antibodies would be localized to the genital tract. These antibodies can prevent the biological function of LIF *in vivo* and prevent successful implantation of the blastocyst. Whether or not the vaccine is reversible could not be determined. The antibodies remaining after immunization did appear to be long-lasting. Given all the previous studies indicating LIF's requirement for implantation in mice, and the likely possibility that the same is true in humans, LIF appears to be a promising target for CV development.

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APPENDIX

Table A1. Chemokines and Growth Factors Involved in the Establishment of Pregnancy

	Protein	Size	Human Gene	Role	Species
Chemokines	CCL-2 (MCP-1)	~11 kDa	<i>CCL2</i>	recruits monocytes, macrophages and T cells in the endometrium [31-34]	human/mouse
	CCL-3 (MIP1 α)	7.9 kDa	<i>CCL3</i>	recruits macrophages[35]	human/mouse
	CCL-4 (MIP-1 β)	7.62 kDa	<i>CCL4</i>	recruits macrophages and NK cells; promotes trophoblast migration [36, 37]	human
	CCL5 (RANTES)	8 kDa	<i>CCL5</i>	recruits macrophages; high levels negatively affect fertilization [31, 34, 35]	human/mouse
	CCL-7 (MCP-3)	8.5 kDa	<i>CCL7</i>	recruits macrophages and NK cells; implantation requires a downregulation [37, 38]	mouse
	CXCL1 (GRO1; KC)	~11 kDa	<i>CXCL1</i>	upregulates the inflammatory response [35, 39]	human/mouse
	IL-8 (CXCL8)	8.5 kDa	<i>IL8</i>	regulates expression of inflammatory response genes [39, 40]	human
	CX3CL1 (fractalkine)	90 kDa	<i>CX3CL1</i>	recruits macrophages and NK cells; promotes trophoblast migration; regulates gene expression for adhesion [36, 41, 42]	human
EGF Family	Amphiregulin (AREG)	9.5-16.5 kDa	<i>AREG</i>	regulated by LIF, important in implantation [43, 44]	mouse
	Epidermal growth factor (EGF)	~6 kDa	<i>EGF</i>	stimulates trophoblast migration/invasion [45, 46]	human/mouse
	Heparin binding EGF-like growth factor (HB-EGF)	22 kDa	<i>HB-EGF</i>	regulated by LIF; promotes development of blastocyst, motility, attachment and invasion [43, 47]	human/mouse
	Transforming growth factor α (TGF α)	17 kDa	<i>TGFA</i>	increases the rate of blastocoel expansion [48]	mouse
Growth Factors	Acrogranin/progranulin	68 kDa	<i>GRN</i>	promotes blastocyst hatching, adhesion and outgrowth [49, 50]	mouse
	Basic fibroblast growth factor (FGF2, bFGF)	18-22 kDa	<i>FGF2</i>	prepares blastocyst for migration [51]	mouse
	Connective tissue growth factor (CTGF)	~38 kDa	<i>CTGF</i>	regulates uterine function [52, 53]	human/mouse
	Hepatocyte growth factor (HGF)	78 kDa	<i>HGF</i>	regulates cytotrophoblast differentiation and depth of invasion [54]	human
	Platelet-derived growth factor (PDGF-A)	16 kDa	<i>PDGFA</i>	promotes trophoblast outgrowth [55]	mouse
	Prokineticin 1 (PROK1)	9.7 kDa	<i>EGVEGF</i>	promotes expression of implantation-related genes (i.e. LIF) [56, 57]	human
	Vascular endothelial growth factor (VEGF-A)	45 kDa	<i>VEGFA</i>	maintains corpus luteum[58]	human

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Table A2. Cytokines Involved in the Establishment of Pregnancy

	Protein	Size	Human Gene	Role	Species
Interleukins	IL-1 α	18 kDa	<i>IL1A</i>	induces changes for adhesion and invasion [59, 60]	human/mouse
	IL-1 β	17.5 kDa	<i>IL1B</i>	induces changes for adhesion and stimulates IL-8 production [59, 61, 62]	human/mouse
	IL-1 receptor antagonist (IL-1Ra)	17 kDa	<i>IL1RN</i>	prevents adhesion [63]	mouse
	IL-6	26 kDa	<i>IL6</i>	stimulates leptin secretion and metalloproteinase activity [64]	human/mouse
	IL-10	18 kDa	<i>IL10</i>	decreases cytotoxic activation of uNK cells [65]	human/mouse
	IL-11	23 kDa	<i>IL11</i>	receptor signaling required for decidua development [66-68]	human/mouse
	IL-12	75 kDa	<i>IL12A/IL12B</i>	Immunomodulatory [69]	human
	IL-15	18 kDa	<i>IL15</i>	regulates IL-8 expression and uNK cells [37]	human
	IL-18	18 kDa	<i>IL18</i>	increases perforin expression and cytolytic potentials of uNK cells [70]	human
	IL-23	21 kDa	<i>IL23A/IL12B</i>	immunomodulatory, regulates IL-8 expression [71, 72]	human/mouse
	IL-27	27 kDa	<i>EBI3/IL30</i>	Immunomodulatory [71]	mouse
	leukemia inhibitory factor (LIF)	26 kDa	<i>LIF</i>	regulates expression of genes important in implantation [44, 73]	human/mouse
CSFs	Granulocyte colony-stimulating factor (G-CSF)	~19 kDa	<i>CSF3</i>	recruits macrophages to the uterus to prepare it for implantation [74]	mouse
	Macrophage colony-stimulating factor (M-CSF)	~36 kDa	<i>CSF1</i>	recruits macrophages to the uterus to prepare it for implantation [31, 74]	mouse
	Granulocyte macrophage CSF (GM-CSF)	14.4 kDa	<i>CSF2</i>	enhances proliferation and viability of blastomeres [75, 76]	mouse
TGF β Superfamily	Activin A	24-28 kDa	<i>INHBA</i>	promotes decidualization; prevents activation of T cells [77-79]	mouse
	Macrophage inhibitory cytokine (MIC-1)	25 kDa	<i>GDF15</i>	regulates trophoblast migration/invasion and decidualization [37, 80]	human
	Transforming growth factor β 1 (TGF β 1)	25 kDa	<i>TGFB1</i>	regulate embryo development [81, 82]	human/mouse
	Transforming growth factor β 2 (TGF β 2)	25 kDa	<i>TGFB2</i>	regulate embryo development [81]	human
	Transforming growth factor β 3 (TGF β 3)	25 kDa	<i>TGFB3</i>	promotes a regulatory T cell response [83]	mouse
TNF Family	Tumor necrosis factor α (TNF α)	25 kDa	<i>TNF</i>	immunomodulatory, has deleterious effects at high levels [84-86]	human/mouse
	tumor necrosis factor-like weak inducer of apoptosis (TWEAK)	17 kDa	<i>TWEAK</i>	controls cytotoxicity, possibly through regulation of IL-15 and IL-18 [71, 87]	human/mouse
Hormones	Growth hormone (GH)	22 kDa	<i>GH1/GH2</i>	affects quality of embryo and fertilization rate [88]	human
	Prolactin (PRL)	24 kDa	<i>PRL</i>	promotes decidualization [89]	human/mouse

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Table A3. Integrins and Other Factors Involved in the Establishment of Pregnancy

	Protein	Size	Human Gene	Role	Species
Integrins	$\alpha 4\beta 1$ integrin	~280 kDa	<i>ITGA4/ITGB1</i>	important in implantation and decidualization [90]	human
	$\alpha 5\beta 1$ integrin	~265 kDa	<i>ITGA5/ITGB1</i>	essential for the migration of extravillous trophoblasts (IFG-I-induced) [91]	human
	$\alpha 9\beta 1$ integrin	~230 kDa	<i>ITGA9/ITGB1</i>	important in implantation [54]	human
	$\alpha v\beta 3$ integrin	~230 kDa	<i>ITGAV/ITGB3</i>	involved in EVT migration (IGF-I-induced), important in implantation and decidualization ¹⁸ , [90, 92]	human
Other Factors	Adrenomedullin	6 kDa	<i>ADM</i>	involved in invasion and pinopode formation [93-95]	human/mouse
	α -fetoprotein	70 kDa	<i>AFP</i>	inhibits the immune response [96]	mouse
	Cochlin (COCH)	~60 kDa	<i>COCH</i>	regulated by LIF; marker for uterine receptivity? [97]	mouse
	Corticotropin-releasing hormone (CRH)	~5 kDa	<i>CRH</i>	promotes implantation by regulating FasL expression [98, 99]	human/mouse
	Cyclooxygenase-2 (COX-2)	72 kDa	<i>PTGS2</i>	synthesizes prostaglandins; required for fertilization, implantation and decidualization [100]	mouse
	Cytosolic phospholipase A2 α (cPLA _{2α})	85 kDa	<i>cPLA2α</i>	provides arachidonic acid for synthesis of PGs by COX2; deficiency results in abnormal spacing and delayed implantation [101]	mouse
	Dickkopf-1 (DKK-1)	~25 kDa	<i>DKK1</i>	required for blastocyst outgrowth and adhesion [102]	mouse
	Glycodelin	28 kDa	<i>PAEP</i>	involved in sperm-oocyte binding and prevention of the inflammatory response [103-105]	human
	Heparan sulfate proteoglycans (HSPG)	>500kDa	n/a	Expressed on the trophectoderm of blastocyst during the attachment phase of implantation [106]	mouse
	Human chorionic gonadotropin (hCG)	37.6 kDa	<i>CGB</i>	responsible for progesterone production and LIF expression; maintains the corpus luteum; also involved in angiogenesis, attachment and immune tolerance [1, 107, 108]	human
	Homeobox A10 (HOXA-10)	~40 kDa	<i>HOXA10</i>	required for decidualization and successful implantation [109-111]	human/mouse
	Homeobox A11 (HOXA-11)	~35 kDa	<i>HOXA11</i>	required for uterine stromal and glandular cell differentiation [112]	mouse
	Immunoresponsive gene 1 homolog (IRG1)	~52 kDa	<i>IRG1</i>	regulated by progesterone and LIF; important for implantation [44, 113]	mouse
	Insulin	5.8 kDa	<i>INS</i>	increases cell proliferation of early stage embryos [114, 115]	mouse
	Insulin-like growth factor I (IGF-I)	7.65 kDa	<i>IGF1</i>	increases number of cells in inner cell mass [116]	mouse
	Insulin-like growth factor II (IGF-II)	7.5 kDa	<i>IGF2</i>	involved in oocyte maturation and development of the embryo to blastocyst stage [117, 118]	human/mouse
	Insulin-like growth factor binding protein 1 (IGFBP-1)	~25 kDa	<i>IGFBP1</i>	limits trophoblast growth and inhibits IGF-I activity [54, 119]	human
	Insulin-like growth factor binding protein 2 (IGFBP-3)	~40 kDa	<i>IGFBP3</i>	upregulated by LIF; involved in oocyte maturation and embryo development [44, 118]	human/mouse
	Interferon-induced 17 kDa protein (ISG15)	17 kDa	<i>ISG15</i>	induced in the endometrium in response to the implanting conceptus; immunomodulatory? [120]	human/mouse
	Leptin	16 kDa	<i>LEP</i>	involved in blastocyst development; mediates the invasiveness of the cytotrophoblast [60, 121, 122]	human/mouse
	Lysophosphatidic acid receptor 3 (LPA3)	40 kDa	<i>LPAR3</i>	regulates uterine receptivity [123, 124]	mouse
	L-selectin	43 kDa	<i>SELL</i>	plays an early role in the homing of leukocytes the uterus, regulating uterine receptivity [125, 126]	human/mouse
	Mucin 1 (MUC-1)	>300 kDa	<i>MUC1</i>	involved in embryo attachment [1, 127]	mouse/human
	Oviduct-specific glycoprotein (OVGP1; MUC-9)	120 kDa	<i>OVGP1</i>	enhances binding of sperm to the zona pellucida [128]	human
	Platelet activating factor (PAF)	~524 kDa	n/a	stimulates early embryo development [129, 130]	human/mouse
	Preimplantation factor (PIF)	0.6-1.8 kDa	n/a	regulates immunity, promotes adhesion and invasion, and regulates apoptotic processes[8, 9]	human
	Prostaglandin E ₂ (PGE ₂)	352 kDa	n/a	involved in the inflammatory response in the endometrium required for implantation [1]	human
	Secreted phosphoprotein 1 (SPP1)	44 kDa	<i>SPP1</i>	allows for attachment to the luminal epithelium; induces focal adhesions [131, 132]	mouse
	Trophinin	69 kDa	<i>TRO</i>	involved in activation of trophectoderm for adhesion [133]	human/mouse

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CURRICULUM VITAE

Angela R. Lemons

112 Orchard Crossings, Morgantown, WV 26505 | 304-641-6050 | anbates@hsc.wvu.edu

OBJECTIVE

To obtain a research position in industry or government that will allow me to design and carry out experiments and possibly supervise research projects. I am a fast learner and am eager and willing to learn new techniques.

EDUCATION

West Virginia University School of Medicine, Morgantown, WV

M.S. in Microbiology, Immunology and Cell Biology

2011

Thesis: "Contraceptive effect of a vaccine targeting leukemia inhibitory factor that is vital for implantation"

Relevant Course Work:

Immunology & Microbial Pathogenesis

Advanced Immunology

Molecular Genetics

Cell Signaling Metabolism

Scientific Writing

Statistics Biomedical Sciences

Seminar -presented once every year

Journal club -presented one every semester

Cumulative GPA – 3.48

Baldwin Wallace College, Berea, OH

B.S. in Biology

2008

Areas of Concentration: Microbiology and Immunology

Graduate of the Honors Program

Minor: Chemistry

Cumulative GPA – 3.50 Cum Laude

Pymatuning Valley High School, Andover, OH

2004

Cumulative GPA – 4.00

AWARDS

Pymatuning Valley High School Valedictorian Scholarship

2004

J. Wendell Marvin Scholarship

2004

Baldwin Wallace Achievement Award

2004-2008

Baldwin Wallace Presidential Scholarship

2004-2008

West Virginia University School of Medicine. Morgantown, WV

Top Presentation at the OB-GYN Annual Research Day

2011

POSITIONS HELD

- Graduate Researcher**, West Virginia University, Morgantown, WV **Aug 2008 - Dec 2011**
Member of the reproductive immunology and molecular biology lab in the department of microbiology, immunology and cell biology developing a contraceptive vaccine targeting leukemia inhibitory factor in mice.
- Laboratory Assistant**, Baldwin Wallace College, Berea, OH **Aug 2006 - May 2008**
Member of the microbiology and immunology laboratory. Assisted with both the microbiology and immunology labs preparing media and other reagents for as well as assisting during the lab sessions.
- Student Researcher**, Baldwin Wallace College, Berea, OH **May 2007 - May 2008**
*Member of the microbiology and immunology laboratory. Conducted a research project expressing and purifying Ada protein from *Brucella abortus* in an *E. coli* system for future analysis of Ada function.*

TEACHING EXPERIENCE

- West Virginia University School of Medicine, Morgantown, WV
Teaching Assistant – to Valerie Watson in “Medical Microbiology” **2010-2011**
Instructed the Medical Laboratory Science laboratory section along with one other teaching assistant, assisted with preparatory work prior to each lab, proctored lab and lecture exams, graded laboratory reports, quizzes and practical exams.

PRESENTATIONS

- Baldwin Wallace College, Berea, OH
Senior Seminar **2008**
Oral Presentation: “Purification of *Brucella abortus* Ada for *in vitro* analysis of function”
- West Virginia University School of Medicine, Morgantown, WV
Van Liere Convocation **2011**
Oral Presentation: “Contraceptive vaccine development using leukemia inhibitory factor that is vital for implantation”
- West Virginia University School of Medicine, Morgantown, WV
OB-GYN Annual Research Day **2011**
Oral Presentation: “Contraceptive vaccine development using leukemia inhibitory factor that is vital for implantation”
- West Virginia University School of Medicine, Morgantown, WV
Van Liere Annual Research Day – Presented a poster **2011**
- West Virginia University School of Medicine, Morgantown, WV
School of Pharmacy Research Day – Presented a poster **2011**

PUBLICATIONS

- “Contraceptive vaccines targeting factors involved in establishment of pregnancy”
American Journal of Reproductive Immunology, 66 (1): 13 – 25 **2011**

SKILLS AND QUALIFICATIONS

I have several years of experience working in a research laboratory and am skilled in the following:

Conjugation techniques	Vaccine development/Immunization
Animal models (mouse)	Antibody analysis (ELISA)
Bacterial transformation	Protein expression in bacterial system
Cell culture	PCR
Primer design	Polyacrylamide gel electrophoresis
Western blot	Agarose gel electrophoresis
Protein quantification assays	Antibody and protein purification

I am also proficient with computers and computer software:

Windows 7/Windows Vista	Microsoft Office (Word, Excel, PowerPoint)
Adobe Photoshop	Sigma Plot
Endnote	DNA sequence assembly/alignment software

REFERENCES

John B. Barnett, Ph.D.
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Professor, Department of Microbiology, Immunology & Cell Biology
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