Accelerated immune senescence and reduced response to vaccination in ovariectomized female rhesus macaques

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Abstract Aging is associated with a general dysregulation in immune function, commonly referred to as “immune senescence”. Several studies have shown that female sex steroids can modulate the immune response. However, the impact of menopause-associated loss of estrogen and progestins on immune senescence remains poorly understood. To help answer this question, we examined the effect of ovariectomy on T-cell homeostasis and function in adult and aged female rhesus macaques. Our data show that in adult female rhesus macaques, ovariectomy

increased the frequency of naïve CD4 T cells. In contrast, ovariectomized (ovx) aged female rhesus macaques had increased frequency of terminally differentiated CD4 effector memory T cells and inflammatory cytokine-secreting memory T cells. Moreover, ovariectomy reduced the immune response (T-cell cytokine and IgG production) following vaccination with modified vaccinia ankara in both adult and aged female rhesus macaques compared to ovary-intact age-matched controls. Interestingly, hormone therapy (estradiol alone or in conjunction with

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progesterone) partially improved the T-cell response to vaccination in aged ovariectomized female rhesus macaques. These data suggest that the loss of ovarian steroids, notably estradiol and progesterone, may contribute to reduced immune function in post-menopausal women and that hormone therapy may improve immune response to vaccination in this growing segment of the population.

Keywords Aging · Immune senescence · Ovariectomy · T cells · Vaccine · Estrogen · Progestin

Introduction

Aging is associated with a general decline in immune system function, commonly referred to as ‘immune senescence’. Hallmarks of immune senescence include loss of naïve T cells and the accumulation of differentiated memory T cells, decreased ratio of CD4:CD8 T cells, decreased frequency of B cells (Larbi et al. 2008), and a heightened inflammatory state which is believed to contribute to the onset or exacerbation of several age-related morbidities such as atherosclerosis, Alzheimer’s disease, and sarcopenia (De Martinis et al. 2005). The most profound impacts of immune senescence are increased susceptibility to infectious diseases and decreased efficacy of vaccination (McElhaney and Effros 2009; Weinberger et al. 2008). Despite intense research, we do not yet fully understand the contribution of other age-related physiological changes to immune senescence. Several studies have shown that female sex hormones modulate immune response during infection and autoimmune diseases (Ansar Ahmed and Talal 1989; Gillgrass et al. 2003; Gillgrass et al. 2005; Kaushic et al. 1998; Marx et al. 1996; Smith et al. 2000; Trunova et al. 2006). However, the impact of ovarian steroids loss associated with menopause on immune senescence and the immune response to infection or vaccination remains unknown.

Clinical studies have shown that patients, including pre-menopausal women, who have undergone a total abdominal hysterectomy including a bilateral oophorectomy experience a decrease in the CD4:CD8 T-cell ratio and in B cells numbers (Giglio et al. 1994; Kumru et al. 2004). Interestingly, these changes are reversed by hormone therapy (HT)

(Giglio et al. 1994; Kumru et al. 2004). Additional studies have reported that post-menopausal women (between the age of 52 and 70) taking HT for at least 12 months prior to the study have increased numbers of B cells and better T-cell proliferative responses than post-menopausal women who are not undergoing any HT (Porter et al. 2001). Moreover, post-menopausal women (>48 years and time since menopause >1.6 years) have higher plasma levels of the inflammatory cytokines TNF α and IFN γ compared to pre-menopausal subjects (Deguchi et al. 2001; Vural et al. 2006a; Vural et al. 2006b), and HT results in a decrease in serum level of TNF α (Vural et al. 2006a), IFN γ (Deguchi et al. 2001), and IL-6 (Saucedo et al. 2002). Similarly, ovariectomy increases IL-6 plasma levels in adult female rhesus macaque, and this change is reversed upon estradiol administration (Keller et al. 2001). These observations suggest that the loss of ovarian steroids contributes to immune senescence and that HT might delay some of these changes. Furthermore, some of the age-related chronic diseases exacerbated by menopause, such as osteoporosis, are linked to the age-related accumulation of memory T cells and increased production of inflammatory cytokines (Ginaldi et al. 2005). These observations establish a complex interaction between immune senescence and menopause. However, a major caveat of the clinical studies is the inability to discriminate between the effects of age versus those induced by loss of ovarian steroids. Furthermore, no studies to date have directly investigated the impact of ovarian steroids loss on the immune responses to vaccination in aged post-menopausal women.

Like women, adult female rhesus macaques menstruate and eventually undergo menopause, which is characterized by a similar marked decline in circulating levels of estradiol and progesterone (Downs and Urbanski 2006). To address this question, in the present study we used adult and aged female rhesus macaques to investigate the impact of ovariectomy on T-cell homeostasis and the immune response to vaccination. We found that ovariectomy resulted in a diminished immune response to vaccination in both adult and aged female rhesus macaques. Furthermore, we found that HT improved some aspects of the immune response to vaccination in aged post-menopausal animals.

Materials and methods

Animals

Female rhesus macaques (*Macaca mulatta*) were cared for by the Division of Animal Resources at the Oregon National Primate Research Center in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals. The animals were housed indoors under controlled conditions: 24°C temperature, 12L:12D photoperiods (lights on at 0700 h), and regular meals at 0800 h and 1500 h (Purina High Protein Monkey Chow; Purina Mills Inc., St. Louis, MO, USA) supplemented with fresh fruit and vegetables; fresh drinking water was available ad libitum. All experiments were carried out in accordance with the regulation of the Institutional Animal Care and Use Committee.

Analysis of T-cell homeostasis in Figs. 1 and 2 was conducted using a cross-sectional analysis of blood samples collected from 31 intact adult female rhesus macaques (9–11 years of age) who exhibited normal menstrual cyclicity, 26 ovariectomized (ovx) adult female rhesus macaques (9–12 years old), 40 intact aged female rhesus macaques (18–25 years old), and 16 aged ovx females (18–23 years of age). The duration of ovx ranged from 6 to 36 months prior to the sample collection.

For the vaccination studies, five adult intact females and five adult ovx animals were randomly selected from the above group (Figs. 3, 4, and 5). The aged animals used for the vaccination studies were 21–28 years of age (average age 24 years, two animals were 27 years of age and only one animal was 28 years of age) at the time of the first vaccination. They were ovariectomized 3–4 years prior to the vaccination studies and randomized to HT or control groups. There were four intact females, nine ovx females, and 10 ovx females receiving hormone therapy (HT) either in the form of estradiol alone or combination estradiol + progestin therapy. Animals in the HT group received hormones continuously since ovariectomy. To mimic circulating sex-steroid levels across the menstrual cycle, estradiol was delivered continuously through a subcutaneous Silastic implant, whereas progesterone was administered orally each day for the first 14 days of each month as

previously described (Kohama et al. 1992). In animals receiving estradiol only, estradiol levels were 106 pg/ml on average. In animals receiving combined HT, estradiol levels were 109 pg/ml and progesterone levels were 1.6 pg/ml.

Analysis of T-cell subsets and T-cell response to CD3 stimulation

PBMC were isolated by centrifugation of whole blood over a density gradient (Histopaque; Sigma-Aldrich, St. Louis, MO, USA). To delineate T-cell subsets, PBMC were stained with antibodies directed against CD4, CD8b, CD28, and CD95 to delineate Na, CM, and EM T-cell subsets as described previously (Messiaoui et al. 2006). All antibodies were purchased from Biolegend (San Diego, CA, USA) with the exception of CD8b, which was purchased from Beckman Coulter (Brea, CA, USA). Samples were acquired using the LSRII instrument and analyzed by FlowJo software (TreeStar, Ashland, OR, USA).

To measure T-cell cytokine production in response to CD3 stimulation, PBMC were stimulated with soluble anti-CD3 mAb (1 µg/ml, clone FN18; Invitrogen) in the presence of Brefeldin A (Sigma) to block cytokine secretion. At the end of the incubation, PBMC were stained with antibodies directed against surface molecules CD8, CD4, CD28, and CD95. The cells were then fixed and permeabilized as per manufacturer's recommendation (Biolegend). Finally, the cells were stained with antibodies against IFNγ and TNFα (Biolegend), and analyzed as described above.

Vaccination protocol

MVA virus was propagated in chicken embryonic fibroblast cells (CEF; Charles River Avian Services) and purified by ultracentrifugation over a 36% sucrose gradient as described for other orthopox viruses (Manes et al. 2008). Virus titers were determined by standard plaque assay on CEF cells. Animals were vaccinated by intradermal/intramuscular inoculation of the flank with 10^8 pfu twice at day 0 and day 30. Blood samples were obtained on days 7, 14, and 30 after each immunization. PBMC and plasma were isolated by centrifugation of whole blood over density gradient.

Fig. 1 Effect of ovariectomy on T-cell subset frequency in adult and aged female rhesus macaques. **a** Frequency of naïve (Na), central (CM), and effector memory (EM) CD4 and CD8 T cells was determined using flow cytometry. **b** Frequency of Na, CM, and EM CD8 T cells in adult intact and ovx females. **c**

Frequency of Na, CM, and EM CD4 T cells in adult intact and ovx females. **d** Frequency of Na, CM, and EM CD8 T cells in aged intact and ovx females. **e** Frequency of Na, CM, and EM CD4 T cells in aged intact and ovx females. * $p < 0.05$

Measuring frequency of proliferating T- and B-cell subsets

To measure T-cell proliferative response following MVA vaccination, PBMC were stained with antibodies to

surface markers CD4, CD8b, CD28, and CD95 to delineate Na, CM, and EM T-cell subsets as described above, and with anti-CD20 to detect B cells. The cells were then fixed and the nuclear membrane permeabilized as per the manufacturer's recommendation prior to

Fig. 2 Effect of ovariectomy on IFN γ and TNF α production by T cells. **a** Frequency of CM and EM T cells that secrete IFN γ and TNF α in response to CD3 stimulation was determined by intracellular cytokine staining and flow cytometry. Percentage of responding cells was calculated by adding quadrants 1, 2, and 3. **b** Frequency of IFN γ - and TNF α -

producing CD8 T cells in adult intact and ovx females. **c** Frequency of IFN γ - and TNF α -producing CD4 T cells in adult intact and ovx females. **d** Frequency of IFN γ - and TNF α -producing CD8 T cells in aged intact and ovx females. **e** Frequency of IFN γ - and TNF α -producing CD4 T cells in aged intact and ovx females. * $p < 0.05$

staining with anti-Ki67 (BD Pharmingen, San Diego, CA, USA). Samples were acquired using the LSRII instrument and data analyzed by FlowJo.

Measuring frequency of MVA-specific T cells

To measure frequency of MVA-specific T cells, PBMC were stimulated with vaccinia virus (VV, WR strain) (multiplicity of infection=1), anti-CD3 (positive control),

or media (negative control) overnight after which Brefeldin A was added for an additional 6 h as described previously (Hammarlund et al. 2003). Cells were then harvested and stained with surface antibodies CD4, CD8b, CD28, and CD95 to delineate CM and EM T-cell subsets. The cells were subsequently permeabilized and stained with antibodies directed against IFN γ and TNF α . Samples were acquired using the LSRII instrument and data analyzed by FlowJo.

Fig. 3 Impact of ovariectomy on T-cell proliferation following MVA vaccination. **a** T-cell proliferation was assessed by flow cytometry based on frequency of cells expressing the nuclear protein Ki67, which increases day 7 post-vaccination (*lower row*) compared to day 0 (*top row*). **b, c** Frequency of

proliferating CD8 CM (**b**) and EM (**c**) T cells in adult intact and ovx females. **d, e** Frequency of proliferating CD4 CM (**d**) and EM (**e**) T cells in adult intact and ovx females. Arrow indicates MVA vaccination * $p < 0.05$

Measuring IgG end-point titers

Plasma antiviral IgG titers were measured by ELISA using plates coated with VV-WR viral lysate, which is

recognized by MVA-specific antibodies. Threefold dilutions of plasma obtained at different days post-vaccination were incubated in triplicates VV-WR virus lysate-coated ELISA plates for 1 h prior to

Fig. 4 The frequency of vaccine-specific T cells in intact and ovx adult females. **a** Frequency of MVA-specific T cells was measured using intracellular cytokine staining and flow cytometry. Representative examples of CD8 CM and EM T-cell responses on day 0 and day 42 (14 days after the booster) are shown here. Percentage of responding cells is calculated by adding the frequencies of cells in quadrants (Q) 1, 2, and 3.

b Frequency of MVA-specific CD8 CM in intact and ovx adult females. **c** Frequency of responding EM CD8 T cells in intact and ovx adult females. **d** Frequency of MVA-specific CD4 CM in intact and ovx adult females. **e** The frequency of MVA-specific CD4 EM T cells in intact and ovx adult females. Arrow indicates booster MVA vaccination. No MVA-specific T cells were detected on day 0 (first MVA vaccination) * $p < 0.05$

Fig. 5 B-cell response following MVA vaccination in adult intact and ovx females. **a** Kinetics and magnitude of B-cell proliferation in response to MVA vaccination in intact and ovx

animals. **b** IgG end-point titers in intact and ovx females post-MVA vaccination. Arrow indicates MVA vaccination * $p < 0.05$

washing, staining with detection reagents (HRP-anti-IgG), and addition of chromogen substrate to allow for detection and quantitation of bound antibody molecules. Log-log transformation of the linear portion of the curve was then carried out with 0.1 OD units the cut-off to calculate end-point titers. Each plate always contained a positive control sample that is used to normalize the ELISA titers between assays. Plasma collected prior to vaccination served as the negative control to ensure assay specificity.

Statistical analysis

The data in Figs. 1 and 2 were analyzed using pairwise comparison and Student's *t* test to measure significance. The data in Figs. 3, 4, 5, 6, 7, and 8 were evaluated using a mixed linear model, more specifically repeated-measures analysis of variance with intact or OVX or HT as between-group factor and time points as within-group factor. As with typical repeated-measures experiments, two measurements taken at adjacent times are more highly correlated than two measurements taken several time points apart. Therefore, we used a Bayesian information criterion to determine the optimal correlation within subject. Outcomes were evaluated using contrast *t* tests with false discovery rate adjustment to control the family-wise error rate due to multiple comparisons.

Results

Impact of ovariectomy on the frequency of naïve T cells

We first investigated the impact of ovariectomy on T-cell homeostasis. CD4 and CD8 T cells can be subdivided into three major subsets using antibodies directed against surface markers CD28 and CD95: (1) naïve T cells (Na, CD28+CD95-), (2) central memory T cells (CM, CD28+CD95+), and (3) effector memory T cells (EM, CD28-CD95+) as illustrated in Fig. 1a (Jankovic et al. 2003; Pitcher et al. 2002). We have previously shown that, as described for humans (Pawelec et al. 2009), increasing age results in a decreased frequency of naïve T cells and an accumulation of memory cells, especially EM T cells in rhesus macaques (Jankovic et al. 2003). We therefore compared the frequency of Na, CM, and EM CD4 and CD8 T cells in intact and ovariectomized (ovx) adult and aged female rhesus macaques (Fig. 1).

We found that ovariectomized (ovx) adult animals had a higher frequency of Na CD4 T cells and a concomitantly reduced frequency of CD4 CM (Fig. 1c, * $p < 0.05$) (Fig. 1b). On the other hand, ovx aged female rhesus macaques had similar frequencies of Na CD8 (Fig. 1d) and CD4 (Fig. 1e) T cells as intact cycling aged animals. However, the frequency of CD4 EM cells were higher in ovx animals, whereas the frequencies of CM cells

Fig. 6 T-cell proliferation following MVA vaccination in intact, ovx, and hormone therapy recipient aged female rhesus macaques. T-cell proliferation of CD4 CM (a) and EM (b) or

CD8 CM (c) and EM (d) T cells in intact, ovx, and HT animals. Arrow indicates MVA vaccination

were lower ($*p < 0.05$) (Fig. 1d, e). These data suggest that ovariectomy results in an increased conversion to terminally differentiated T cells in aged ovx female rhesus macaques.

Effect of ovariectomy on inflammatory cytokine production by T cells

We next evaluated the effect of ovariectomy on the frequency of inflammatory cytokine-producing T cells. We previously showed that increased production of inflammatory cytokines by memory T cells following polyclonal stimulation is a hallmark of immune senescence in rhesus macaques (Jankovic et al. 2003; Messaoudi et al. 2006). To that end, we

measured the frequency of CM and EM CD4 and CD8 T cells that secreted IFN γ and TNF α following polyclonal stimulation with anti-CD3 using intracellular cytokine staining (ICS) as shown in Fig. 2a. Although the frequency of CD8 (Fig. 2b) and CD4 CM and EM T cells (Fig. 2c) that secreted these inflammatory cytokines appeared to be slightly lower in ovx adult females compared to intact age-matched females, this difference did not reach statistical significance. In contrast to these findings, the frequencies of IFN γ and/or TNF α producing CD8 (Fig. 2d) and CD4 (Fig. 2e) memory T cells were significantly higher in aged ovx animals compared to age-matched intact females (Fig. 2d, e; $*p < 0.05$).

Fig. 7 Modulation of T-cell responses to MVA by hormone therapy (HT). **a, b** Frequency of MVA-specific CD8 CM and EM T cells in intact, ovx, and HT animals. **c, d** CD4 CM and CD4 EM T-cell response in intact, ovx, and HT animals

following MVA vaccination. * $p < 0.05$ HT versus OVX and HT versus intact in both CD4 CM and EM. Arrow indicated booster MVA vaccination. No MVA-specific T cells were detected on day 0 (first MVA vaccination)

The effect of ovariectomy on the immune response to vaccination in adult females

Overall, it seems that ovariectomy had a positive impact on T-cell homeostasis in adult animals by increasing the frequency of CD4 naïve T cells. On the other hand, ovariectomy appears to accelerate T-cell aging in old animals, as indicated by the frequency of EM CD4 T cells and increased CD8 and CD4 T-cell production of inflammatory cytokines. We therefore investigated whether ovariectomy impacts the generation of the immune response following vaccination. Five adult intact and five adult ovx animals were immunized with 10^8 plaque-forming units (pfu) of modified vaccinia ankara (MVA, a highly attenuated form of vaccinia

virus) intramuscularly twice at a 1-month interval. Following antigen encounter, T cells undergo a dramatic proliferative burst that can be measured by determining the number of T cells expressing the cell cycle-associated nuclear protein Ki67 using flow cytometry. A representative example of Ki67 staining from one animal is shown in Fig. 3a where the frequency of CD4 CM and CD8 CM expressing Ki67 increases on day 7 post-vaccination compared to day 0 (Messaudi et al. 2009). T cells in all four T-cell subsets (CD8 CM, CD8 EM, CD4 CM, and CD4 EM) from intact adult females underwent a robust proliferative burst 7 days after the first immunization and a smaller burst 7 days after the booster dose (35) (Fig. 3b–e). In sharp contrast, T cells from ovx adult

Fig. 8 Changes in the antibody response to vaccination by HT. **a** B-cell proliferation following MVA vaccination in intact, ovx, and HT animals following MVA vaccination. **b** IgG end-point

titers in intact, ovx, and HT animals following MVA vaccination. * $p < 0.05$ intact versus OVX; no significant differences were detected between intact and HT

females showed a significantly reduced T-cell proliferative response on day 7 post-vaccination (Fig. 3b–e). In fact, we did not detect an increase in Ki67+ T cells in ovx females until day 28 or day 35 (Fig. 3b–e). Moreover, in all subsets except the CD4 EM (Fig. 3e), the peak of the proliferative burst never reached that achieved by T cells in intact animals.

We also measured the frequency of MVA-specific T cells at different time points after vaccination by measuring the frequencies of T cells producing IFN γ and/or TNF α following stimulation with MVA by ICS (Hammarlund et al. 2005). A representative example is shown in Fig. 4a, which illustrates that a robust IFN γ or TNF α response can be detected following but not before vaccination in response to MVA antigens. This assay specifically detects MVA-specific T cells since stimulation of PBMCs with another virus (simian varicella virus) that was not used in the vaccination does not result in IFN γ and/or TNF α production (data not shown). Overall, intact adult females generated a greater frequency of MVA-specific T cells at almost every time point examined in adult ovx females compared to intact females in all four T-cell subsets (Fig. 4).

We then investigated whether ovariectomy resulted in an altered B-cell response to vaccination. Similar to T cells, B cells undergo a robust proliferative burst following vaccination or infection that can also be measured by flow cytometry using Ki67 as a marker. B cells in intact animals underwent a proliferative burst on day 14 following primary MVA vaccination and a

second burst 7 days after the booster vaccination (day 35) (Fig. 5a). In contrast, proliferation of B cells in ovx female rhesus macaques was significantly delayed compared to intact animals and was only detected 28 days after primary vaccination (Fig. 5a). This delay in proliferation suggests a delay in antibody production. To test this hypothesis, we measured the MVA-specific IgG antibody response using end-point ELISA. MVA-specific IgG titers were negligible following primary vaccination (Fig. 5b), most likely due to the attenuated nature of MVA. However, following booster vaccination, IgG titers were significantly lower in ovx animals compared to intact animals (Fig. 5b). These observations indicate that the immune response to vaccination is compromised in ovx females.

Modulation of the immune response to vaccination in aged macaques by hormone therapy

Given that ovariectomy resulted in a dampened T- and B-cell response to MVA vaccination in adult ovx female rhesus macaques, we next investigated whether HT could improve immune response to vaccination in aged ovx female rhesus macaques. For these studies, three groups of aged female rhesus macaques were immunized with 10^8 pfu MVA intramuscularly twice at a 1-month interval: (1) intact cycling females (4), (2) ovx females (9), and (3) ovx females that have been continuously receiving HT (10). These animals were on average 24 years of age at the time of vaccination and had been ovariectomized 3–4 year prior to these studies. The HT

group consisted of a mixture of animals: five received only estrogen, in the form of subcutaneous estradiol implants, as previously described (Kohama et al. 1992); five received estradiol implants as well as micronized progesterin, administered orally for 14 consecutive days every 28 days. All animals received their respective hormone treatment continuously since ovariectomy.

Following MVA vaccination, we measured the magnitude and kinetics of the T-cell response. As expected, aged animals generated a lower T-cell proliferative burst than adult animals as illustrated by the lower frequency of Ki67+ T cells following vaccination (Fig. 6 compared to Fig. 3). T-cell proliferation was detected 7 days after primary and then again 7 days after booster vaccination (day 37) in all three groups. No differences in T-cell proliferation were detected in the animals receiving estradiol alone or estradiol and progesterin. These animals were therefore grouped into a single HT group. No differences in T-cell proliferation were detected between intact, ovx, and HT animals (Fig. 6a–d).

Although no significant differences in T-cell proliferation were detected, there were some small differences in frequency of responding T cells between the experimental groups (Fig. 7). Overall, at most of the time points examined, intact animals seem to generate a more robust response than ovx animals, and at several time points, HT animals generated a higher T-cell response than ovx animals. More specifically, no differences in the frequency of responding T cells within the CD8 CM subset were detected (Fig. 7a). In the CD8 EM subset, intact animals generated the most robust response at every time point with the exception of days 7 and 60. At every time point, the *p* value for the difference between intact and ovx animals was 0.09 when adjusted for false discovery rate and <0.05 when the adjustment was not applied (Fig. 7b). On day 7 after booster immunization, the HT animals generated the largest CD4 CM and EM response ($p < 0.02$ after adjustment compared to ovx and intact animals). The CD4 CM response of the HT group was marginally higher than that of the ovx group (adjusted *p* value = 0.09) on days 14 and 60 post-vaccination (60 = 30 post-booster). Intact animals generated marginally higher responses (adjusted *p* value = 0.09) within the CD4 CM subset on days 14, 30, and 37.

Analysis of the B-cell response following MVA vaccination revealed no differences in either the kinetics or the magnitude of B-cell proliferation (Fig. 8a). A

higher IgG titer was measured in intact aged animals on day 37 compared to ovx but not HT animals.

Discussion

A large body of literature suggests that female sex hormones play a critical role in immune function (Ansar Ahmed et al. 1985). Therefore, it is very likely that the loss of estrogen and progesterone associated with menopause could modulate the age-related decline in immune function commonly referred to as immune senescence. However, this question has not been investigated. In this study, we examined the impact of ovariectomy (ovx) on T-cell homeostasis and the immune response to vaccination in young and aged female rhesus macaques. We found that whereas ovx adult animals have a higher frequency of CD4 naïve T cells, ovx aged female rhesus macaques had increased frequency of terminally differentiated CD4 memory T cells. Moreover, ovx aged female rhesus macaques showed increased inflammatory cytokine production by T cells compared to intact aged animals. These data suggest that ovariectomy may accelerate some aspects of immune senescence in aged females. We also report that both adult and aged ovx animals generated a reduced T- and B-cell response to vaccination compared to ovary-intact females. These observations strongly suggest that the loss of ovarian steroids results in diminished T-cell response to vaccination/infection. Finally, data presented here suggest that hormone therapy partially improves immune response to vaccination in aged ovx females.

Similar observations were made in rodents where studies showed that gonadectomy and the ablation of sex steroids in young male and female rodents increases the production of new naïve T cells by the thymus (Lynch et al. 2009; Perisic et al. 2010). Although the difference was not statistically significant, we noted a small decrease in inflammatory cytokine production by T cells from ovx adult female rhesus macaques, which is also consistent with the notion that ablation of sex steroids can rejuvenate the immune system in young animals (Holland and van den Brink 2009). Previous clinical studies have shown no differences in the frequency of naïve and memory T cells in post-menopausal women who are receiving hormone therapy compared to post-menopausal women who

are not, suggesting that estrogen deficiency does not modulate T-cell subset frequency in older women (Kamada et al. 2000). Similarly, our data show no increase in the frequency of total memory T cells in aged ovx animals. Rather, we observed a shift within the memory T-cell population towards increased prevalence of terminally differentiated (effector) memory T cells, suggesting that ovx aged females might be experiencing accelerated conversion into terminally differentiated memory T cells. Our observation that a higher frequency of T cells from ovx aged animals produces inflammatory cytokines is also consistent with clinical studies that have shown that post-menopausal women have higher levels of pro-inflammatory cytokines (Vural et al. 2006a, b).

More importantly, we found that ovx adult and aged animals generate significantly reduced T- and B-cell responses following vaccination. Specifically, T- and B-cell proliferation was significantly delayed and reduced in magnitude; IgG titers were reduced and the frequency of MVA-specific T cells was significantly lower in adult ovx animals compared to intact age-matched animals. The differences in the immune response to MVA between intact and ovx animals were less striking in aged female rhesus macaques, which suggests that the impact of age might be bigger than that of ovarian steroid loss. Nevertheless, these results strongly suggest that, regardless of age, the loss of ovarian steroids reduces immune response to infection. We also report that aged ovx females receiving HT generated an immune response that was either comparable or at times bigger than that of non-treated ovx females. Similarly, previous studies in adult female mice have shown that ovariectomy results in a reduced immune response to malaria and E or E + P supplementation rescued this defect (Cernetich et al. 2006; Klein et al. 2008). Ovariectomy also reduced the immune response to *Bacillus brasiliensis* in adult female rats (Pinzan et al. 2010). Interestingly in our studies, HT did not restore the magnitude of the T- and B-cell responses to levels measured in intact animals. There are two main differences between previously published and our current findings that could explain this discrepancy: (1) the species used, rodent versus non-human primates; and (2) the age of the animals, young adult versus aged. It is also possible that additional ovarian steroids could play a critical role in the generation of a protective immune response. An additional possi-

bility is that the extreme advanced age of these animals (24 years; median life span of rhesus in captivity 25 years) could have dampened the impact of HT on T- and B-cell function. Our studies highlight the need for additional studies to investigate the impact of the dose, duration, and route of administration of estradiol and progesterone on immune function in aged post-menopausal women.

A large body of evidence supports the finding that the female sex hormones estradiol and progesterone modulate the immune response to infection. For instance, estradiol administration to ovariectomized female mice protected them from HSV-2 infection (Gillgrass et al. 2005). Similarly, estradiol pre-treatment can protect female rhesus macaques from SIV transmission (Smith et al. 2000). Interestingly, vaccination studies in humans indicated that vaginal immunizations might be more effective for induction of genital tract antibodies if performed during the mid-follicular phase of the menstrual cycle (Kozlowski et al. 2002). Whether ovarian steroids directly or indirectly (by acting on innate immune cells) influence T- and B-cell function is an area of active investigation. Several studies have shown that lymphocytes express estrogen (ER) and progesterone (PR) receptors (Ansar Ahmed et al. 1985). One quantitative study revealed that ERs are differentially expressed in PBMC subsets (Phiel et al. 2005). Specifically, CD4⁺ T cells express relatively high levels of ER α compared with ER β , whereas CD8⁺ T cells express low but comparable levels of both ERs and B cells express high levels of ER β mRNA but low levels of ER α . Other studies have also shown that innate immune cells also express ERs and PRs (Hughes et al. 2008; Lambert et al. 2005). The observation that both T- and B-cell responses are compromised in ovx animals suggests that ovarian steroids modulate an early event in the initiation of the immune response such as antigen presentation and/or T-cell priming. However, it is also possible that ovarian steroids influence both the initiation as well as the amplification of the immune response to infection. Indeed, a recent study showed that signaling through the estrogen-related receptor ERR α was required for IFN γ production and efficient clearance of the bacterium *Listeria monocytogenes* by macrophages (Sonoda et al. 2007). Estrogen was shown to increase survival of B cells through the upregulation of the anti-apoptotic molecule Bcl-2 (Evans et al. 1997). Taken together, data presented

here suggest that ovariectomy and the associated withdrawal of ovarian steroids in aged female rhesus macaques result in changes associated with accelerated immune senescence, notably decreased response to vaccination. Moreover, HT might improve the immune response to vaccination in ovariectomized aged female rhesus macaques. These findings have significant implications for designing interventions to improve immune function in post-menopausal women.

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