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Targeting regulatory T cells to improve vaccine immunogenicity in early life

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Human newborns and infants are bombarded with multiple pathogens on leaving the sterile intra-uterine environment, and yet have suboptimal innate immunity and limited immunological memory, thus leading to increased susceptibility to infections in early life. They are thus the target age group for a host of vaccines against common bacterial and viral pathogens. They are also the target group for many vaccines in development, including those against tuberculosis (TB), malaria, and HIV infection. However, neonatal and infant responses too many vaccines are suboptimal, and in the case of the polysaccharide vaccines, it has been necessary to develop the alternative conjugated formulations in order to induce immunity in early life. Immunoregulatory factors are an intrinsic component of natural immunity necessary to dampen or control immune responses, with the caveat that they may also decrease immunity to infections or lead to chronic infection. This review explores the key immunoregulatory factors at play in early life, with a particular emphasis on regulatory T cells (Tregs). It goes on to explore the role that Tregs play in limiting vaccine immunogenicity, and describes animal and human studies in which Tregs have been depleted in order to enhance vaccine responses. A deeper understanding of the role that Tregs play in limiting or controlling vaccine-induced immunity would provide strategies to improve vaccine immunogenicity in this critical age group. New adjuvants and drugs are being developed that can transiently suppress Treg function, and their use as part of human vaccination strategies against infections is becoming a real prospect for the future.

Keywords: regulatory T cells, vaccines, infants, neonates, immunogenicity, immune modulation, adjuvants

INTRODUCTION

The infant immune system is uniquely adapted to meet the challenges of early life (Kollmann et al., 2012). The newborn emerges from an immune-protected environment into a world where they constantly encounter new antigens. There is therefore a need to have a series of immunoregulatory mechanisms in place in order to prevent excessive inflammation and tissue damage. At the same time the infant needs to develop immune memory upon pathogen encounter in order to be protected against future challenge. The newborn has little immunological memory, and neonates and infants are heavily reliant on innate immunity to protect them against antigenic challenge as discussed in a series of comprehensive review articles (Levy, 2007; Ghazal et al., 2013; Levy and Wynn, 2014).

In this review we discuss the regulatory factors that infants employ to suppress or control their developing immunity. We will focus on regulatory T cells (Tregs) in particular, and the potential role they play in suppressing or controlling vaccine-induced immunity in early life. We will explore the mechanisms of action used by Tregs to confer suppression, and differences in the phenotypic and functional characteristics between Tregs in infants and adults. We will discuss the role of Tregs in malaria, HIV, and hepatitis C virus (HCV) infections; and briefly describe the results of clinical trials in human infants of vaccines against these three infections. A detailed understanding of the immunoregulatory factors controlling vaccine immunogenicity in early life may provide potential strategies for improving vaccine efficacy in this vulnerable age group. We will discuss immunotherapeutic agents and vaccine adjuvants developed for use in humans that can down-modulate Treg activity and thus enhance vaccine efficacy, demonstrating that this approach is a viable option for the future.

THE INFANT IMMUNE SYSTEM

INNATE IMMUNITY

The innate immune system which acts as the first line of defense against infection is suboptimal at birth, and does not reach full capacity until teenage years. Innate cells express pattern recognition receptors (PRRs) which detect highly conserved pattern associate molecular patterns (PAMPs) expressed by invading pathogens or vaccines, including Toll-like receptors (TLRs) and NOD-like receptors (NLRs). Newborns and young infants have similar levels of expression of these PRRs as adults (Kollmann et al., 2012), however, responses to PRR stimulation are low at birth in part due to diminished innate signaling pathways such as IRF7 translocation (Danis et al., 2008) and TLR3 and 4 signaling (Aksoy et al., 2007). Reactivity to certain PRR agonists, e.g., TLR4 and TLR5 are acquired rapidly, and reactivity of the viral ssRNA sensing TLR7 and TLR8 receptors is robust from birth (Burl et al., 2011), hence TLR7/8 agonists are being investigated

as possible adjuvants to boost immune responses to neonatal vaccines (Dowling et al., 2013). Th2 (IL-6, IL-10) and Th17 (IL-6, IL-23) polarizing cytokines dominate the innate response in early life, while TNF-α and IL-1β responses rise in the first few years of life as the former cytokines decline (Belderbos et al., 2009; Kollmann et al., 2009; Nguyen et al., 2010; Burl et al., 2011). Infant dendritic cell (DC) function is also suboptimal (De Wit et al., 2004; Goriely et al., 2004; Aksoy et al., 2007), and NK cells (Guilmot et al., 2011) and neutrophil functions (Carr, 2000) are less potent than in adults. Low complement levels in neonatal plasma are thought to increase susceptibility to certain bacterial infections, and lead to impaired adaptive immunity (Levy, 2007).

ADAPTIVE IMMUNITY IN INFANCY

Infant T cell immunity

The adaptive immune system is characterized by minimal immunological memory at birth, since the newborn has been relatively protected from antigenic exposure *in utero*, and most of their T cells are of a naïve phenotype. Furthermore, high levels of TGF-β, progesterone and prostaglandin E2 *in utero* required to prevent the mother developing Th1 alloreactivity to her fetus (Philbin and Levy, 2009), alongside poor innate Th1 support (Langrish et al., 2002), result in the newborn having intrinsically skewed Th2-type immunity from birth. Additionally, the Th17 biased innate immunity in infants also results in a Th17 adaptive bias. This bias against Th1 immunity results in an increased vulnerability to microbial infections and suboptimal reactivity to many vaccines. Despite this, infants have been shown to stimulate adult level Th1 type immune responses to BCG vaccination (Marchant et al., 1999) and are thus capable of robust Th1 immunity. However, neonatal BCG vaccination results in a Th17 biased mycobacterial response compared to those receiving BCG at $4\frac{1}{2}$ months of age (Burl et al., 2010), in keeping with the Th17 bias described above.

Infant B cell immunity

Newborn infants acquire IgG antibodies transplacentally from their mothers which provide protection against infections encountered in early life, while the other immunoglobulin subclasses are unable to cross the maternal–placenta interface. The maternally acquired antibody (MAb) levels wane over the first 6 months of life and are usually absent by 1 year of age. Several studies suggest that MAbs inhibit humoral responses to infant vaccines; including live measles vaccine (Albrecht et al., 1977) and oral poliomyelitis vaccine, and non-live vaccines including pertussis (Burstyn et al., 1983; Englund et al., 1995), tetanus and diphtheria toxoids (Bjorkholm et al., 1995), Hib conjugate vaccine (Claesson et al., 1989; Daum et al., 1991) and hepatitis A vaccine (Kanra et al., 2000); while other studies report no influence of MAbs on responses to these vaccines (Gans et al., 1998; Siegrist et al., 1998; Sallusto et al., 1999). Responses may still be protective even if MAb inhibition occurs (Jones et al., 2014), and while MAbs may interfere with the generation of humoral responses to vaccination, T cell responses do not seem to be similarly affected (Siegrist, 2003).

Human neonatal antibody responses are delayed in onset, of shorter duration, achieve lower peak levels, and have lower affinity than adults. The isotype distribution also differs, with IgG1 and IgG2 levels peaking at ∼3–4 years of age, and IgG4 only reaching adult levels at 4–6 years of age, while IgG3 is stable from birth (Ngamphaiboon et al., 1998). Histological studies of infant splenic tissue show that the marginal zone does not reach full development until 2 years of age, which alongside low complement levels (Zandvoort and Timens, 2002; Kruetzmann et al., 2003) and low expression of CD21 (complement receptor 2; Griffioen et al., 1993), would account for the delayed antibody response to T cell independent glycoproteins and polysaccharide antigens, including encapsulated bacteria such as *Streptococcus pneumoniae* and *Haemophilus influenzae*, and thus poor reactivity to polysaccharide vaccines (Adkins et al., 2004).

IMMUNOREGULATORY FACTORS IN NEONATAL AND INFANT PLASMA

Neonatal and infant plasma contain a number of immunoregulatory factors that serve to maintain Th2 polarization, and limit pro-inflammatory innate and adaptive immunity. Newborns and infants have high levels of plasma adenosine, an endogenous purine metabolite with immunosuppressive properties. Adenosine causes mononuclear cells to produce cAMP, which acts as a second messenger to inhibit TLR-stimulated production of pro-inflammatory cytokines while polarizing toward IL-10 and Th17 cytokine production (Levy et al., 2006; Power Coombs et al., 2011; Philbin et al., 2012). Neonatal monocytes have increased sensitivity to these effects of adenosine via their adenosine A3 receptors, thus modulation of this system could potentially be used to enhance innate and therefore adaptive pro-inflammatory responses.

Several studies have shown that there are high levels of the immunosuppressive cytokine IL-10 in cord blood (CB; De Wit et al., 2004; Belderbos et al., 2009; Nguyen et al., 2010). IL-10 can be produced by most cell types of the immune system, including antigen presenting cells (APCs), granulocytes, and Th1, Th2 and many regulatory T cell subsets. IL-10 acts at a number of stages of an immune response in order to control inflammation. It inhibits the production of pro-inflammatory cytokines and chemokines by monocytes, macrophages and DCs, leading to increased IL-10 production by various T cell subsets. It suppresses both Th1 and the more recently described "Th1+Th17" cells, while enhancing $CD4^+FOXP3^+$ (forkhead box P3) regulatory T cell survival and activity, and promoting IgG and IgA class switching by B cells (Banchereau et al., 2012).

HUMAN REGULATORY T CELL SUBTYPES AND THEIR MODES OF ACTION

Regulatory T cells are unique subpopulations of T cells that play a major role in immune homeostasis and tolerance (Sakaguchi, 2000; Belkaid et al., 2002; Mills and Mcguirk, 2004; Belkaid, 2007). Although Tregs have been shown to be beneficial in preventing an over-exuberant response and immune pathology following encounter with pathogens (Belkaid, 2008; Belkaid and Tarbell, 2009), they have also been shown to limit the favorable effector responses required for sterilizing immunity, thus allowing pathogen persistence (Kao et al., 2010).

THYMUS DERIVED AND PERIPHERAL CD4$^+$FOXP3$^+$ Tregs

The Treg field was invigorated with the discovery of the transcription factor FOXP3 which is vital for the development, function and homeostasis of Tregs (Fontenot et al., 2003; Hori et al., 2003), and is thus considered the master regulator of Tregs. Its importance is further highlighted by patients with mutations in FOXP3 who develop a severe fatal disorder known as immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome (Bennett and Ochs, 2001; Gambineri et al., 2003).

Recently a group of experts in the Treg field introduced a consensus nomenclature for FOXP3$^+$ Tregs. They suggest replacing previously used terms which they describe as being to some extent "inaccurate and ambiguous" (Abbas et al., 2013). They recommend that the subset of FOXP3$^+$ Tregs of thymic origin, which are also known as natural Tregs (nTregs), should be called thymus-derived Tregs (tTregs); while FOXP3$^+$ Tregs that differentiate in the periphery should be called peripherally derived Tregs (pTreg) rather than the previous term inducible Tregs (iTregs). The pTregs are induced in the periphery in response to antigenic stimulation, and possess identical characteristics to tTregs, and therefore both subsets will be described together.

Phenotype of human CD4$^+$ FOXP3$^+$ Tregs

CD4$^+$FOXP3$^+$ Tregs are the most widely studied Treg subset. They were first described as a subset of CD4$^+$ T cells which constitutively express the interleukin 2 (IL-2) receptor alpha-chains (CD25) and can prevent the development of autoimmunity in mice (Sakaguchi et al., 1995).

Determining the precise phenotype of human CD4$^+$FOXP3$^+$ Tregs has proved difficult with many conflicting studies. Since CD25 is transiently expressed on conventional T cells (Hatakeyama et al., 1989), the CD25^{hi} subset is described as a more reliable marker of CD4$^+$FOXP3$^+$ Tregs in humans (Schmetterer et al., 2012). Human FOXP3$^+$ Tregs also tend to express low levels of the IL-7 receptor CD127 (Liu et al., 2006; Seddiki et al., 2006). Therefore the most commonly analyzed phenotypes in human studies are CD4$^+$CD25$^+$FOXP3$^+$ or CD4$^+$CD25$^+$CD127^{lo}. Furthermore, human CD4$^+$FOXP3$^+$ Tregs generally express high levels of the co-inhibitory receptor cytotoxic T lymphocyte antigen 4 (CTLA4; Sansom and Walker, 2006). More recently the chemokine markers CCR4, CCR6, CXCR3, and CXCR10 have been proposed to define four distinct populations of human tTregs, each with distinct functional characteristics (Duhen et al., 2012). Each of these four Treg subsets are thought to co-localize *in vivo* with and regulate a distinct Th subset (Th1, Th2, Th17, Th22) expressing the same chemokine receptors.

CD45RA expression can be used to distinguish tTregs that are naïve or resting (rTregs; FOXP3^{lo}CD45RA$^+$) from the memory subset described as activated Tregs (aTregs; FOXP3^{hi}CD45RA$^-$; Miyara et al., 2009). The memory Tregs can be further subdivided into central memory (Treg$_{CM}$) and effector memory (Treg$_{EM}$) similarly to Th cells, based on the expression of chemokine receptor 7 (CCR7; Sallusto et al., 1999; Tosello et al., 2008).

Certain subpopulations among CD4$^+$FOXP3$^+$ Tregs are more suppressive than others. For example, Tregs expressing the tumor necrosis factor receptor 2 (TNFRII) are thought to represent a highly suppressive CD4$^+$FOXP3$^+$ Treg subset (Minigo et al., 2009). Those CD4$^+$CD25$^+$FOXP3$^+$ Tregs expressing the transmembrane cyclic ADP ribose hydrolase CD38 (mainly thymic derived and in the spleen) have particularly high suppressive activity in a murine model (Patton et al., 2011). CD38 is part of a cascade involved in the production of the immunosuppressive factor adenosine from NAD$^+$ (Horenstein et al., 2013) which can have immunoregulatory properties as discussed earlier. Interestingly, the majority of infant T cells express CD38 (Scalzo-Inguanti and Plebanski, 2011), and as previously mentioned infants also have high plasma levels of adenosine, but a link between these two factors has yet to be explored in neonates and infants.

Mechanisms of CD4$^+$ FOXP3$^+$ Treg mediated suppression

CD4$^+$FOXP3$^+$ Tregs can suppress the proliferation and activation of a multitude of immune cell types including T cells, NK and NKT cells, monocytes, macrophages, B cells, DCs, and eosinophils. They employ a variety of mechanisms to mediate this suppression, and are thought to be flexible in this respect by adapting their mechanism according to their local environment (reviewed by Wing and Sakaguchi, 2012). Both IL-2 and CTLA-4-dependent mechanisms have been described, with CD25 and CTLA-4 knockout mice having a similar phenotype to Foxp3 deficient mice (Wing and Sakaguchi, 2012). It is thought that the constitutive expression of CD25 by CD4$^+$FOXP3$^+$ Tregs allows them to consume the available IL-2, depriving effector T cells (Teffs) and leading to effector cell death (De La Rosa et al., 2004). Those tTregs expressing CTLA-4 can suppress T cell responses via down-modulation of CD28 signaling (Walker, 2013), and reduced co-stimulatory capacity of CD80/86 expressed by DCs (Wing et al., 2011).

A commonly used mechanism of Treg action is the production of soluble inhibitory factors, including either membrane bound or released immunosuppressive cytokines IL-10, TGF-β, and IL-35 (Collison et al., 2007). FOXP3$^+$ Tregs can also generate high concentrations of adenosine (Mandapathil et al., 2010) which binds to the A2a receptor on immune cells activating an immunoinhibitory loop (Sitkovsky and Ohta, 2005) which results in inhibition of T cell proliferation and cytokine production (Raskovalova et al., 2005).

TYPE 1 REGULATORY T CELLS (Tr1)

The Tr1 Tregs are a unique Treg subset that do not rely on the expression of high levels of CD25 or FOXP3 for their function (Levings and Roncarolo, 2000). They are activated in the periphery following antigenic stimulation in the presence of IL-10 (Groux et al., 1997; Vieira et al., 2004). Recently, lymphocyte-activation gene 3 (LAG3) and CD49b have been described to represent specific markers for Tr1 cells (Gagliani et al., 2013).

The Tr1 Tregs are known to produce high levels of the immunosuppressive cytokines IL-10 and TGF-β, some IL-5, low levels of IFN-γ and IL-2, and no IL-4 (Groux et al., 1997). The secretion of IL-10 is the main mechanism by which Tr1 cells are thought to mediate suppression. The IL-10 can be either free or membrane bound, and has been shown to suppress Teff proliferation/activation both directly

and indirectly via a modulation of APC function (Roncarolo et al., 2006). They have also been shown to use cell–cell contact mechanisms (Gregori et al., 2012) and the production of granzyme B and perforin (Gregori et al., 2010) to mediate suppression.

T HELPER TYPE 3 CELLS (Th3)

This unique subset of TGF-β producing Tregs was identified in early studies investigating oral tolerance. They have been shown to suppress the proliferation and activation of Th1 cells and suppress the development of autoimmunity in the mouse model of multiple sclerosis (Chen et al., 1994). They become activated in the periphery upon encounter with a specific antigen, and suppress via the production of the inhibitory cytokine TGF-β. Some studies show that Th3 cells may have a role to play in controlling autoimmunity and allergy in humans (Andersson et al., 2002; Perez-Machado et al., 2003), but the role that this subset plays in the maintenance of immune tolerance in humans is still not clearly defined.

$CD8^+$ Tregs

While $CD4^+$ Tregs have been widely studied in humans, $CD8^+$ Tregs have not received the same attention. However, there is increasing evidence that subsets of $CD8^+$ Tregs also play important immunoregulatory roles, and impaired $CD8^+$ Treg function may lead to autoimmunity (Hu et al., 2004; Lu et al., 2008). The most widely described phenotype for $CD8^+$ Tregs is $CD25^+CD28^-$ (Ciubotariu et al., 1998; Filaci et al., 2004). Other markers include CD122, CTLA-4, GITR, CD38, CD103, and CD8αα (Uss et al., 2006; Simone et al., 2008; Smith and Kumar, 2008; Liu et al., 2014); a host of different CD8 Treg subsets have been described in humans expressing various combinations of these markers (Suzuki et al., 2012). While FOXP3 expression has been described in many CD8 Treg subsets, it may also represent an activation marker rather than acting as a regulatory factor since $CD8^+FOXP3^+$ cells have been found to be minimally suppressive in some studies (Mayer et al., 2011). Mechanisms of action of $CD8^+$ Tregs that have been reported include cell–cell contact mediated suppression, secretion of the suppressive cytokines IL-10 and TGF-β, and induction of APC energy (Suzuki et al., 2008). $CD8^+CD45RA^+CCR7^+FOXP3^+$ cells may represent a discrete subset of CD8 Tregs which interfere with the TCR signaling cascade (Suzuki et al., 2012). More extensive work is required to better understand the origin and role of $CD8^+$ Tregs in immunoregulation and autoimmunity, particularly in humans.

PHENOTYPIC AND FUNCTIONAL DIFFERENCES BETWEEN Tregs IN INFANTS COMPARED TO ADULTS

Distinct qualitative and quantitative differences have been identified between the Tregs in adults and those of infants. Most of the studies in infants have analyzed Tregs in neonatal CB for comparison with adults, and different phenotypic markers have been used to characterize the Tregs in these studies contributing to some discrepancies in the results.

$FOXP3^+$ Tregs have been found in much higher levels at birth compared to adults, whether defined as $CD4^+CD25^+CD127^{lo}$ (Nettenstrom et al., 2013) or $CD4^+CD25^+FOXP3^+$ (Flanagan et al., 2010). Preterm infants have been shown to have higher levels still (Luciano et al., 2014). However, a study comparing $CD4^+CD25^+CD127^{lo}$ Tregs at different age groups, showed slight increases in Treg frequencies with age: 6.10% in CB; 7.22% in adults aged 20–25 years; and 7.5% in adults over the age of 60 years (Santner-Nanan et al., 2008); and another study found that neonates had similar number of cells expressing FOXP3 compared to their mothers, and a lower number of $CD4^+CD25^{bright}$ cells (Ly et al., 2009). The reason for these conflicting results is not known.

Cord blood Tregs have been shown to be predominantly of the $CD45RA^+CD45RO^-$ naïve phenotype in several studies (Kanegane et al., 1991; Wing et al., 2002; Takahata et al., 2004; Ly et al., 2009; Flanagan et al., 2010). Other phenotypic differences between cord and adult Tregs include the observation that CB Tregs are mostly $CD27^+$ and thus at an earlier differentiation state than their mothers; they have a lower apoptotic potential as evidenced by lower CD95/Fas expression than their mothers; and less CD62L suggesting less of a $Treg_{CM}$ lymph node homing phenotype (Flanagan et al., 2010). CB Tregs also express less CCR6 than their matched mothers, which is the chemokine receptor that characterizes the Th17- and Th22-like Tregs (Duhen et al., 2012). Since infants are Th2 biased then their Tregs should be predominantly of a $CCR4^+CCR6^-CXCR3^-$ Th2 Treg phenotype in keeping with the classification discussed previously (Duhen et al., 2012), although this has not been investigated in infants.

In vitro Treg suppression assays are difficult to perform in infants due to the lack of availability of large volumes of blood, combined with the low Treg frequencies in peripheral blood. Studies using CB are easier since large volumes are available for study. Several studies have shown that newborn CB Tregs are highly functional whereby they suppress T cell proliferation and IFN-γ production, further deviating from a Th1 response (Godfrey et al., 2005; Wing et al., 2005). Fan et al. (2012) found that $CD4^+CD25^+$ CB Tregs had a stronger immunosuppressive function than adult blood Tregs following two cycles of polyclonal stimulation. Mayer et al. (2012) found that CB $CD4^+CD25^{hi}$ cells failed to suppress upon TCR activation whereas those freshly purified from adult blood did, but CB Tregs became strongly suppressive after antigen-specific stimulation. Another study found that low FOXP3 expression levels by CB Tregs correlated with minimal suppressive activity, but following expansion there was a significant increase in the suppressive activity of these CB Tregs with a shift from the $CD45RA^+$ to the $CD45RA^-$ phenotype (Fujimaki et al., 2008). It has recently been shown that CB Tregs can be expanded more easily than adult peripheral blood Tregs, and CB Tregs are better suppressors in allogeneic mixed lymphocyte reactions than their adult counterparts (Lin et al., 2014).

Taken together these studies suggest distinct differences between infant and adult Tregs. Overall they seem to be present in higher frequencies than in adults, are more naïve and less differentiated, and are highly suppressive; all supporting an active immunoregulatory role in early life.

THE ROLE OF Tregs IN REGULATING IMMUNITY TO MALARIA, HIV, AND HEPATITIS C VIRUS

Regulatory T cells have been implicated with an immunoregulatory role in both murine and human malaria infections (Scholzen et al., 2010). In mice, ablation of $Foxp3^+$ Tregs led to increased T cell activation and decreased parasitaemia (Abel et al., 2012). *In vivo* depletion of Tregs protected mice from experimental cerebral malaria in a *Plasmodium berghei* model of infection (Wu et al., 2010). A FOXP3 promoter polymorphism in children has been associated with significant parasitaemia in a Congolese study suggesting a Treg role (Koukouikila-Koussounda et al., 2013). Malaria infected red blood cells (iRBCs) induced $CD4^+CD25^{hi}FOXP3^+$ Tregs *in vitro* in healthy human volunteers (Scholzen et al., 2009). In human malaria sporozoite challenge experiments, Tregs have been shown to be induced rapidly after infection, and linked to lower pro-inflammatory cytokines and increased TGF-β production (Walther et al., 2005). Another study showed that malaria antigens can activate latent TGF-β on the surface of aTregs (Clemente et al., 2011). A study of 112 subjects in Kenya (infants to adults) found a correlation between the frequency of $CD4^+CD25^{hi}$ T cells and increased risk of clinical malaria, suggesting Tregs may negatively affect natural immunity to malaria in humans (Todryk et al., 2008). In naturally exposed Gambians $CD4^+FOXP3^+CD127^{lo}$ Tregs during acute infection were inversely correlated with memory responses at 28 days, suggesting suppression of immune memory. In the same study, a $CD4^+FOXP3^-CD45RO^+$ T cell population co-producing IFN-γ and IL-10 was more prevalent among children with uncomplicated malaria than those with severe disease, suggesting a beneficial immunoregulatory role for this IL-10 producing subset, presumably by limiting excessive inflammation (Walther et al., 2009). A role for the highly suppressive $TNFRII^+$ Tregs in malaria parasite survival has been implicated in a study of Indonesian school children (Wammes et al., 2013). Overall, these data support an induction of Tregs during acute malaria infection which can limit the generation of immune memory and increase susceptibility to infection, but also control immunopathology and disease severity.

The role of Tregs in HIV infection remains poorly understood and the data are conflicting, in part due to the different phenotypes used to define Tregs in the various studies. However, the studies do support a regulatory role. For example, combination anti-retroviral therapy (cART) non-responders with persistent CD4 counts <200 cells/μL on therapy had higher peripheral blood Tregs and aTregs than cART responders, with higher $IL\text{-}10^+$ Tregs and lower FOXP3 in lymphoid tissue (Gaardbo et al., 2014). Another longitudinal study also found higher numbers of Tregs associated with immunological non-responders defined as CD4 <500/μL (Saison et al., 2014). A study analyzing multiple Treg phenotypes in HIV infected individuals found evidence of Treg redistribution depending on HIV status (Serana et al., 2014). Untreated viraemic patients with stable CD4 counts had higher proportions of naïve Tregs with decreased $Treg_{CM}$ compared to those on cART and healthy controls (Serana et al., 2014). The study suggests that effective cART restores Treg homeostasis since Treg subpopulations in the cART group were similar to those of healthy donors. Increased proportions and decreased numbers of Tregs associate with progression of HIV (Wang et al., 2013). Treg depletion in a murine chronic retrovirus infection model resulted in reduced viral loads (Dietze et al., 2013). In combination, the data suggest that Tregs may suppress HIV-specific immunity leading to lower CD4 counts and viral persistence.

Hepatitis C virus is characterized by its ability to establish chronic infection in the majority of those infected, and an immunoregulatory role for Tregs in this process has been well described. Chronic HCV patients have increased levels of $CD4^+$ and Tr1 Tregs in peripheral blood which are thought to suppress anti-viral T cell responses leading to viral persistence (Chang, 2007). Certain HCV epitope variants have been shown to induce Tregs in HCV-infected patients (Cusick et al., 2011). Chronic HCV patients have more serum IL-10 than those with resolved infection, which is proposed to play a role in the induction of $CD4^+FOXP3^+$ Tregs in chronic HCV infection (Macdonald et al., 2002; Cusick et al., 2013); and CD49b, a marker for IL-10 producing Tr1 Treg cells, is lower in those who respond to viral therapy, thus suggesting a regulatory role for Tr1 Tregs too (Fabien et al., 2014). Indoleamine 2,3-dioxygenase (IDO) production by stimulated monocyte derived DCs was higher in HCV patients compared to healthy controls, and these DCs were more able to induce Tregs, suggesting a role for this Treg induction pathway in chronic HCV (Higashitani et al., 2013). Expression of the inhibitory signaling pathway molecule T cell immunoglobulin and mucin-domain-protein-3 (Tim-3) is upregulated on both Teff and $CD4^+CD25^+FOXP3^+$ Tregs in chronic HCV, correlating with increased Treg and decreased Teff proliferation, suggesting that the Tim-3 pathway controls the Treg/Teff balance in chronic HCV (Moorman et al., 2012). Viral persistence following acute HCV infection is accompanied by increased plasma Galectin-9 (Gal-9) which is the ligand for Tim-3, alongside expanded Gal-9 expressing Tregs and increased expression of Tim-3 and CTLA-4 on HCV-specific $CD8^+$ T cells (Kared et al., 2013). Thus high levels of Tregs likely contribute to viral persistence in HCV infection, and both $FOXP3^+$ and Tr1 Tregs have been implicated. Mechanisms of Treg induction in HCV may be multifactorial but include HCV antigen driven induction, IL-10, IDO, Gal-9/Tim-3, and CTLA-4.

ROLE OF Tregs IN CONTROLLING VACCINE IMMUNOGENICITY

The role that Tregs play in controlling or limiting vaccine immunogenicity remains to be fully determined. Given that Tregs are induced by natural infections to regulate the inflammatory response, it makes sense that Tregs would be induced as part of the immune response to vaccination, particularly for live attenuated vaccines. One might predict that their induction would play a beneficial immunoregulatory role by preventing an overexuberant immune response to the vaccine. However, most studies suggest that Tregs can interfere with the generation of vaccine-induced immunity. Thus, depletion of Tregs pre-vaccination in murine models has been shown to enhance immune responses to some vaccines. In a DEREG mouse model, which allows for *in vivo* depletion of $Foxp3^+$ Treg cells at any point during an immune response using diphtheria toxin (Lahl and Sparwasser,

2011), Treg depletion led to an enhanced anti-tumor response to vaccination against an established melanoma (Klages et al., 2010). A more recent study showed that the short term depletion of Tregs in DEREG mice greatly enhanced vaccine-induced immunity against a solid tumor; increasing NK cells and CD8 T cell activation and IFN-γ production (Mattarollo et al., 2013). Administration of vaccines with anti-CD25 monoclonal Ab has been shown to induce more durable immunity in mice compared to when the vaccine is administered alone, for both BCG and hepatitis B vaccines, which has been attributed to a depletion of $CD25^+$ Tregs (Moore et al., 2005). Ho et al. (2010) showed that antigen-specific Tregs induced by environmental mycobacteria suppress Th1 immune responses, thus compromising the response to BCG vaccination in mice. They also showed a correlation between the pre-existing Tregs and the subsequent vaccine response. Murine studies of Parkinson's disease have shown that Tregs are induced by BCG vaccination (Lacan et al., 2013).

It is difficult to translate these studies into primates and humans since murine Tregs are not phenotypically identical, and *in vivo* depletion of $FOXP3^+$ Tregs in healthy humans presents logistic and ethical challenges. An oral vaccine against simian immunodeficiency virus (SIV) based on a *Lactobacillus* commensal that favors immune tolerance induction was used to induce T cell tolerance to SIV antigens in macaques (Lu et al., 2012). The vaccine-induced $CD8^+$ Tregs that suppressed $CD4^+$ T cell activation and *ex vivo* SIV replication, and provided sterile protection against an intrarectal SIV challenge in 15 of 16 vaccinated macaques. This strategy is thought to work because $CD4^+$ T cell activation drives the initial phase of viral replication, and provides the proof-of-concept that an oral Treg inducing vaccine could prevent the establishment of HIV infection.

Using a DC-based vaccine in HIV patients undergoing cART, it was shown that depletion of the Tregs *in vitro* significantly enhanced the vaccine-induced anti-HIV-1-specific polyfunctional T cell response, suggesting that Tregs can dampen vaccine-induced immunity (Macatangay et al., 2010). This study also showed a marked increase in the $CD4^+CD25^{hi}FOXP3^+$ Treg numbers following vaccination, however, this increase did not correlate with the effector $CD8^+$ T cell vaccine-induced response. Increased FOXP3 mRNA expression has been demonstrated in malaria vaccinated adults; however, the authors concluded that this might be attributed to the participants being naturally exposed to the malaria parasite rather than as a result of vaccination *per se* (Mwacharo et al., 2009).

Very few studies have looked at the role that Tregs play in controlling vaccine immunogenicity in infants. Our group found no correlation between PPD-specific $CD4^+CD25^+FOXP3^+$ Tregs or $CD4^+IL\text{-}10^+$ Tregs, or PPD stimulated total IL-10 production on the day of BCG vaccination of Gambian infants, and subsequent IFN-γ responses to PPD (Burl et al., 2010). No functional Treg assays were conducted in this study. In another study we found that placental associated malaria (PAM) infection is associated with increased malaria-specific $CD4^+CD25^+FOXP3^+$ Tregs (Flanagan et al., 2010) and that PAM also correlates with decreased immunogenicity of BCG vaccination as evidenced by poorer PPD reactivity persisting to 1 year of age compared to PAM negative children (Walther et al., 2012). Whether the Tregs are the cause of this attenuation of BCG responses is not known.

TARGETING Tregs *IN VIVO* TO ENHANCE VACCINE IMMUNOGENICITY

The cancer research field has made considerable advances in dissecting the role that Tregs play in cancer progression and their role in suppressing responses to cancer vaccines. Moreover, trials conducted in animal models and humans have demonstrated that certain drugs and immunotherapies can transiently decrease Treg frequencies *in vivo* leading to improved anti-tumor Teff functions, and in some cases reduced tumor load. Since Treg depletion can enhance inflammation and autoimmunity then such transient depletion, as opposed to long term effects, is desirable. In low doses, the agent cyclophosphamide transiently decreases Treg frequencies while Teff functions are preserved, leading to enhanced responses to vaccine antigens and improved vaccine immunogenicity in mouse and human cancer vaccine trials (Barbon et al., 2010; Le and Jaffee, 2012). Anti-CD25 monoclonal antibodies, which deplete Tregs *in vivo*, enhanced vaccine efficacy in mouse models of pancreatic carcinoma (Keenan et al., 2014) and melanoma (Tan et al., 2013). Basiliximab and Daclizumab are anti-human CD25 MAbs that cause both decreased number and decreased function of Tregs by blocking IL-2 signaling (Goebel et al., 2000; Kohm et al., 2006; Mitchell et al., 2011). Daclizumab has been used in several human breast cancer vaccine trials where it depleted Tregs and improved effector responses, and furthermore may reprogram naïve Tregs to become IFN-γ producers (Rech and Vonderheide, 2009; Rech et al., 2012). The human monoclonal antibody, Ipilimumab, inhibits Tregs by blocking CTLA-4; and was approved by the FDA in 2011 for use in melanoma patients (Peggs et al., 2009).

Certain innate agonists that are being used as vaccine adjuvants preferentially expand Teff over Tregs, e.g., the TLR3 agonist Poly(I:C) and the TLR9 agonist CpG-ODN; whereas others favor Treg expansion, e.g., the TLR7 agonist imiquimod (Perret et al., 2013). OX40 is part of the TNFR superfamily expressed by Teff and Tregs, and the monoclonal antibody increases Teff function while blocking Treg function. OX40 clones have been humanized as potential agents to enhance the immunogenicity of vaccines against infectious diseases (Voo et al., 2013).

An interesting approach is that of local depletion of Tregs at the site of injection of a vaccine. Chemokine receptor 4 (CCR4) antagonists can be used as vaccine adjuvants to target and decrease local recruitment of $CCR4^+$ Tregs in order to amplify vaccine responses at the site of immunization (Bayry, 2014).

Therefore a number of agents that target Tregs are being used experimentally in humans in order to enhance vaccine efficacy. Some of these are non-toxic and safe for use in humans, offering the future prospect of using this approach to enhance immunogenicity of vaccines against infectious diseases including malaria, HIV, and HCV.

TRIALS OF NOVEL VACCINES AGAINST MALARIA, HIV, AND HCV IN INFANTS

Despite the multiple obstacles to successful infant vaccination discussed above, many vaccines are currently delivered in infancy

with good immunogenicity, including the live BCG, measles and yellow fever vaccines; and the inactivated diphtheria, tetanus, pertussis and hepatitis B vaccines, *H. influenzae b*, and pneumococcal conjugate vaccine. The RTS,S/AS01 malaria vaccine is the most advanced malaria vaccine in human clinical trials, having reached phase III testing in children and infants, with potential licensure in 2015. It reduced clinical and severe malaria by 56 and 47.3% respectively in children aged 5–17 months (Bejon et al., 2008; Olotu et al., 2011); but only 31.3 and 36.6% when administered in three doses with routine Expanded Program on Immunization (EPI) vaccines in the 6–12 week old age group (Rts et al., 2012). Follow up of the 5–17 month old vaccinated group over a 4 year period found protection waned to 16.8%; with waning greater among those with higher malaria exposure suggesting that natural immunity to malaria contributes to the waning (Olotu et al., 2013). Whether exposure induced Tregs play a role in this waning has not been investigated.

Fowlpox and modified vaccinia Ankara (MVA) based malaria vaccines have been tested in 1–6 year olds with no evidence of protective efficacy (Bejon et al., 2007). A blood stage vaccine FMP2.1/AS02A has been tested in Malian children aged 1–6 years with an efficacy of <10% (Laurens et al., 2013). The blood stage alum adjuvanted GMZ2 malaria vaccine elicited good inhibitory antibody levels in pre-school children (Jepsen et al., 2013). Prime-boost strategies based on chimp adenovirus vector priming followed by MVA boosting are being tested in children and infants in Africa, and while results of these trials are not yet available the adult studies have shown unprecedented immunogenicity for malaria exposed populations (Ogwang et al., 2013) and should stimulate good immunity in infants.

Only a few human HIV vaccine trials have been conducted in healthy uninfected and HIV-exposed infants. Immunogenicity was limited among healthy Gambian infants given a single dose of MVA.HIVA, but this was not surprising given that MVA alone is known to be poorly immunogenic (Afolabi et al., 2013). In a Ugandan trial, infants were vaccinated at birth, 4, 8, and 12 weeks of age with ALVAC-HIV vCP1521 (ALVAC) candidate HIV vaccine which induced low level CD4 and CD8 T cell responses at 24 months (Kaleebu et al., 2014).

The target population for HCV vaccines include intravenous drug abusers and health care professionals. However, HCV infection is common throughout the world and mother-to-child transmission is well described (Yeung et al., 2001). Thus an infant HCV vaccine would have its place, particularly in resource poor settings where the anti-viral therapies available are currently not affordable. Both therapeutic and prophylactic vaccines are being developed, and several have now entered phase I/II human clinical trials, mostly of therapeutic vaccines in chronically infected cohorts. These include recombinant protein, peptide, DNA, and vector-based vaccines aimed at producing robust anti-T cell responses (Halliday et al., 2011). As far as we are aware no HCV vaccine trials have been conducted in children.

FUTURE PROSPECTS

There is very little in the literature regarding the role of Tregs in infants in general, and even less in respect to vaccine immunogenicity. The fact that functional Tregs are present in high numbers in infancy and have potent suppressive activity, coupled with poor immunological responses to some vaccines in this vulnerable age group, supports a need to better understand the role they play in controlling the response to childhood vaccines in particular. The data available suggest that Tregs suppress immunity to vaccines, and that they can also be induced by vaccination. We have shown that malaria, HIV, and HCV all use Tregs to evade host immune responses, therefore vaccine-induced Tregs would be predicted to reduce the protective efficacy of vaccines against these infections. A better understanding of the role that Tregs and other immunoregulatory factors play in contributing to poor vaccine immunogenicity in childhood would help with the design of better vaccines. Studies in cancer patients have shown that transient Treg inactivation or depletion is a viable approach to enhancing vaccine efficacy. A number of Treg modifying agents are available for use in humans, therefore this approach is a very real prospect for the future and may be particularly applicable to neonates and infants.

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