Therapeutic Effects of Human Amniotic Epithelial Cells in a Murine Model of Multiple Sclerosis

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Abstract

Multiple sclerosis (MS) is an inflammatory demyelinating disease that has 1/1000 prevalence in the Western world. Current therapies for MS are only partially effective and are accompanied by undesirable side effects. In this thesis I examined a newly emerged placental-derived cell, amniotic epithelial cell (hAEC), as cell therapy for a myelin oligodendrocyte glycoprotein (MOG) peptide-induced MS model of experimental autoimmune encephalomyelitis (EAE). I tested the effects of hAEC in preventing or intervening EAE disease course. In both instances I found milder neurological signs, reduced inflammation in the spinal cord accompanied by lowered peripheral immune responses against MOG peptide. In searching for mechanisms by which hAEC mediate their therapeutic roles, I found that hAEC inhibited antigen-nonspecific and antigen-specific splenocyte proliferation in a dose-dependent manner. Transforming growth factor-β (TGF-β) and prostaglandin E2 (PGE2) are essential for this suppression *in vitro*. Because MS is thought to be a T cell-mediated disease, the ability to modulate T cell response is key to control MS progression. I found that splenic T cells from hAEC-treated EAE mice showed elevated IL-5 production, and in the intervention model there was also elevated production of IL-2 and IL-5. Peripheral immune cell populations in spleens and lymph nodes were mainly unchanged except for elevated regulatory T cells found in the intervention model after corticosteroid-induced remission followed by hAEC treatment. There were also decreased secretions of MOG-specific antibodies in sera of mice in both the prevention and intervention models, reflecting the alleviated peripheral autoimmune responses after hAEC treatment. hAEC were found in the lung but not other organs after intravenous delivery to EAE mice. My data suggest that hAEC may have potential as

cell therapy for MS to prevent and to alleviate relapse of MS. Compared to primary hAEC, expanded hAEC in culture showed morphological changes, reduced suppressive capacity and lower production of immunomodulatory molecules. hAEC-derived hepatocyte-like cells exhibit immunogenicity, which is not evident in primary hAEC, but still retain immunomodulatory properties *via* secreted factors. These findings suggest that hAEC may also be applied as cell therapy for organ-specific diseases and tissue transplantation.

Abbreviations

AB/H	Antibody-high strain
ARR	Annual relapse rate
BALT	Bronchus-associated lymphoid tissue
BBB	Blood-brain barrier
CD	Clusters of differentiation
CFA	Complete Freund's adjuvant
CNS	Central nervous system
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
FasL	Fas ligand
FDA	Food and Drug Administration (US)
FoxP3	Forkhead box P3
hAEC	Human amniotic epithelial cell
hAM	Human amniotic membrane
HEL	Hen egg lysozyme

HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
IgG	Immunoglobulin G
IFN-β	Interferon-β
IFNγ	Interferon-γ
IL-2	Interleukin-2
IL-5	Interleukin-5
IL-10	Interleukin-10
IL-12	Interleukin-12
MAG	Myelin-associated glycoprotein
MBP	Myelin basic protein
MHC	Major histocompatability complex
MIF	Migration inhibitory factor
MLR	Mixed lymphocyte reaction
MOG	Myelin oligodendrocyte glycoprotein
MP4	MBP-PLP fusion protein
MRI	Magnetic resonance imaging
mRNA	Messenger RNA (RNA: ribonucleic acid)

MS	Multiple sclerosis
MSC	Mesenchymal stem cell
NO	Nitric oxide
PBMC	Peripheral blood mononuclear cell
PGE2	Prostaglandin E2
PLP	Proteolipid protein
PML	Progressive multifocal leucoencephalopathy
PPMS	Primary-progressive MS
PT	Pertussis toxin
RRMS	Relapsing-remitting MS
SPMS	Secondary-progressive MS
SSEA-4	Stage-specific embryonic antigen-4
Sox-2	Sex-determining region box 2
TCR	T cell receptor
TGF-β	Tumor necrosis factor-β
Th1	Type 1 helper T cell
Th17	T helper 17 cell
TMEV	Theiler's murine encephalomyelitis virus
TNFα	Tumor necrosis factor-α

TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cell

List of Publications and Conferences

■ Publications:

Pratama G, Vaghjiani, V, Tee JY, **Liu YH**, Chan J, Tan C, Murthi P, Gargett C, Manuelpillai, U. Changes in Culture Expanded Human Amniotic Epithelial Cells: Implications for Potential Therapuetic Applications. PLoS ONE, 2012; 6(11): e26136.

Liu YH, Vaghjiani V, Tee JY, To K, Cui P, Oh DY, Manuelpillai U, Toh BH, Chan J. Amniotic Epithelial Cells from the Human Placenta Potently Suppresses a Mouse Model of Multiple Sclerosis. PLoS ONE, 2012; 7(4):e35758.

Tee JY, Vaghjiani V, **Liu YH**, Murthi P, Chan J, Manuelpillai U. Immunogenicity and Immunomodulatory Properties of Hepatocyte-like Cells Derived from Human Amniotic Epithelial Cells. Curr Stem Cell Res Ther. 2013 Jan;8(1):91-9.

Liu YH, Chan J, Vaghjiani V, Murthi P, Manuelpillai U, Toh BH. Human Amniotic Epithelial Cells Suppress Relapse of Corticosteroid-remitted Experimental Autoimmune Disease. Cytotherapy, 2013 (In Press).

■ Conference:

2009: 10th FIMSA Advanced Immunology Training Course (Queensland, Australia).

2009: Annual Scientific Meeting of Australasian Society of Immunology (Queensland, Australia).

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2012: YH Liu, *et al*. Amniotic epithelial cells from the human placenta suppress a mouse model of multiple sclerosis. **International Placenta Stem Cell Society (IPLASS) 2nd meeting (Vienna, Austria), oral presentation.** (Abstract included in Journal of Tissue Engineering and Regenerative Medicine, Sep 2012; 6, Suppl 1, p458)

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Chapter 1: General Introduction

1.1 Introduction to Autoimmunity

Autoimmune diseases affect approximately 5% of the population in Australia and New Zealand (Australasian Society of Clinical Immunology and Allergy: http://www.allergy.org.au/patients/autoimmunity/autoimmune-diseases). They are due to immune responses mediated by lymphocytes against distinct autoantigens. These lymphocyte responses are normally prevented by multiple mechanisms to maintain an immune system that fights foreign materials and ignores self. During lymphocyte generation, random recombination generates lymphocyte clones having diverse receptor specificity including those that are potentially harmful by recognizing autoantigens; but in the mature lymphocyte repertoire, most of these harmful clones are removed by clonal deletion before they can reach the mature stage (Goodnow, Sprent et al. 2005). Even if they escape clonal deletion and do mature, the subsequent checkpoints will make them non-reactive by the lack of costimulatory signals or by inhibitory signals from soluble molecules secreted by suppressor lymphocyte populations (Nossal 2001; Kronenberg and Rudensky 2005).

1.1.1 Central and Peripheral Tolerance

Lymphocytes are developed in primary lymphoid organs (thymus and bone marrow) and distributed to peripheral lymphoid organs (spleen, lymph node and gut-associated lymphoid tissues). Tolerance mechanisms employed by primary lymphoid organs are defined as "central tolerance". For lymphocytes that escaped this tolerance mechanism, they could be further regulated by "peripheral tolerance" in the peripheral/secondary lymphoid tissues such as lymph nodes, tonsils, spleen, and Peyer's patches. Any defects in these mechanisms could result in autoimmunity (Goodnow, Sprent et al. 2005).

In the primary lymphoid organs, precursors of T and B lymphocytes have universal genes that undergo genetic recombination within the V(D)J segments to generate distinct clones with unique antigen specificity and, contribute to the vast receptor diversities so as to recognize all possible antigens. The mechanism of central tolerance reside on controlling self-reactive immature lymphocytes, which "experience" strong high affinity binding of their receptors to autoantigens expressed in the thymus and bone marrow. These cells may be triggered to die (clonal deletion) (Surh and Sprent 1994) or undergo further epitope editing during development (*via* V(D)J recombination or somatic hypermutation) (Lang, Arnold et al. 1997; Li, Woo et al. 2004; von Essen, Kongsbak et al. 2012). Therefore, only cells which are unable to bind to autoantigens will survive in this so-called "negative selection" process (Morris and Allen 2012). Mature, lower-affinity self-reactive clones leaving the primary lymphoid organs are controlled by "peripheral tolerance" mechanisms such as clonal anergy (Goodnow, Crosbie et al. 1988), clonal suppression (by regulatory T cells or inhibition by

co-inhibitory molecules), apoptosis, and even the transition to a tolerated/regulatory lineage (Figs. 1 and 2). Autoimmunity arises when these central and/or peripheral tolerance mechanisms break down, leading to the immune system recognising autoantigens, followed by activation and proliferation of self-reactive clones, and resulting in damage to self tissues and organs (Schuetz, Niehues et al. 2010).

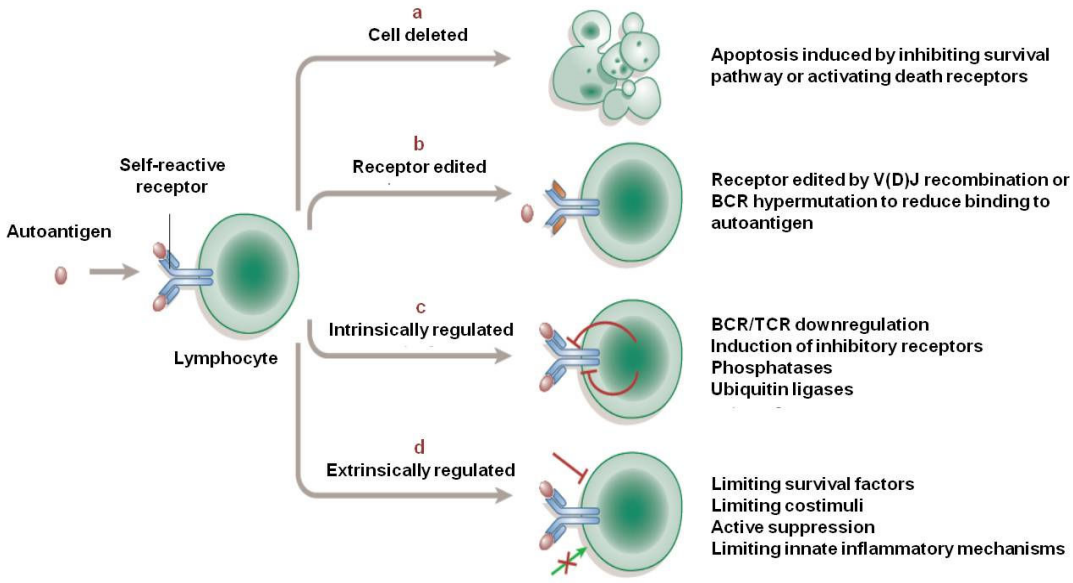

Figure. 1. Regulation of self-reactive lymphocytes: the cellular strategies employed during T and B cell differentiation.

Damage of autoimmunity from self-reactive T or B cells can be prevented by "censor" mechanisms including: a) apoptosis (programmed cell death); b) specificity of lymphocyte receptor editing through genetic recombination (T cells) or point mutation (B cells); c) changes in intrinsic signal pathways in self-reactive lymphocytes that inhibit receptor activity or downregulation of lymphocyte receptors; d) self-reactive lymphocytes receiving less external signals (limited survival factors, costimuli, or inflammatory mediators) or extra suppression signals (from soluble factors or regulator cells). Adapted from (Goodnow, Sprent et al. 2005).

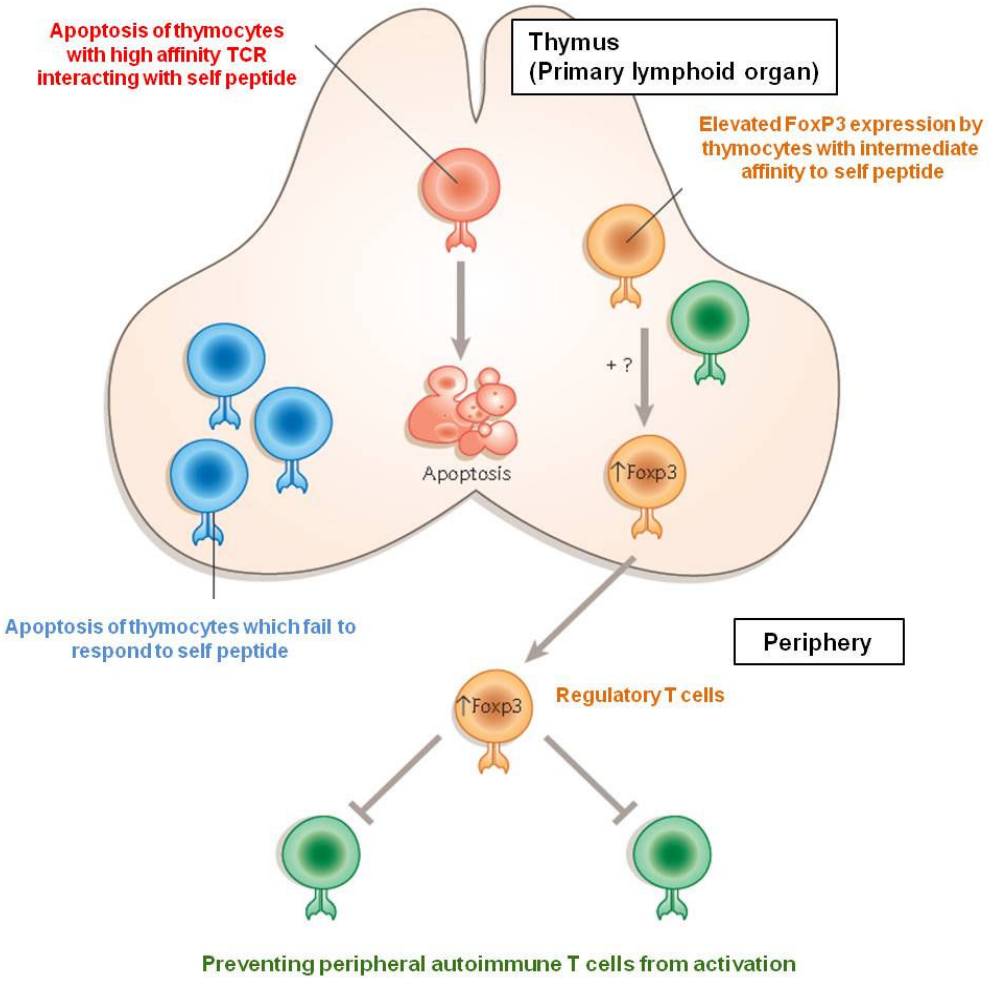

Figure. 2. Selection leads to different fates of thymocytes.

Thymocytes develop in the central lymphoid organ to T cells. Some with high affinity T cell receptor (TCR) specific to self peptide are deleted (red cells). Some with the TCR that do not respond to self peptide undergo apoptosis (blue cells). Those with TCR that interact with self peptide weakly are positively selected into conventional $CD4^+$ and $CD8^+$ lineages. Others with TCR which moderately interact with self peptide induce regulatory T cell (Treg) lineage (orange cells) and prevent activation of autoreactive T cells in the periphery. Adapted from Kronenberg (Kronenberg and Rudensky 2005).

1.2 Autoimmune Diseases

For each of the autoimmune diseases, there are usually genetic, environmental, and pathological factors that contribute to the etiology of the disease and disease progression. Once autoimmunity begins, it could become a self-perpetuating chronic inflammatory disease driven by immune responses to the distinct autoantigens widespread in tissues/organs (Tabas and Glass 2013). Autoimmune diseases can be generally classified as organ-specific or systemic. Organ-specific autoimmune diseases include diseases that only affect specific organs or localized tissues of the body, such as type 1 diabetes mellitus and multiple sclerosis, with the former primarily affecting pancreatic islets and the latter affecting the myelin sheath of the central nervous system. Systemic autoimmune diseases affect multiple organs with systemic lupus erythematosus, in which antibodies against ubiquitous antigens attack multiple organs in the body, being a prime example.

1.3 Multiple Sclerosis

Multiple sclerosis (MS) is considered a T cell-mediated neurological demyelinating disease affecting more than 2.1 million people worldwide (National MS Society (USA): http://www.nationalmssociety.org). Treatments for MS are available but not highly effective. It affects the central nervous system (CNS) to cause visual disturbances, motor and sensory impairments, balance issues, and cognitive deficits (Repovic and Lublin 2011). With its initial cases having the pathological features of demyelination,

glial scars, and sparing of axons in lesions described and reviewed by Jean Martin Charcot in 1868, MS was later identified as a distinct disease (Putnam 1943). The manifestation of MS is variable among individuals, but it often consists a long silent phase in clinical symptoms. There are around 85% of patients who have their disease phase starting from reversible intermittent episodes of neurological disability, usually described as “attacks” or “relapses”. This reversible phase, called relapsing-remitting MS (RRMS), lasts between 5 to 30 years. It usually turns into an irreversible exacerbation with fewer attacks to cause physical and cognitive deterioration, called secondary-progressive MS (SPMS) (Ransohoff 2012). On the other hand, there are 15% of MS patients who have the primary-progressive MS (PPMS) from the onset of disease, with rare or no relapses throughout their disease course (Sospedra and Martin 2005). The clinical presentations of MS include oligoclonal immunoglobulin G (IgG) in cerebrospinal fluid, dissemination of lesions in brain and spinal cord, and the attacks, according to McDonalds’ criteria (McDonald, Compston et al. 2001; 2004). Although these different MS phenotypes exist, there is some evidence showing similarities in pathology of MS patients from distinct phenotypes (Ransohoff 2012).

Studies have shown that genetic predisposition, environmental risk factors and infections contribute to the as yet unclear disease etiology (Hillert and Olerup 1993; Soldan and Jacobson 2001; Kalman, Albert et al. 2002; Ascherio, Munger et al. 2010). Geography, vitamin D and UV are factors known to affect MS susceptibility as the incidence of MS increases in countries further from the equator. Gender issues are also involved as females are more prone to MS than males, and sex hormones and /or sex-linked genes are linked to susceptibilities to MS (Voskuhl 2002; Alonso and Hernan 2008; Chiarini, Serana et al. 2012). The pathology of MS, including demyelination, is similar to some infectious neurological diseases caused by viruses

therefore the infection may also contribute to pathogenic responses (Fazakerley and Walker 2003). Autoreactive type 1 helper T cell (Th1) targeting myelin sheath in the CNS serve as a main role to mediate the destructive autoimmune responses (McFarland and Martin 2007; Fugger, Friese et al. 2009).

1.3.1 Pathogenesis of Multiple Sclerosis

Even though the detailed mechanism of MS pathogenesis is not entirely known today, it is hypothesized that MS is an autoimmune disease starting from abnormal immune responses to environmental pathogens in genetically predisposed individual, and the defects in immunoregulatory mechanisms further proceed disease process (Bar-Or, Oliveira et al. 1999; Ermann and Fathman 2001; Jacobson and Cross 2001; Kenealy, Pericak-Vance et al. 2003). The disease progress of MS is outlined in Fig. 3. The immune responses toward CNS components initiate MS pathogenesis and cause damage to local cells and tissues. The pathological hallmarks of MS that occur in CNS including blood-brain barrier (BBB) leakage, perivascular inflammation, oligodendrocyte damage or cell death, demyelination, glial scar formation and axonal loss (Frohman, Racke et al. 2006). These features are described in the following paragraphs.

Cerebral endothelial cells build the tight junction to prevent entry of harmful substances from bloodstream to cerebrospinal fluids. This barrier is called blood-brain barrier (BBB). In MS the release of inflammatory cytokines and chemokines was found parallel with the compromised state of BBB, enabling leukocytes to pass across

the barrier and hence initiate CNS inflammation resulting from BBB leakage (Minagar and Alexander 2003). The outcomes of CNS inflammation can sometimes be beneficial to promote neuro-regeneration and neuro-protection, but in most cases it is detrimental to incur tissue injury (Yong 2009).

Following BBB leakage, recruitment of lymphocytes and monocytes/macrophages passing the BBB initiates and exacerbates CNS inflammation and therefore these cells can be seen in large numbers as inflammatory infiltrates, usually appearing in periventricular white matter of the brain. Plaques in focal areas in the white matter can be seen by magnetic resonance imaging (MRI) due to inflammatory-mediated demyelination (Horowitz, Kaplan et al. 1989). The existence of lesions is the cause of reversible disability in patients.

Oligodendrocytes are responsible for myelin formation. In MS, it is proposed that apoptosis cascades are triggered in oligodendrocytes, leading to oligodendrocyte depletion and therefore the damaged myelin cannot be repaired. Besides the damage to oligodendrocytes, neuroantigen-specific antibodies and complement may also cause demyelination and therefore exposing axons that had been protected by myelin (Lucchinetti, Bruck et al. 2000). The repair process of myelin, called remyelination, occurs after demyelination or sometimes together with demyelination (Franklin and Ffrench-Constant 2008).

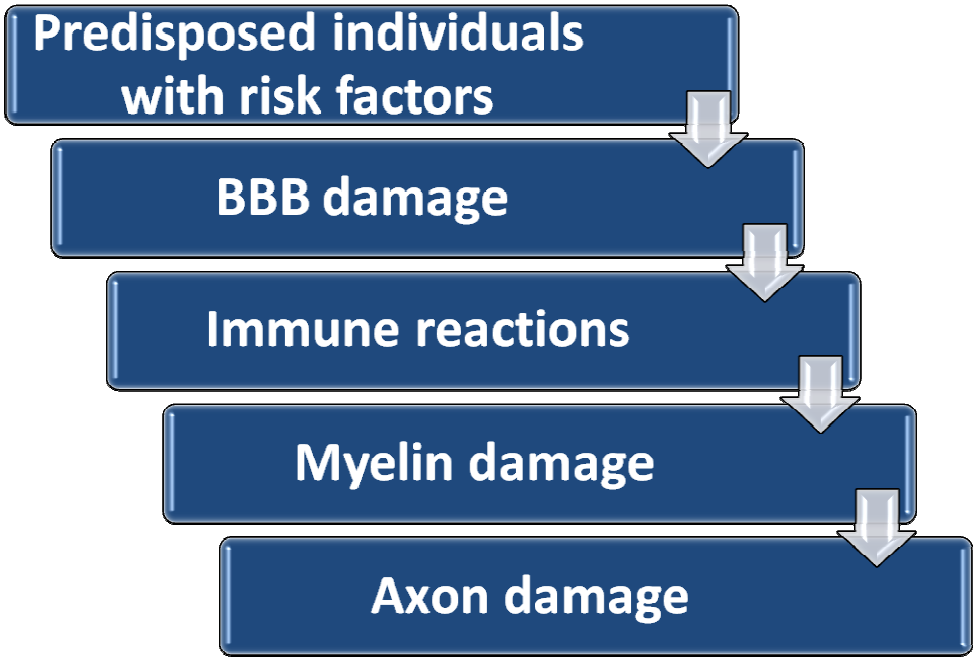

Figure. 3. Current view of MS disease progression. The immune-mediated disease occurs in genetic predisposed individuals exposed to environmental risk factors. With break of blood-brain barrier integrity, immune cells enter the CNS and cause damage to local cells and tissues. Adapted from Murray (Murray 2009).

Axonal loss is usually considered the direct consequences of demyelination. The extent of axonal damage correlates with inflammatory infiltrate within early MS lesions. Axonal loss accumulates silently in patients with remitting-relapsing phase of MS, until it reaches a threshold of axonal loss that develops into irreversible neurological damage in the CNS. At this stage, patients who have increased disability enter the progressive phase of MS (Dutta and Trapp 2007). The key concept of myelin loss that causes axon damage is illustrated in Fig. 4.

In histopathology which obtained from post-mortem examination, PPMS patients have shown more extensive spinal cord lesions, while SPMS patients have bigger brain lesions. PPMS patients also have more inflammatory demyelination lesions, while SPMS patients have more extensive remyelination (Kipp, van der Valk et al. 2012).

The inflammatory reaction in MS lesions is associated with the upregulation of a variety of Th1 and Th17 cytokines such as IL-2, IL-12, IFN-γ, and TNF-α. They are also found in the cerebrospinal fluid of MS patients. Hence it is suggested that MS is Th1 and Th17 mediated autoimmune disease (Pittock and Lucchinetti 2007).

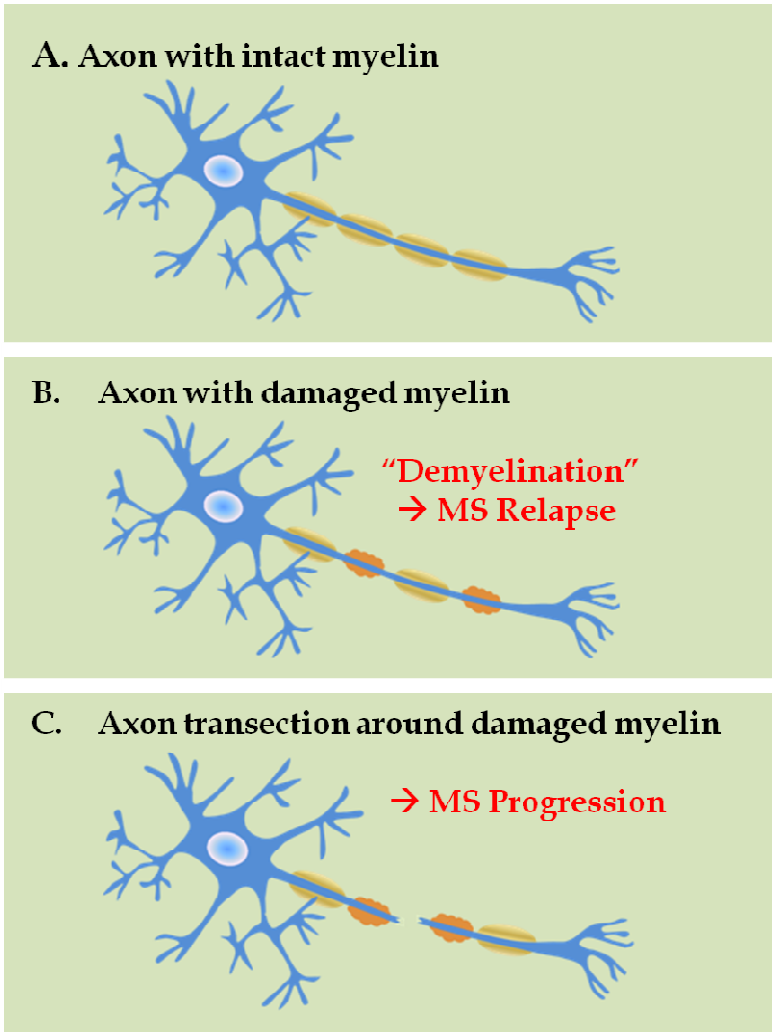

Figure. 4. Myelin loss causes axon damage in MS.

A) Normal axons (blue) are protected by surrounding myelin sheath (yellow) and are able to transmit nerve impulses. B) The start of MS triggers immune responses toward CNS myelin and therefore the loss of myelin, called demyelination (orange), occurring as relapses in patients with relapsing-remitting MS. C) The demyelinated axons are transected, mostly occurring in later stage of chronic MS when the disease has entered a progressive course in which patients have irreversible neurological disabilities.

1.3.1.1 B cell-mediated Responses

It has been suggested that B cells and antibodies have pathogenic roles in MS. Increased intrathecal IgG in patients with MS has been observed in most patients. Measurement of these elevated antibodies along with oligoclonal IgG bands in the cerebrospinal fluid is the important immune parameter in the diagnosis of MS. This substantially increased IgG in cerebrospinal fluid may be important in pathogenesis, as autoantibody levels have been linked to MS disease severities both in patients and in animal model (Iglesias, Bauer et al. 2001; Weber, Hemmer et al. 2011). Also, neuroantigen-specific autoantibodies found in MS lesions are shown to be essential for demyelination (Lucchinetti, Bruck et al. 1996). B cells undergoing clonal expansion have also been found in MS lesions and in cerebrospinal fluid (Baranzini, Jeong et al. 1999; Colombo, Dono et al. 2000). Use of anti-CD20 monoclonal antibodies in MS patients for B cell depletion provided the direct evidence to support the above mentioned studies. However, other antibodies targeting B-cell-activating factor and its receptor have shown the opposite results from MS patients, making the role of B cells in MS pathogenesis uncertain (Hauser, Waubant et al. 2008; Hartung and Kieseier 2010).

1.3.1.2 T cell-mediated Responses

Autoreactive $CD4^+$ T helper 1 cells are considered the main players in MS pathogenesis (Stinissen, Raus et al. 1997; McFarland and Martin 2007). T cell involvement is evidenced by cell composition in the cerebrospinal fluid (Zhang,

Markovic-Plese et al. 1994). The numbers of myelin-reactive T cells are similar between patients with MS and healthy persons, but those myelin-reactive T cells in patients with MS appear more of the memory or activated phenotype (Lovett-Racke, Trotter et al. 1998; Scholz, Patton et al. 1998). Similarly, in the animal model of MS, activation of myelin-specific $CD4^+$ Th1 cells that produce interferon-γ have been shown to mediate the disease (Ando, Clayton et al. 1989). Th1 and Th17 cells exhibit pro-inflammatory phenotype while Th2 cells exhibit anti-inflammatory phenotype, and studies have provided the evidence that Th1 and Th17 cell responses together mediate the pathogenesis of MS (Bettelli, Sullivan et al. 2004; Damsker, Hansen et al. 2010). But the imbalance of Th1/Th2 cytokines cannot fully explain the overall pathogenesis in the murine MS model, as mice deficient in some molecules related to Th1 differentiation are susceptible to developing the disease in the animal model of MS (Ferber, Brocke et al. 1996; Becher, Durell et al. 2002; Gran, Zhang et al. 2002). Moreover, activated Th2 cells were shown to have pathogenic role in some settings (Lafaille, Keere et al. 1997), making the absolute role of each T cell subset more difficult to define.

Regulatory T cells play a key role in suppressing effector T cells. In MS patients, both the reduced numbers of peripheral regulatory T cells and the loss of transcription factor Forkhead box P3 (FoxP3) expression in regulatory T cells have been observed in different reports when compared to healthy individuals (Viglietta, Baecher-Allan et al. 2004; McKay, Swain et al. 2008; Venken, Hellings et al. 2008). In addition, the effector T cells from some patients have shown resistance to the inhibition from regulatory T cells (Schneider, Long et al. 2013). Altered Treg function may worsen disease in MS patients because of breakdown of tolerance to myelin protein components (Viglietta, Baecher-Allan et al. 2004). It has also been shown that MS therapy has changed the

distribution of natural Treg in patients (Chiarini, Serana et al. 2012). Therefore, the maintenance of Treg to control local inflammation should be one of the important issues in MS pathogenesis.

1.3.1.3 The Role of Myelin in the Central Nervous System

The components of myelin include myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), and myelin-associated glycoprotein (MAG), shown in Fig. 5. Because myelin insulates the nerve and protects it from injury, any immune response targeting the components of myelin may cause destruction of myelin (demyelination), damage of neurons, and severe neurological inflammation in the CNS. The structure of axons surrounded by myelin sheath which extends from the oligodendrocytes, is shown in Fig. 6.

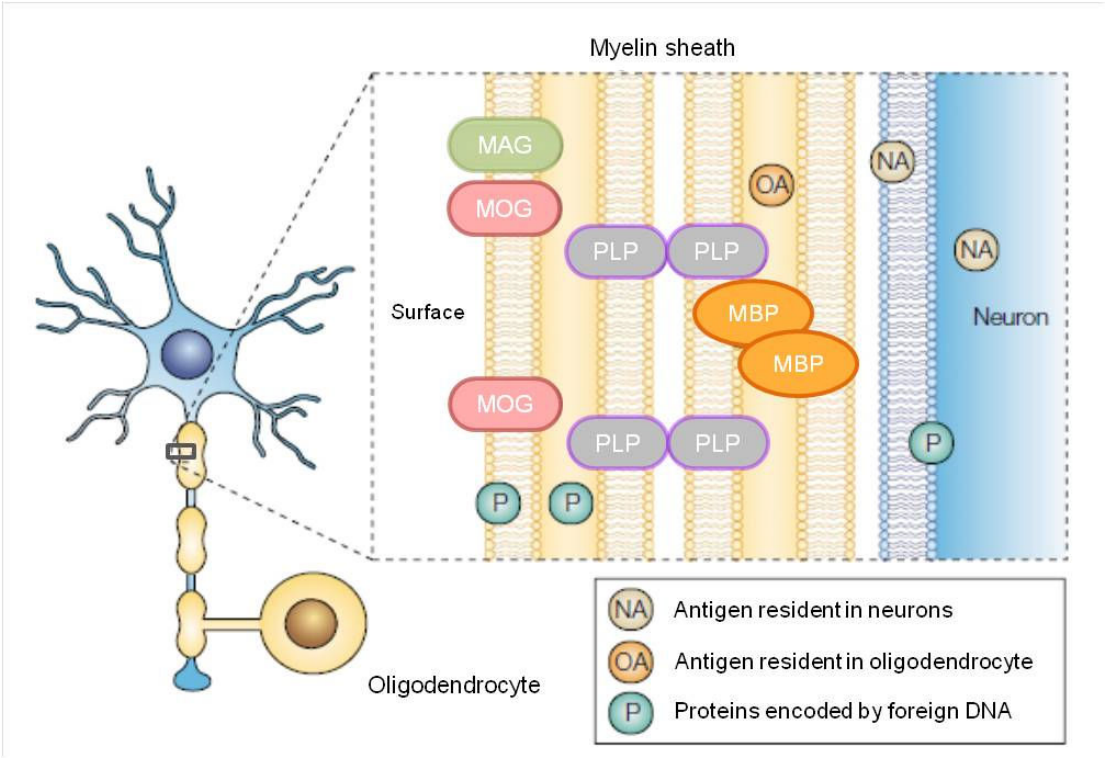

Figure. 5. Components of myelin sheath.

The different protein components of myelin sheath can serve as autoantigens and be attacked by the immune system during the pathogenesis of MS (MAG: myelin-associated glycoprotein; MOG: myelin oligodendrocyte glycoprotein; PLP: proteolipid protein; MBP: myelin basic protein). Adapted from Hemmer *et al* (Hemmer, Archelos et al. 2002).

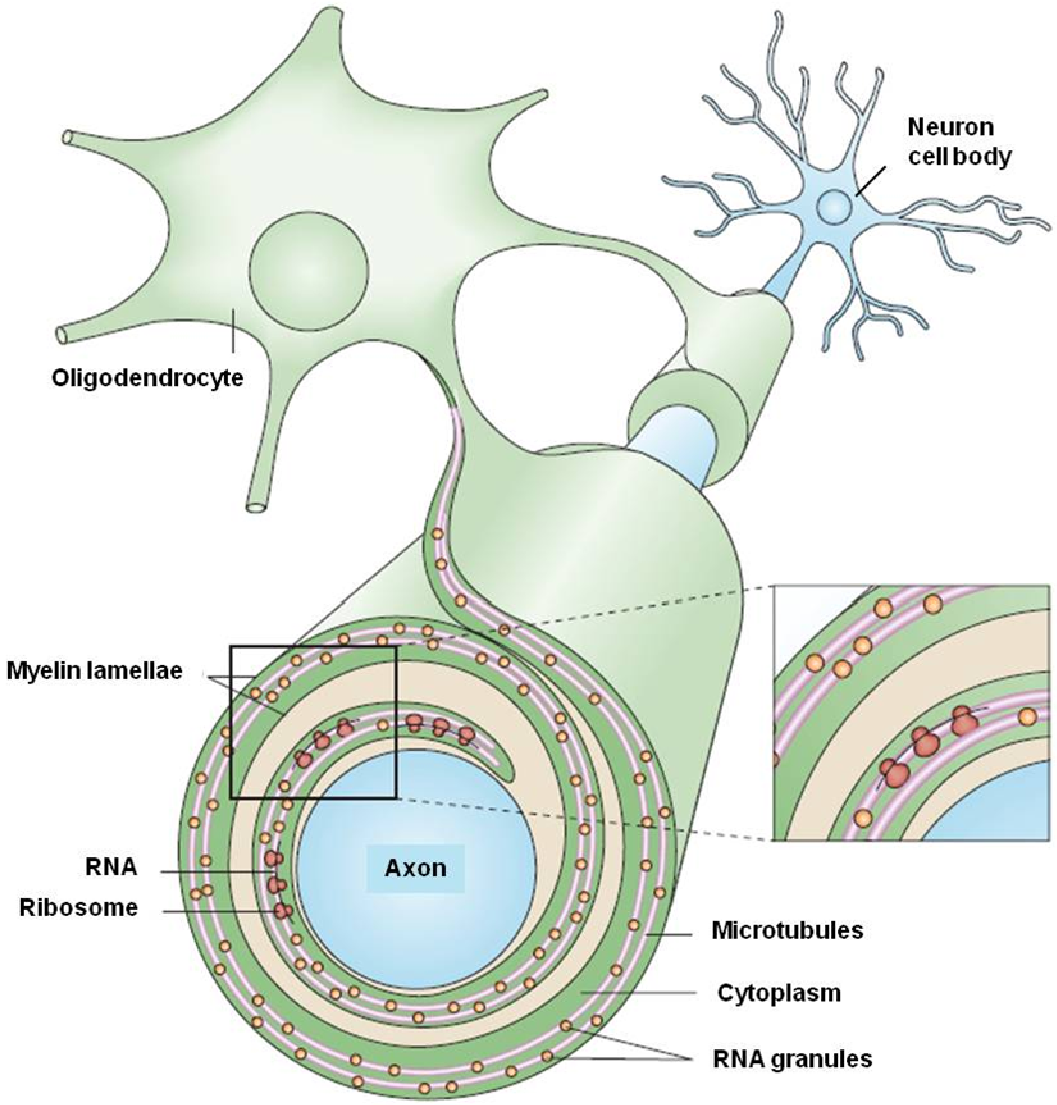

Figure. 6. Axon is insulated by myelin sheath produced by oligodendrocyte.

Neuron cell body has an elongated axon wrapped with myelin lamellae produced by the oligodendrocyte. Myelin proteins are generated *via* microtubule-based transport from the nucleus to the distant myelin lamellae. Adapted from Sherman *et al* (Sherman and Brophy 2005).

1.3.2 Animal Model of Multiple Sclerosis: Experimental Autoimmune Encephalomyelitis

Considering the difficulties of studying MS in humans, animal models of MS, are widely used for research. Different types of MS models have been generated to mimic part of the disease, but some of these models are only suitable for studying part of the disease. For example, cuprizone model is ideal for studying de- and re-myelination, but lacking T cell infiltrates in CNS (Kipp, Clarner et al. 2009). TMEV model, which is induced by inoculation of Theiler's murine encephalomyelitis virus, helps investigating the relationship between virus infection and CNS autoimmunity (Tsunoda and Fujinami 2010). The most commonly used MS model is experimental autoimmune encephalomyelitis (EAE) which represents many parts of the disease, both pathologic and immunological (Batoulis, Recks et al. 2011). The first EAE model was generated in the 1930s by immunization of CNS homogenate in non-human primates (Rivers and Schwentker 1935). Today, EAE can be induced in different wild-type species with injection of myelin components in emulsion with suitable adjuvants, or *via* passive transfer of encephalitogenic T cells (Stromnes and Goverman 2006; Miller and Karpus 2007).

EAE induction methods using myelin components are called "active method", while those using the transfer of antigen-specific T lymphocyte to induce EAE are called "passive method". In active methods of EAE induction, adding adjuvants while introducing neuroantigens may alter the responsiveness of the immune system and the blood barrier in CNS. On the other hand, passive methods, without the effect of introducing extra substances, involve transfer of neuroantigen-specific T lymphocytes

cultured *in vitro* (Voskuhl 1996). The above mentioned conditions could be disadvantages when studying T lymphocyte behaviors in the specific model.

Besides induced EAE, spontaneous EAE models can be generated in neuroantigen-specific T cell receptor transgenic mice (Krishnamoorthy, Holz et al. 2007). The details of disease course, type of introduced autoantigens, animal strains, similarities to human MS, and shortcomings of some commonly used murine EAE models are discussed in Table 1. Selected models are actively induced by neuroantigens, while other transgenic mouse models exhibit spontaneous disease course.

One of the most common EAE model is generated by immunization of myelin oligodendrocyte glycoprotein (MOG) peptide along with an emulsified solution that contains complete Freund's adjuvant (CFA) and heat-killed *Mycobacterium tuberculosis*, which boosts the proinflammatory response. Injections of pertussis toxin in C57Bl/6 female mice are required to promote EAE possibly *via* assisting T cells to enter CNS across blood-brain barrier and augmenting MOG-specific responses (Hofstetter, Shive et al. 2002; Chen, Winkler-Pickett et al. 2006). It induces a chronic, relapsing and progressive disease with CNS demyelination which is triggered by a mixed Th1- and Th17- immunity (Tigno-Aranjuez, Jaini et al. 2009; Kuerten and Lehmann 2011). Beside the consensus T cell involvement, this model also suggests an important role of autoantibodies in disease development (Taneja and David 2001).

MOG-induced EAE is considered a straight-forward model of MS. The severity of EAE is correlated with frequencies of MOG-specific T cells before disease onset, and MOG-specific T cell populations are maintained during the disease course. For the

other commonly used relapsing-remitting EAE model induced by proteolipid protein (PLP), PLP-induced SJL mice, the correlation is not as clear as MOG-induced EAE mice and the frequencies of PLP-specific T cells fluctuate during the relapses. So there may be more difficulties to relate EAE severity by constantly monitoring T cell response in other models (Kuerten and Lehmann 2011).

EAE is like human MS in that the pathogenesis involves T helper cells-induced immune responses (Stinissen, Raus et al. 1997; Hafler, Slavik et al. 2005; Domingues, Mues et al. 2010). Therefore, passive method as adoptive transfer of CNS antigen-activated T cells has been tested and shown to be sufficient to induce EAE in mice (Zamvil and Steinman 1990; Engelhardt 2006). Activation of CNS-reactive T cells initiates local microglia expansion and recruitment of blood-borne monocytic cells (Hickey, Hsu et al. 1991; Swanborg 1995). These cells secrete pro-inflammatory cytokines and participate in demyelination (Benveniste 1997). Monocytic infiltration into the CNS is correlated with progression of clinical disease and blocking their infiltration prevents EAE progression (Ajami, Bennett et al. 2011). Therefore, monitoring the existence and quantity of these infiltrated cells is important in animal models of multiple sclerosis.

Animals with EAE display clinical and pathological characteristics that are similar to MS in humans, such as paralytic symptoms, demyelination, perivascular mononuclear cell infiltrate in the CNS, and neuroantigen-specific T cell responses (Irani 2005; Steinman and Zamvil 2006). Several approved treatments for MS have been developed in the EAE model prior to clinical trials, showing that EAE is valuable to development of possible therapies (Ridge, Sloboda et al. 1985; Yednock, Cannon et al. 1992; Steinman and Zamvil 2006). However, it is important to aware of some differences

between commonly used EAE and MS. For example, there is no gender bias in MOG-induced EAE in C57BL/6 mice (Okuda, Okuda et al. 2002).

Mouse strain	Disease course	Immunogen + adjuvant	Similarities to human MS	Shortcomings of the model	Reference
PL/J	Acute monophasic	MBP Ac_{1-11} (or Ac_{1-9}) + CFA and PT	Demyelination, axonal damage	No spontaneous disease, no chronic relapsing disease	(Zamvil, Mitchell et al. 1986)
C57Bl/6	Chronic progressive	MOG_{35-55} + CFA and PT	$CD4^+$ T, $CD8^+$ T, Treg, Th17, B cells, antibodies, DC, microglia/macrophage, complement, demyelination, axonal damage	No spontaneous disease, lack of dynamic CNS inflammation and B cell infiltration	(Mendel, Kerlero de Rosbo et al. 1995; Oliver, Lyon et al. 2003)
C57Bl/6 TCR^{MBP} transgenic	Optic neuritis	None	Brain and cerebellum-involvement	Artificial transgenic background	(Bettelli, Pagany et al. 2003)
C57Bl/6 $TCR^{MOG}{}_X$ IgH^{MOG} transgenic	Acute monophasic	None	Spontaneous onset, optico-spinal disease, T and B cell involvement	Artificial transgenic background	(Bettelli, Baeten et al. 2006; Krishnamoorthy, Lassmann et al. 2006)
C57Bl/6 (MP4-induced EAE)	Acute or chronic monophasic	MP4/Apogen + CFA with HEL	B cell-dependent	No spontaneous disease, no chronic relapsing disease	(Kuerten, Javeri et al. 2008)
SJL MOG-specific TCR transgenic	Remitting-relapsing	None	B cell-dependent	Artificial transgenic background	(Pollinger, Krishnamoorthy et al. 2009)
SJL	Relapsing progressive	TMEV infection	$CD4^+$ T cell	No spontaneous disease	(Olson, Croxford et al. 2001)
SJL	Remitting-relapsing	$PLP_{139-151}$ + CFA and PT	$CD4^+$ T, Treg,Th17, DC, demyelination, axonal damage	No spontaneous disease	(McRae, Kennedy et al. 1992)
Biozzi AB/H	Remitting-relapsing	Spinal cord homogenate + CFA and PT	$CD4^+$ T, microglia/macrophage, demyelination, axonal damage	No spontaneous disease	(Baker, O'Neill et al. 1990)
B10.PL-$H2^u$ TCR^{MBP} transgenic	Acute monophasic	None	T cell response, spinal cord inflammation	background, no chronic relapsing disease	(Goverman, Woods et al. 1993)

Table. 1. Comparison of selected murine EAE models.

(Abbreviations: CNS, central nervous system; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; PLP, proteolipid protein; MP4, MBP-PLP fusion protein; CFA, complete Freund's adjuvant; PT, pertussis toxin; TMEV, Theiler's murine encephalomyelitis virus; HEL: hen egg lysozyme; Treg, regulatory T cells; DC, dendritic cells; TCR, T cell receptor; AB/H, antibody-high strain.)

1.4 Current Therapies for Multiple Sclerosis

MS can be treated with immunosuppressive agents, immunomodulatory drugs and humanized monoclonal antibodies. These therapies are only partially effective and often accompanied by undesirable side effects (Saidha, Eckstein et al. 2012). I will discuss some representative therapies that are used to treat MS patients, as well as briefly introduce the newly-developed drugs.

1.4.1 First-line therapies

The following therapies are common first-line disease-modifying therapies, including corticosteroids, IFN-β and glatiramer acetate:

Corticosteroids

Corticosteroids are among the few strategies targeted to treat attacks, rather than slowing down the disease progression. Short-term treatment with corticosteroids, such as intravenous methylprednisolone, prednisolone, or oral prednisone, is widely used for treating of MS exacerbations (Frohman, Shah et al. 2007). Corticosteroids reduce inflammation to control recurrent relapses and were shown to prevent cytokine-induced cell death on oligodendrocyte (Melcangi, Cavarretta et al. 2000), reduces expression of adhesion molecules and proinflammatory cytokines, and also the number of circulating lymphocytes (Burton, O'Connor et al. 2009). Short-term use of corticosteroids has been reported to be associated with none or few minor adverse effects in clinical trials of MS (Barnes, Bateman et al. 1985; Durelli, Cocito et al. 1986;

Milligan, Newcombe et al. 1987; Barnes, Hughes et al. 1997; Martinelli, Rocca et al. 2009). Most of the adverse events are not lethal, such as gastrointestinal symptoms, but the rarely occurred infections due to immunosupression could be life-threatening (Berkovich 2013).

Interferon-β (IFN-β)

This immunomodulator IFNβ-1b has been applied as one of the major MS treatments since its approval by the Food and Drug Administration (FDA) in 1993. The close family member IFNβ-1a also has been widely used for MS patients since its approval in 1996. They reduce the frequency of relapses and was the first approved medication to do so (Bakshi 2013). Their mechanisms of action rely on interaction with matrix metalloproteinases and adhesion molecules to reduce blood-brain barrier permeability and inhibit trafficking of T cells into CNS. These result in downregulation of proinflammatory cytokines, upregulation of anti-inflammatory cytokines, induction of apoptosis and increase of the regulatory T cell population (Sega, Wraber et al. 2004; Graber, McGraw et al. 2010; Kieseier 2011). However, about 30-50% of MS patients did not respond well to IFN-β treatment (Axtell, de Jong et al. 2010). Additionally, IFN-β could also be given together with other disease-modifying drugs for better therapeutic result (Tullman and Lublin 2005).

Glatiramer Acetate

Glatiramer acetate is a synthetic random copolymer of amino acids mimicking myelin basic protein components. Synthesis of the first form of this drug, designated copolymer 1, originally aimed to understand how myelin basic protein induce MS in

animal model but surprisingly suppressed MS in mice and proceeded to clinical trial (Arnon 1996; McGraw and Lublin 2013). Glatiramer acetate is a standardized combination of four kinds of amino acids in a fixed ratio (Carter and Keating 2010). Its mechanisms of action involves binding to major histocompatability complex (MHC) class II on antigen presenting cells to induce anti-inflammatory Th2 response, and stimulate neurotrophin secretion in the CNS to promote neuronal repair (Blanchette and Neuhaus 2008). Glatiramer acetate is often used for early treatment to reduce annual relapses and number of lesions, mainly by blocking the effector function of myelin-specific T cells through bystander suppression mediated by Th2 cells (Yong 2002; Tintore 2009).

Glatiramer acetate is usually well-tolerated but exhibit only modest effect. There has been longstanding interest in exploring combination therapies of glatiramer acetate and other disease-modifying drugs (Tullman and Lublin 2005). For example, glatiramer acetate and IFN-β combined treatment were shown to have additive effect, which was superior to each single therapy (Milo and Panitch 1995; McGraw and Lublin 2013).

Alemtuzumab

Alemtuzumab is a humanised monoclonal antibody targeting CD52 and results in lymphocyte depletion. Patients with relapsing-remitting MS treated with alemtuzumab in clinical trials experience repopulation of T and B cells that skews the immune system, together with increase in regulatory T cell numbers and anti-inflammatory cytokine production (Jones, Phuah et al. 2009; Klotz, Meuth et al. 2012). Alemtuzumab has shown superior therapeutic effect compared to IFN-β therapy even though there is notable high risk of thyroid-associated autoimmunity (Jones, Phuah et al. 2009; Cohen, Coles et al. 2012; Coles, Fox et al. 2012; Coles, Twyman et al. 2012).

1.4.2 Second-line therapies

The following are some approved second-line therapies: mitoxantrone, natalizumab, and fingolimod. They are generally more effective and convenient to deliver when compared to first line therapies IFN-β or glatiramer acetate, but unfortunately have additional safety problems. Therefore their application is recommended to highly active MS patients only, especially to patients who fail to respond to IFN-β therapy or has entered to secondary progressive phase of MS (European Medicines Agency: www.ema.europa.eu). Alternatively, some combination therapies using a second-line medication together with a first-line medication appear to be more effective than single medication in patients with relapsing-remitting MS (Tullman and Lublin 2005; Rudick, Stuart et al. 2006).

Mitoxantrone

Approved for MS in 2000, mitoxantrone is a cytotoxic immunosuppressant that is used for treating progressive forms of MS. It is an anthrecenedione that interfere DNA replication (Durr, Wallace et al. 1983). Mitoxantrone reduces clinical attack rates, MRI activity, and progression of disability of patients. These properties make mitoxantrone superior to other cytotoxic immunosuppressants (Hartung, Gonsette et al. 2002). Mitoxantrone is also able to shift the cytokine balance toward Th2 profile and that is one of the possible mechanisms for mitoxantrone to alleviate MS disease symptoms (Vogelgesang, Rosenberg et al. 2010). However, it may cause late congestive heart failure or myelodysplastic syndrome and leukemia, and currently it is only applied to severe MS patients (Ghalie, Edan et al. 2002; Marriott, Miyasaki et al. 2010).

Natalizumab

Natalizumab is a humanized monoclonal antibody that targets the α4β1 integrin, expressed by mononuclear white cells for attaching to the vessel wall for entry into the inflamed CNS (Ramos-Cejudo, Oreja-Guevara et al. 2011; Chataway and Miller 2013). Therefore, natalizumab decreases CNS inflammation mediated by neuroantigen-specific T cell and consequently reduces relapse rate and slow the progression of disability. However, because it prevents the egress of all leukocytes outside the bloodstream, patients would have higher risk of infection. The known association of natalizumab and progressive multifocal leucoencephalopathy (PML), the opportunistic brain infection caused by the JC virus, was the reason that natalizumab was temporarily withdrawn from the market (Tan and Koralnik 2010; Bloomgren, Richman et al. 2012).

Fingolimod

Fingolimod, the first oral drug for MS, is a sphingosine-1-phosphate receptor modulator that can prevent lymphocyte egress from lymph nodes, thereby reducing the infiltrating lymphocytes in CNS and showed its clinical efficacies (Scott 2011). However, fingolimod is complicated with adverse events such as cardiac arrhythmias, infections, skin cancer, and sudden death (Cohen, Barkhof et al. 2010; Lindsey, Haden-Pinneri et al. 2012).

MS treatments cannot cure the disease but generally help treating MS attacks, managing symptoms, and reducing progress of the disease. There is no best therapy as shown clearly in the above paragraphs, and prescriptions usually struggle with possible

side effects or have to compromise with lower efficacy. In the following table (Table 2), selective MS therapies are compared with their adverse events and efficacies. Some pipeline therapies which currently are under development are discussed together with their status in clinical trials.

Current therapies for MS				
Drug	**Target patient**	**Efficacy**	**Adverse events**	**FDA approval**
IFN-β	RRMS	Lower	Anaphylaxis (IFNβ-1a), lymphopaenia risk (IFNβ-1b), immunogenicity reported	1993 (IFNβ-1b), 1996 (IFNβ-1a)
Glatiramer acetate	RRMS	Lower	Usually well-tolerated	1996
Mitoxantrone	SPMS, RRMS, worsening RRMS	Higher	Cogestive heart failure, myelodysplastic syndrome, leukemia	2000
Natalizumab	Monotherapy for RRMS	Higher	Progressive multifocal leucoencephalopathy, immunogenicity reported	2004 (Initial)
Fingolimod	Relapsing forms of MS	Higher	Cardiac arrhythmias, asystole, infections, skin cancer, sudden death	2010
Teriflunomide	Relapsing forms of MS	Higher	Potential for liver damage, infections	2012

MS pipeline products				
Drug	**Target patient**	**Efficacy**	**Adverse events**	**Trial phase**
Alemtuzumab	RRMS	71% reduction in disability and 74% reduction in ARR	Immune thrombocytopenic purpura, autoimmune hyperthyroidism, Goodpasture syndrome, B cell chronic lymphocytic leukemia	3
Laquinimod	RRMS	40% reduction in lesions	Dose-dependent increase in liver enzymes, Budd-Chiari syndrome	3
Dimethyl fumarate	RRMS	~50% reduction in ARR	Headache, flushing, nausea, diarrhea	3
Daclizumab	RRMS	72% reduction in lesions	Injection-site reactions, rash, infections	3
Ocrelizumab	RRMS	~90% reduction in lesions	Infection, systemic inflammatory response, infusion-site reactions	2

Table. 2. Current available disease-modifying therapies and pipeline therapies for MS.

Abbreiviations: FDA, US Food and Drug Administration; IFN, interferon; RRMS, relapsing-remitting MS; ARR, annual relapse rate. Adapted partly from Saidha (Saidha, Eckstein et al. 2012).

1.4.3 Stem Cell Therapy

Hematopoietic stem cells (HSC) from bone marrow or peripheral blood, and mesenchymal stem cell (MSC) are the major stem cell types that have entered clinical trials for MS, based on successful results in animal models (Burt, Burns et al. 1995; Connick, Kolappan et al. 2011). HSC therapy has been used for the last one and half decades to more than 600 patients, usually with severe forms of MS refractory to conventional therapies (Mancardi, Sormani et al. 2012; Atkins and Freedman 2013).

1.4.3.1 Hematopoietic stem cells

HSC therapy can reduce or eliminate ongoing clinical relapses, halt further progression, and reduce the burden of disability in patients having aggressive highly active multiple sclerosis, to whom other treatments with disease-modifying agents fail to work (Atkins and Freedman 2013).

To prevent rejection by immune cells in HSC transplant, prior conditioning to patients ready to receive the transplants is necessary. Therefore, there are additional issues to be considered in HSC therapy, such as safety and toxicity of immuosuppressants/conditioning regimens, possible infections from these regimens, and chronic graft-versus-host disease due to the transplant. HSC graft usually contains 3-5% HSC only and with large numbers of immune cells in the graft. Immune cell load

in the graft is the reason to cause rejection of the graft, therefore some conditioning regimens with lymphocyte depletion is preferable in transplantation protocols (Gress, Emerson et al. 2010; Atkins and Freedman 2013). These are the main shortcomings of using HSC transplant in treating human diseases.

1.4.3.2 Mesenchymal stem cells

The other cell type that have entered clinical trials, mesenchymal stem cells, are multipotent non-hematopoietic precursor cells, that are an alternative cell type for cell-based therapies (Payne, Siatskas et al. 2011). MSC have been shown to inhibit myelin-specific T cell activation and antibody production, decrease inflammation, CNS pathology, and promote repair (Zappia, Casazza et al. 2005; Gerdoni, Gallo et al. 2007; Constantin, Marconi et al. 2009; Bai, Lennon et al. 2012). MSC were found to provide clinical benefits without their necessary presence in the affected organs, with most of the cells lodged within the lungs (Prockop and Youn Oh 2011; Roddy, Oh et al. 2011). Although MSC is generally considered as poorly immunogenic (Tse, Pendleton et al. 2003), there is evidence of cellular mass formation in CNS of mice (Grigoriadis, Lourbopoulos et al. 2011).

None of the currently approved stem cell/precursor cell therapies are perfect. The procurement of these cells from bone marrow is invasive, cells may require further cell expansion and conditioning before transplantation, which is relatively time-consuming and costly. The ideal stem cell should be readily available in abundant numbers, highly purified, and with consistent differentiation potential to specific lineages; while

tumorigenicity, immunogenicity and ethical issues from the cells are minimized. From this perspective a newly emerging stem cell-like cell, human amniotic epithelial cell (hAEC), will be discussed in the following paragraphs for their potential for cell therapy for MS.

1.5 Human Amniotic Epithelial Cells

Human amniotic epithelial cells (hAEC) are obtained from the amnion of the placenta. The human placenta comprises the decidua, villous placenta, umbilical cord and the amnion and chorion. The amnion is derived from the embryo (Fig. 7). hAEC arise from the epiblast of the embryo at about 8 days after fertilization and before gastrulation of the embryo. The epiblast cells migrate outwards and form a monolayer lining the amnion membrane which encloses the amniotic cavity and the embryo (Akle, Adinolfi et al. 1981). The hAEC have stem cell-like features, are readily accessible in large quantities from otherwise discarded gestational tissue after childbirth. Isolation of hAEC post-parturition for clinical applications avoids extended *ex vivo* expansion or ethical concerns arising from bone marrow and embryo-derived stem cells, therefore the cost and time involved for cell expansion can be minimized (Miki 2011).

Besides the advantages described above, hAEC express low levels of Class IA human leukocyte antigens (HLA) and lack of Class II antigens, which may potentially reduce the risk of immune-rejection after hAEC transplantation. This makes them a tolerogenic cell type (Ilancheran, Michalska et al. 2007; Parolini, Alviano et al. 2008). Previous studies have shown that hAEC also have immunomodulatory properties and

inhibit mixed lymphocyte reactions and mitogen stimulated T cell proliferation (Bailo, Soncini et al. 2004; Wolbank, Peterbauer et al. 2007) where some of these effects may be attributed to secreted factors (Li, Niederkorn et al. 2005). hAEC also exhibits anti-inflammatory and anti-fibrotic effects in tissue fibrosis models and ameliorate neurological diseases in animal studies (Cargnoni, Gibelli et al. 2009; Manuelpillai, Tchongue et al. 2010; Parolini and Caruso 2011).

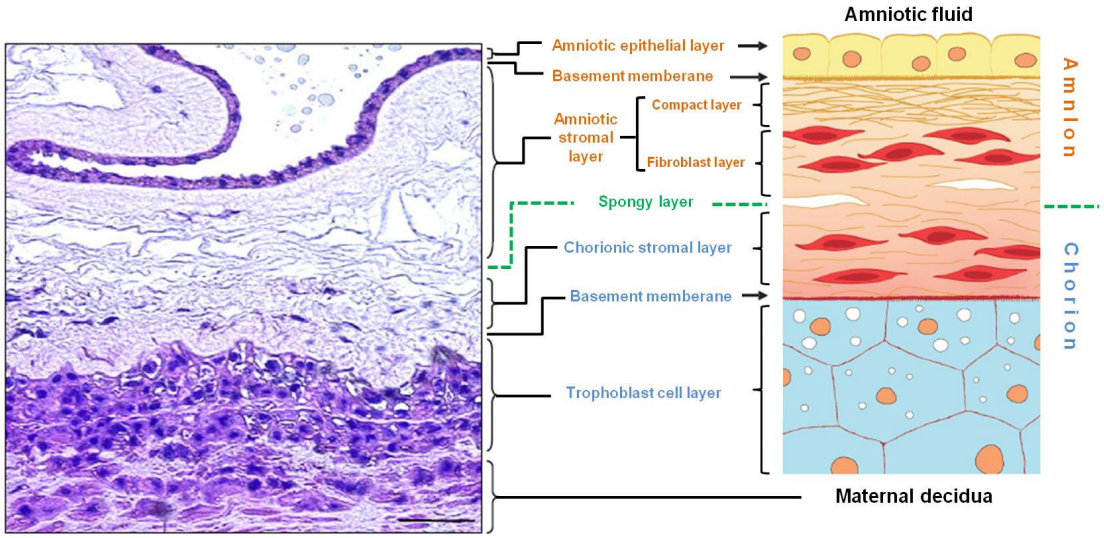

Fig. 7. Human term fetal membranes.

Haematoxylin-eosin stained cross section of fetal membranes shows composition of amnion and chorion shown on the left. Scale bar = 50 µm. Schematic representation on the right illustrates the different layers of amnion and chorion, showing amnion enclosing amniontic fluid, and chorion adherent onto maternal decidua. The amnion consists of a single layer of epithelial cells (AEC) resting on basement membrane and stromal layer. Going further toward the maternal side across the spongy layer is the chorion, consisting of stromal layer (reticular layer) and trophoblast cell layer. Maternal decidual cells adherent to the chorion can also be seen from the picture. Adapted from Ilancheran *et al* and Dobreva *et al* (Ilancheran, Moodley et al. 2009; Dobreva, Pereira et al. 2010).

1.5.1 hAEC in Regenerative Medicine and Other Applications

Human amniotic membrane (hAM) have been utilized for various clinical applications, such as ophthalmic and skin reconstruction, and sometimes as dressings to stimulate healing of skin/ocular wounds, facilitating wound repair and anti-inflammation in these cases (Gajiwala and Gajiwala 2004; Tosi, Massaro-Giordano et al. 2005; Tejwani, Kolari et al. 2007). In various studies hAM has shown their abilities to secrete anti-inflammatory cytokines and to display anti-bacterial, anti-viral anti-angiogenic and pro-apototic characteristics (Mamede, Carvalho et al. 2012). hAEC, as the important component in the hAM, have shown some of these features of hAM (Li, Niederkorn et al. 2005; Niknejad, Khayat-Khoei et al. 2013). hAEC transplantation was shown to be beneficial in experimental ophthalmic restorations as well, suggesting hAEC as a therapy for these applications (He, Alizadeh et al. 1999; Parmar, Alizadeh et al. 2006). In cell culture hAEC could form feeder layers to support the growth of other stem cells as well as maintaining pluripotency of these cells (Liu, Cheng et al. 2010; Liu, Cheng et al. 2012). More recently hAEC have also been shown to be effective for tendon and salivary gland regeneration in animal models (Barboni, Russo et al. 2012; Zhang, Huang et al. 2013). The ease of accessibility, low antigenicity, repair capacity and immunomodulatory properties of hAEC make them a suitable cell type in these pre-clinical applications.

1.5.2 Stem Cell Characteristics of hAEC

hAEC differ from other parts of the placenta that they originate from pluripotent embryonic epiblast cells which is believed to enable hAEC to maintain stem cell features of embryonic stem cells. They express embryonic stem cell markers octamer-binding protein-4 (Oct4), Nanog, stage-specific embryonic antigen-4 (SSEA-4) (Ilancheran, Michalska et al. 2007; Parolini, Alviano et al. 2008), pluripotency associated markers sex-determining region box 2 (Sox-2), Tra-1-60 and Tra-1-80, in addition to a panel of lineage-associated genes (Izumi, Pazin et al. 2009; Manuelpillai, Moodley et al. 2011). hAEC possess the plasticity of pre-gastrulation embryonic cells and differentiate into lineages representing cells originating from all three germ layers, including liver, pancreas (endoderm), cardiomyocytes (mesoderm), and neural cells (ectoderm), and thus are suitable for pre-clinical cell replacement models (Miki, Lehmann et al. 2005; Ilancheran, Michalska et al. 2007; Diaz-Prado, Muinos-Lopez et al. 2010).

hAEC also have immune-privileged properties like other placental cells, expressing low levels of MHC Class IA molecules and produce immunomodulating cytokines/chemokines and their receptors, as well as express some surface markers. They form spheroid structures that retain stem cell properties, and do not require other cell-derived feeder layers to maintain Oct-4 expression. They do not express telomerase and hence have the advantage over many stem cell types in being non-tumorigenic. All the above characteristics are beneficial for hAEC to be applied for therapeutic use.

1.5.3 Immunomodulatory Properties of hAEC

In the past few years, hAEC transplantation in animal models has shown encouraging results in treating lung and liver fibrosis models. The beneficial immune regulatory effects of hAEC transplantation in these disease models were shown to reduced local inflammation, apoptosis and pro-inflammatory cytokines (Kong, Cai et al. 2008; Cargnoni, Gibelli et al. 2009; Manuelpillai, Tchongue et al. 2010; Liu, Vaghjiani et al. 2012; Moodley, Vaghjiani et al. 2013; Vosdoganes, Lim et al. 2013). hAEC have the ability to produce some immunomodulating cytokines/chemokines and their receptors and hence may alter immune cell function. hAEC secrete a range of immunosuppressive molecules. For example, human leukocyte antigen-G (HLA-G), prostaglandin E2 (PGE2) and transforming growth factor-β (TGF-β), factors which can inhibit activities of T cells and induce regulatory T cell populations (Naji, Durrbach et al. 2007; Carosella, HoWangYin et al. 2008; Sreeramkumar, Fresno et al. 2012; Tran 2012). Other molecules mediating immunomodulation have also been found in hAEC culture supernatants, include IL-1β and nitric oxide (Wichayacoop, Briksawan et al. 2009). hAEC have also been shown to directly influence immune cells such as macrophages (Manuelpillai, Lourensz et al. 2012).

1.5.4 hAEC for the Treatment of Neurological Disorders

hAEC may have potential for treating neurological degenerative diseases because hAEC are able to secrete neurotransmitters, nerve growth factors and neurotrophic

factors (Elwan and Sakuragawa 1997; Sakuragawa, Misawa et al. 1997; Uchida, Inanaga et al. 2000; Uchida, Suzuki et al. 2003). In some cases, these neurotrophic factors secreted by hAEC were linked to inhibition of axonal damage, reduction of CNS inflammation and promotion of neuroprotection (Venkatachalam, Palaniappan et al. 2009; Stroet, Linker et al. 2013; Yawno, Schuilwerve et al. 2013). hAEC enhanced neural differentiation of neural stem cells and umbilical cell-derived mesenchymal stem cells (Meng, Chen et al. 2007; Yang, Xue et al. 2013). All the above studies suggest that hAEC transplantation may be useful for the treatment and repair of inflammatory neurological diseases. Indeed, successes have been shown in a few disease models. hAEC ameliorated the rotational asymmetry in rat, supported axon growth and enhanced repair in spinal cord injury model, and ameliorated a Parkinson's disease model by increased production of neurotrophic factors that enhanced local repair (Sankar and Muthusamy 2003; Kong, Cai et al. 2008; Yang, Xue et al. 2009). In this thesis, I propose to explore the potential of hAEC to treat a mouse MS model, as this has not previously been tested.

1.6 Proposed Mechanism for hAEC to Treat Autoimmune Diseases

For tissue/organ transplantation, there is less chance for hAEC to be rejected by the immune system of the recipients. (Akle, Adinolfi et al. 1981; Adinolfi, Akle et al. 1982). Other cell types in the placenta such as trophoblast cells which lack certain HLA expression, the expression of specific alloantigens HLA-G on trophoblast cells is

considered one of the mechanisms that promote maternal-fetal tolerance during pregnancy (Menier, Riteau et al. 2000; Seavey and Mosmann 2008). This leads to the question of whether similar expressions of HLAs enable hAEC to induce tolerance in other occasions. The absence of CD80 (B7.1) and CD86 (B7.2) expression in hAEC accounts for the other part of tolerogenic phenotype (Banas, Trumpower et al. 2008), because recognition of CD80 and CD86 is a necessary signal for T cell activation in adaptive immunity. Besides the mechanism which passively avoids T cell activation, soluble factors secreted by hAEC may actively suppress or modulate immune responses. This immune-privileged cell type hAEC has mRNA and/or protein expression of TNFα, Fas ligand (FasL), TNF-related apoptosis-inducing ligand (TRAIL), TGF-β, and macrophage migration-inhibitory factor (MIF) (Li, Niederkorn et al. 2005). As the consequence of the expression profile, hAEC culture supernatants was shown to inhibit the activation of T cells and B cells, migration of macrophages, and to induce apoptosis in T cells and B cells. It is noteworthy that all the immune-privileged tissue will express FasL (Bohana-Kashtan and Civin 2004), and FasL is the apoptosis-related factors in this study which was shown to be responsible in the induction of apoptosis of T and B cells (Li, Niederkorn et al. 2005). TGF-β, IL-10, PGE2, and HLA-G, which are immunosuppressive factors and important in suppressing lymphocytes, are produced abundantly by hAEC and were suggested to be the inhibitory molecules accounts for reduced lymphocyte activities in mixed lymphocyte reaction (MLR) and peripheral blood mononuclear cell (PBMC) (Ueta, Kweon et al. 2002). From MSC studies which suppress allogeneic T cell proliferation mediated by secreted factors similar working mechanism based on secreted immunomodulatory factors of hAEC may appear in disease models (Di Nicola, Carlo-Stella et al. 2002; Selmani, Naji et al. 2008).

1.7 Hypothesis

Primary hAEC have potential as an alternative cell-based therapy for multiple sclerosis by exerting their immunomodulatory properties to suppress MOG-specific immune responses and alleviate MOG-induced EAE in mice.

1.8 Specific Aims

1. To examine the capacity of hAEC to suppress murine T cells from normal mice and EAE mice.
2. To determine the capacity of hAEC to attenuate murine EAE in both a preventive model and an intervention model.
3. To understand mechanisms by which hAEC achieve their therapeutic effects.
4. To compare primary hAEC with cultured or differentiated hAEC with respect to phenotypic and functional characteristics and to suggest suitable applications for different types of hAEC.

Chapter 2. Amniotic Epithelial Cells from the Human Placenta Potently Suppress a Mouse Model of Multiple Sclerosis

Multiple sclerosis (MS) is an inflammatory demyelinating disease affecting about 2.5 million people around the world. Current therapies for MS struggle with efficacy and adverse reactions, but none of the available therapeutic strategies can cure the disease. Stem cell therapies for MS have shown some promising results in clinical trials of hematopoietic stem cell (HSC) and mesenchymal stem cell (MSC), but both cell types have shortcomings of invasive procurement procedure and cell number limits. Here I explore the potential of an alternative pluripotent cell type, human amniotic epithelial cells (hAEC), in treating a murine model of MS named experimental autoimmune encephalomyelitis (EAE). hAEC have advantages over HSC and MSC because abundant cells can be obtained through a non-invasive procedure from each amnion membrane after parturition. In the very first test hAEC showed their suppressive ability to T lymphocyte proliferation, which is essential for EAE development. This data suggested that hAEC may have potential to alleviate T cell-mediated pathology. I then delivered hAEC through intravenous injection to EAE-induced mice and found the treatment ameliorated clinical signs, CNS pathology and peripheral autoimmune response. My *in vitro* experiment also suggests that hAEC use immunomodulatory molecules TGFβ and PGE2 in suppressing murine splenic T cell proliferation. Intravenously delivered hAEC remained in the lungs of EAE-induced mice after one

week. This is the first study which successfully evaluates the potential of hAEC in treating a MS disease model.

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Amniotic Epithelial Cells from the Human Placenta Potently Suppress a Mouse Model of Multiple Sclerosis

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Abstract

Human amniotic epithelial cells (hAEC) have stem cell-like features and immunomodulatory properties. Here we show that hAEC significantly suppressed splenocyte proliferation *in vitro* and potently attenuated a mouse model of multiple sclerosis (MS). Central nervous system (CNS) $CD3^+$ T cell and $F4/80^+$ monocyte/macrophage infiltration and demyelination were significantly reduced with hAEC treatment. Besides the known secretion of prostaglandin E2 (PGE2), we report the novel finding that hAEC utilize transforming growth factor-β (TGF-β) for immunosuppression. Neutralization of TGF-β or PGE2 in splenocyte proliferation assays significantly reduced hAEC-induced suppression. Splenocytes from hAEC-treated mice showed a Th2 cytokine shift with significantly elevated IL-5 production. While transferred CFSE-labeled hAEC could be detected in the lung, none were identified in the CNS or in lymphoid organs. This is the first report documenting the therapeutic effect of hAEC in a MS-like model and suggest that hAEC may have potential for use as therapy for MS.

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Introduction

Multiple sclerosis (MS) is a T cell-mediated demyelinating disease affecting over two million people worldwide with no cure available [1,2]. Myelin oligodendrocyte glycoprotein (MOG) [3,4] induced experimental autoimmune encephalomyelitis (EAE) is an animal model extensively used to study the pathogenesis of MS by inducing paralytic symptoms, and demyelination in the CNS accompanied by perivascular mononuclear cell infiltration [5,6,7].

Mesenchymal stem (stromal) cells, which can inhibit T cell expansion, are being trialed as a therapy for MS [8]. We explored the potential of human amniotic epithelial cells (hAEC) to suppress a mouse model of MOG-induced EAE. hAEC originate from pluripotent embryonic epiblasts, express some embryonic and mesenchymal stem cell markers [9,10,11,12], and are isolated from the amniotic membrane of the human placenta. hAEC can be obtained in large amounts without extended *ex vivo* expansion or ethical concerns compared to bone marrow and embryo derived stem cells. They have stem cell-like features and can differentiate into lineages representing cells originating from the three germ layers [10,11], and express low levels of Class IA human leukocyte antigens (HLA) and lack Class II antigens which may potentially reduce the risk of immune-rejection after transplantation [10,12]. Previous studies have shown that hAEC also have immunomodulatory properties and inhibit mixed lymphocyte reactions and mitogen stimulated T cell proliferation [13,14] where some of these effects may be attributed by secreted factor(s) [15]. Besides having effect on T cells, hAEC have been shown to secrete neurotrophic substances [16,17] suggesting that hAEC transplantation may be useful for the treatment and repair of inflammatory neurological diseases. Overall, the ease of accessibility, low antigenicity, repair capacity and immunomodulatory properties make hAEC an important cell type for regenerative medicine.

Here, we show that intravenous hAEC transplantation potently ameliorated MOG-induced EAE, significantly reduced $CD3^+$ T cells and $F4/80^+$ monocyte/macrophage infiltration and demyelination within the central nervous system (CNS). We also showed that hAEC secreted transforming growth factor-β (TGF-β) and prostaglandin E2 (PGE2) in primary culture. Blocking TGF-β using a neutralizing antibody or PGE2 by indomethacin significantly reduced the suppression of splenocyte proliferation by hAEC. In addition, splenocytes from hAEC-treated mice produced significantly more Th2 cytokine IL-5 compared to control. Injected CFSE-labeled hAEC were detected in the lung but none were detectable in the CNS or peripheral lymphoid organs. We suggest that hAEC may have potential for treating MS due to their immunosuppressive effects and improvement seen within the CNS of the mouse model of MS.

Materials and Methods

Ethics Statement

The study was approved by Southern Health Human Research Ethics Committee and the Institutional Review Board of Monash University. Informed, written consent was obtained from each

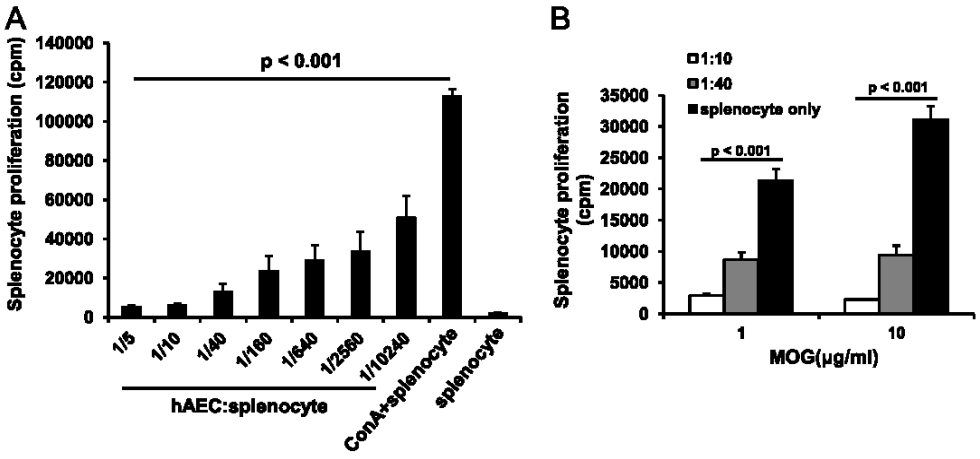

Figure 1. Dose-dependent suppression of splenocyte proliferation by hAEC. (**A**) ConA (5 μg/ml) stimulated proliferation of splenocytes from naive mice was dose-dependently inhibited by hAEC in hAEC:splenocyte ratios ranging from 1:5 to 1:10240 (n = 5). (**B**) Proliferation of splenocytes from EAE mice stimulated by 1 μg/ml and 10 μg/ml MOG peptide was inhibited by hAEC (n = 3) at hAEC:splenocyte ratios of 1:10 and 1:40. Splenocyte proliferation is expressed as counts per minute (cpm) of ^{3}H-thymidine incorporation. All data are means±SEM.
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patient prior to amnion membrane collection. Tissues were retrieved from placentae delivered by healthy women with a normal singleton pregnancy undergoing elective cesarean section at term (37 40 weeks gestation; n = 30). Animal experimentation was approved by the Animal Ethics Committee, Monash University (approval number MMCB 2009/16).

hAEC isolation and culture

Cell isolation, culture and characterization were as described previously [10,18]. Briefly, amnion membranes were cut into small pieces and digested twice in 0.05% trypsin:EDTA (Gibco) for 40 min at 37°C. Following inactivation of trypsin with newborn calf serum, dispersed cells were washed in DMEM/F12 medium (Gibco) and erythrocytes lysed in hypotonic solution. Batches (n = 15) >99% positive for the epithelial markers cytokeratin-7 and 8/18 (Dako, Denmark) by flow cytometry and displaying a cobblestone epithelial morphology in culture were used for *in vivo* and *in vitro* studies [19].

EAE induction and treatment

EAE was induced in female C57BL/6 mice 8 12 weeks old by immunization of 200 μg $MOG_{35\ 55}$ peptide (GL Biochem, China) and 350 ng pertussis toxin (Sigma-Aldrich) given on the day of immunization and repeated 2 days later [7]. $MOG_{35\ 55}$ peptide was emulsified with 100 μl complete Freud's antigen (CFA; Sigma-Alderich) and 4 mg/ml *Mycobacerium tuberculosis* (Difco Laboratories) in PBS. Two million hAEC (primary cells, passage 0, pooled from 4 donors) in basal media were administered intravenously on day 9 after immunization in 3 independent experiments, while control littermates remained untreated (hAEC-treated mice, n = 25; EAE control mice, n = 26, in total 3 experiments). Animals were monitored daily and neurological impairment scored on an arbitrary clinical score: 0, no clinical sign; 1, limp tail; 2, limp tail and hind limb weakness; 3, severe hind limb paresis; 4, complete hind limb paresis; 5, moribund or death [6,7]. All studies were performed with approval of the local animal ethics committee. As required by animal ethics, mice were euthanized upon reaching a score of 3.

Detection of immunoregulatory molecules produced by hAEC

hAEC (n = 7 donors) were cultured for 7 days in complete DMEM-F12 and then 2 days more in serum-deprived DMEM-F12 medium. TGF-β1, interleukin-10 (IL-10) and hepatocyte growth factor (HGF) were measured using ELISA kits (R&D Systems). Nitric oxide (NO) production was detected using Griess reagent system (Promega). PGE2 was detected by EIA kit (Cayman Chemical). Cytokine and PGE2 measurements are expressed as pg/ml/million hAEC.

Splenocyte suppression assays and cytokine detection

Mouse splenocytes were used as responders for mitogen/antigen stimulation. Gamma-irradiated hAEC (20 Gy) as suppressor cells were co-cultured at different ratios with splenocytes (5×10^{5}) in 96-well plates. For antigen non-specific suppression, splenocytes from naïve mice were stimulated with the mitogen ConA (5 or 10 μg/ml, Sigma-Aldrich). For antigen-specific suppression, splenocytes from EAE-induced mice were stimulated with $MOG_{35\ 55}$ peptide (1, 10, or 100 μg/ml). Proliferation was assessed by ^{3}H-thymidine incorporation as described earlier [6]. Blocking assays were performed by adding anti-human TGF-β monoclonal antibody (0.4 μg/ml, R&D Systems) or the PGE2 inhibitor indomethacin (1 nM, Sigma-Aldrich) to hAEC/splenocyte cultures. In some experiments, 72 hr culture supernatants were collected for mouse cytokine detection (IL-4, IL-5, IL-10, IL-17, IFN-γ, GM-CSF, TNF-α) by flow cytometry according to the manufacturer's instructions (Bender MedSystems/FlowCytomix, eBioscience).

Detection of anti-MOG antibodies

Anti-MOG antibodies were detected in mouse sera as described previously [6]. Sera were collected from mice at the end-point of *in vivo* experiments, and tested at 1:300 dilutions in 96-well plates coated with 5 μg/ml of $MOG_{35\ 55}$ peptide. Anti-MOG antibodies bound to MOG were detected by horseradish-peroxidase conjugated goat anti-mouse IgG (Dako, Denmark) and then developed by TMB ELISA substrate (Thermo Scientific). Mean absorbance of samples analyzed in triplicate was calculated minus

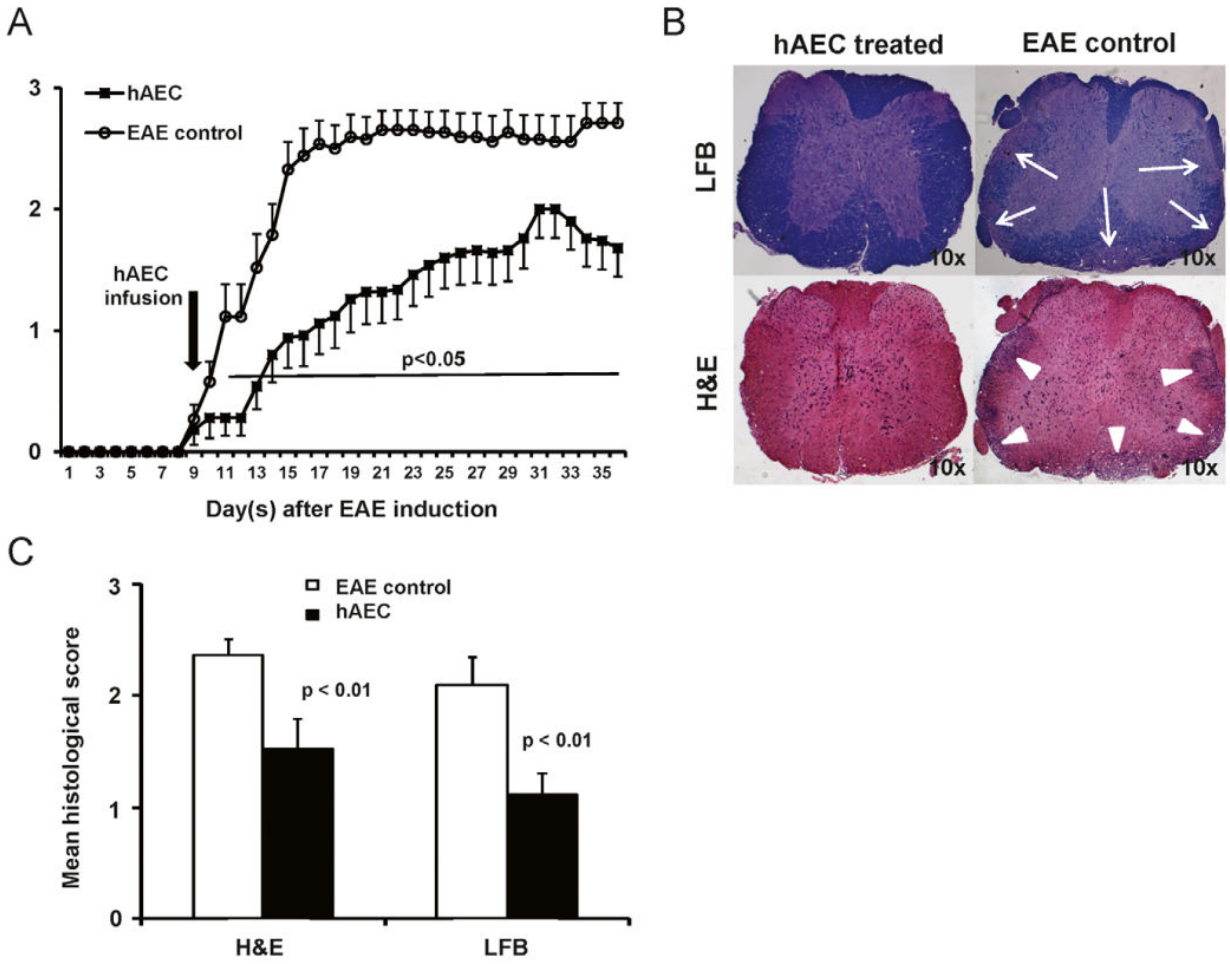

Figure 2. hAEC infusion potently ameliorated EAE and reduced spinal cord pathology. EAE was induced by immunization with 200 μg MOG_{35-55} peptide in 100 μl CFA followed by 350 ng pertussis toxin and the clinical scores were evaluated. (**A**) hAEC (2×10^6) injected intravenously on day 9 after EAE induction ameliorated disease development (n = 25 in total) while control animals developed EAE (n = 26 in total). Data shown are combined results of three independent experiments each using pooled hAEC from n = 4 donors. (**B**) Spinal cord sections were stained by H&E and LFB for assessment of cellular infiltrates and demyelination, respectively. Representative spinal cord sections from hAEC-treated mice showed intact myelin sheath (blue color) from LFB staining and no cellular infiltrate from H&E staining. EAE control sections show regions of demyelination (arrows) and cellular infiltrate (arrow heads). (**C**) hAEC-treated mice (n = 9) showed significantly lower histological scores in both H&E and LFB assessments compared to EAE control (n = 5). All data are means±SEM.
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values from uncoated controls using VICTORE X Multilabel Counter (Perkin-Elmer).

Flow cytometry

Cells from spleen and inguinal lymph nodes which were harvested from hAEC-treated and EAE control mice were stained for 30 min at 4°C with PB-, PE-, or APC-conjugated monoclonal antibodies: CD4 (clone RM4 5, 1 μg/ml, BD), CD8 (clone 53-6.7, 1 μg/ml, BD), CD19 (clone 1D3, 0.25 μg/ml, eBioscience), CD25 (clone not specified, 1 μg/ml, BD), Foxp3 (clone FJK-16s, 1 μg/ml, APC anti-mouse/rat staining kit, eBioscience), or matched isotype control IgG (BD or eBioscience). The percentage of stained cells was analyzed by CANTO flow cytometer (BD). Fc receptors of splenocytes were blocked prior to antibody staining by anti-mouse CD16/CD32 (clone 2.4G2, 2.5 μg/ml, BD) for at least 15 min at 4°C.

Histology

Histological assessment of spinal cord was performed as previously described [6]. On average, 20 sections (5 μm) taken 20 μm apart from each mouse were examined. The extent of inflammation and demyelination was evaluated blinded with hematoxylin and eosin (H&E) and Luxol fast blue (LFB) stains, respectively [6,7]. For inflammation, evaluation was performed using H&E-stained sections and scored as follows: 0, no inflammation; 1, cellular infiltrate only in the perivascular areas and meninges; 2, mild cellular infiltrate in parenchyma; 3, moderate cellular infiltrate in parenchyma; 4, severe cellular infiltrate in parenchyma. Myelin breakdown was assessed as pale staining with LFB and scored as follows: 0, no demyelination; 1, mild demyelination; 2, moderate demyelination; 3, severe demyelination.

For immunostaining, CNS paraffin sections (5 μm) were dewaxed and rehydrated treated with standard antigen retrieval

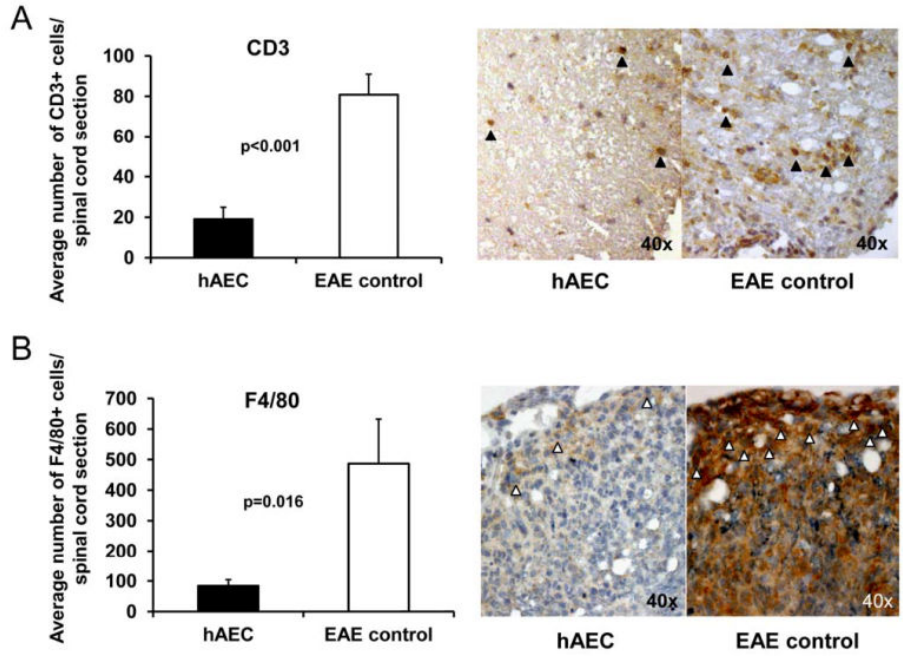

Figure 3. Reduced CD3$^+$ T cells and F4/80$^+$ monocytes/macrophages in CNS. Average number of CD3$^+$ T cells (**A**) and F4/80$^+$ cells (**B**) enumerated per spinal cord section (mean of 3 sections per mouse) showed significant reduction in hAEC-treated mice (n = 8) compared to EAE control (n = 4). Photomicrographs showing representative CD3 (black arrowheads) and F4/80 (white arrowheads) staining in hAEC-treated and EAE control spinal cords. All data are means±SEM.
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protocol (0.01 M citrate buffer [20]). Endogenous peroxidase activity was quenched by adding 0.6% H_2O_2. Non-specific binding was minimized by CAS protein blocking solution (Invitrogen). Sections were incubated with anti-CD3 (Abcam) or anti-F4/80 (eBioscience) antibodies. Appropriate secondary antibodies were used and detected using DAB (Vector Laboratories). Slides were counterstained with hematoxylin. Three spinal cord sections were analyzed per mouse.

hAEC tracking

After harvesting, primary hAEC from n = 4 donors were pooled and labeled with CFSE (Invitrogen) as described previously [19]. Briefly, CFSE was dissolved in DMSO and further diluted 1000 times with DMEM-F12, then added to the hAEC suspension (1×10^6 cells/ml) at a final concentration of 10 μM. After incubation at 37°C for 10 min, the staining was quenched by addition of 5 volumes of DMEM/F12. The CFSE-labeled hAEC were then washed, resuspended in DMEM-F12 medium and intravenously injected into MOG immunized mice (n = 5). Each mouse received 2×10^6 cells in 200 μl medium. Seven days later, mice were killed and organs were collected. Single cells from 1/3 of each spleen and the right inguinal lymph node were analyzed by acquiring 1.5 2×10^6 live cell events using flow cytometry while the remaining spleens, the left inguinal lymph nodes, lungs, the livers, the brains and spinal cords were frozen in OCT and sectioned (5 μm thick) and examined for CFSE positive cells. Between 9 16 frozen tissue sections were analyzed for each organ/mouse.

Statistical Analysis

Data are presented as mean±SEM and evaluated by one-way ANOVA with Tukey's test or Student's T-test. A P value <0.05 was considered significant.

Results

hAEC suppress *in vitro* splenocyte proliferation

Since T cell-mediated responses are thought to be crucial for MS development, we assessed hAEC for their capacity to suppress T cell proliferation *in vitro* and then assessed their potential to relieve MS-like symptoms in a mouse model *in vivo*. We found that hAEC potently suppressed the proliferation of splenocytes from naive mice stimulated with 5 μg/ml ConA. Dose-dependent suppression of ConA stimulation of splenocytes was observed at hAEC:splenocyte ratios ranging from 1:5 to 1:10240. Suppression of 95% was observed at hAEC:splenocyte ratios of 1:5 and 1:10, and even at hAEC:splenocyte ratio of 1:10240 suppression of 55% was still observed (Fig. 1A). hAEC also exerted similar immunosuppressive effects in a MOG antigen-specific setting. Splenocytes from MOG-induced diseased mice showed vigorous proliferation to MOG peptide re-stimulation (Fig. 1B). hAEC potently inhibited these proliferative responses by 60 90% at hAEC:splenocyte ratios of 1:10 and 1:40 at different MOG peptide concentrations (1 and 10 μg/ml) (Fig. 1B). Thus, hAEC exert potent suppression of splenocyte proliferation in both antigen non-specific and antigen-specific manner.

hAEC ameliorate EAE and improve CNS histopathology

Given the potent immunosuppressive effect of hAEC *in vitro*, we then assessed their *in vivo* therapeutic effects by injecting 2×10^6 hAEC into mice at day 9 post-EAE induction. hAEC infusion ameliorated EAE in mice from three independent experiments with significance detected in mean scores after day 11 ($p<0.05$). The hAEC-treated mice (n = 25) had mild or delayed EAE compared to their littermate controls (n = 26, Fig. 2A).

Examination of spinal cords from hAEC-treated mice showed no or minimal inflammatory cell infiltration and myelin loss while

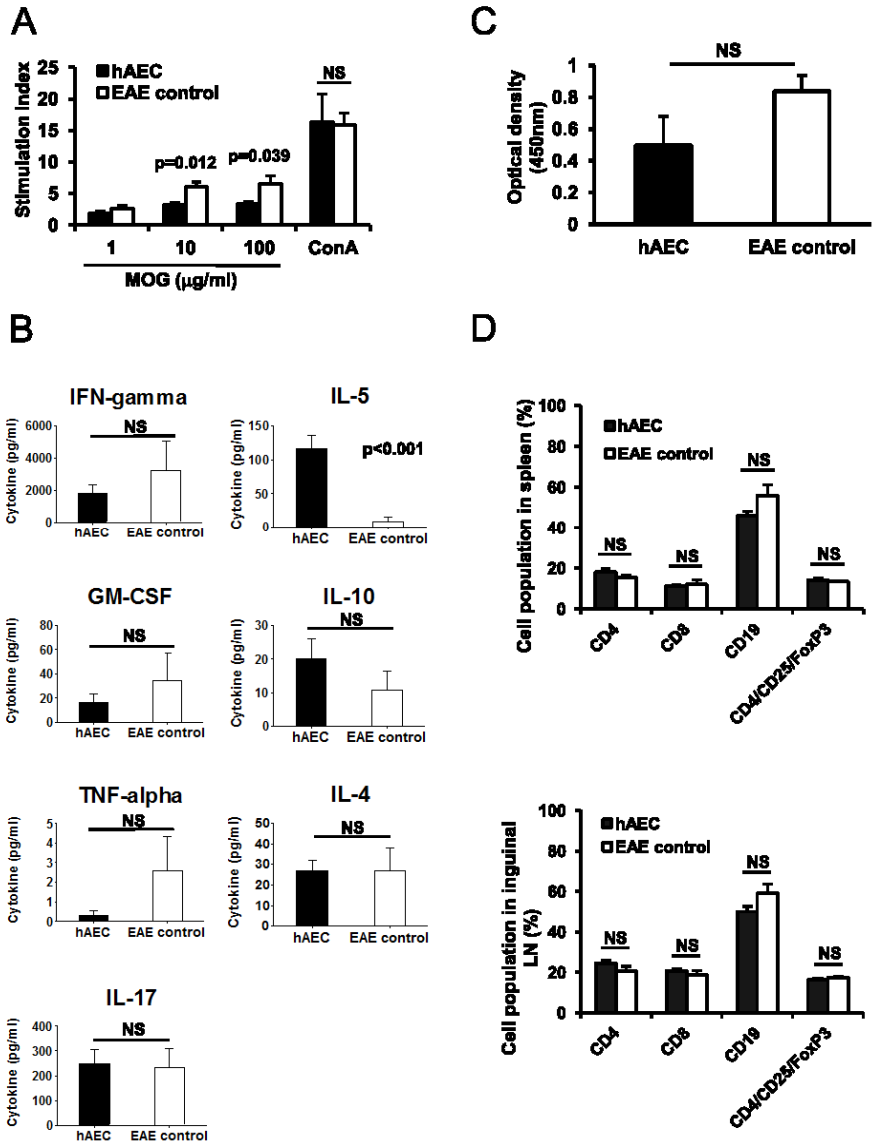

Figure 4. hAEC-treated mice showed reduced splenocyte proliferation and Th2 cytokine shift following stimulation *in vitro* with MOG peptide but serum MOG antibody and lymphocyte populations in spleen and lymph nodes remained unaffected. (**A**) Splenocytes from MOG-immunized and hAEC-treated mice (n = 8) stimulated with MOG peptide proliferated significantly lower than EAE control mice (n = 5) at 10 and 100 µg/ml MOG peptide. ConA (10 µg/ml) stimulated proliferation from both groups was similar. (**B**) Cytokines in supernatants from splenocyte cultures in (A) stimulated with 10 µg/ml of MOG for 72 hr were analyzed. IL-5 was significantly elevated. (**C**) MOG-specific antibodies in sera of hAEC-treated mice (n = 8) and EAE control (n = 5) were not significantly different. (**D**) FACS analysis of T cell subsets ($CD4^+$, $CD8^+$, $CD4^+CD25^+$ $FoxP3^+$) and B cells ($CD19^+$) from the spleen (upper panel) and from inguinal lymph node (lower panel) showed no significant differences between hAEC-treated mice (n = 9) and EAE control (n = 5). All data are means ± SEM. NS: Not significant.
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EAE control mice showed extensive cellular infiltration and demyelination (Fig. 2B). Blinded quantitation of these parameters via a validated histological scoring system [6,21] showed that these differences in cellular infiltration and demyelination were significant ($p<0.01$; Fig. 2C). Inflammatory infiltrates such as T cells and monocytes into CNS play important roles in the pathogenesis of EAE [1,2,22]. Thus, we further investigated the cellular infiltrate in the spinal cords for the presence of inflammatory cells. We found significant reduction in the numbers of $CD3^+$ T cells ($p<0.001$; Fig. 3A) and $F4/80^+$ monocytes/macrophages ($p = 0.016$; Fig. 3B) in the spinal cords from hAEC-treated mice compared to controls.

Clinical improvement is associated with decreased immune response to MOG and a Th2 shift

Since hAEC potently suppressed lymphocyte-mediated EAE development, we tested whether lymphocytes from hAEC-treated mice had reduced responsiveness to *ex vivo* MOG stimulation. We found that splenocytes from hAEC-treated mice proliferated significantly less than EAE control mice after MOG peptide

stimulation (10 and 100 μg/ml), but their antigen non-specific proliferation to the mitogen ConA (10 μg/ml) remained similar (Fig. 4A). Splenocytes from hAEC-treated mice appeared to produce less Th1 cytokine IFN-gamma and less inflammatory cytokines GM-CSF and TNF-α; although the reduction did not reach statistical significance (Fig. 4B). In contrast, the Th2 cytokines IL-5 and IL-10 appeared increased with the increase in IL-5 reaching a significance of $p<0.001$; however, IL-4 remained unchanged. There was also no significant difference in IL-17 levels (Fig. 4B). We also examined the effect of hAEC on B cells by measuring anti-MOG antibody in serum from hAEC-treated mice and from EAE control mice. hAEC treatment led to a reduction in MOG-specific autoantibodies in sera but it failed to reach statistical significance (Fig. 4C).

To determine whether hAEC transplantation altered the proportion of T and B lymphocyte subpopulations in peripheral lymphoid organs, we analyzed cells from the spleen and inguinal lymph node by flow cytometry. We showed that there were no significant differences in the percentages of $CD4^+$, $CD8^+$, $CD19^+$, and $CD4^+CD25^+FoxP3^+$ [23,24,25] lymphocyte sub-populations between hAEC-treated and the control group (Fig. 4D).

hAEC utilize TGF-β and PGE2 for immunosuppression

Soluble factors have been suggested to be utilized by hAEC to modulate immune responses [15]. We detected TGF-β1 (86.09±26.66 pg/ml/million hAEC) and PGE2 (85.33± 30.55 pg/ml/million hAEC) in serum-deprived hAEC-conditioned culture medium but were unable to detect HGF, NO or IL-10. The result suggested that TGF-β and PGE2 might be important factors produced by hAEC that suppressed the proliferation of stimulated splenocytes. Thus, we set up two co-culture settings using hAEC and splenocytes from EAE-diseased mice stimulated with MOG peptide or from naïve mice stimulated with ConA. We added neutralizing antibody to block TGF-β or indomethacin to inhibit PGE2 in the co-culture. The presence of TGF-β neutralizing antibody or indomethacin significantly abrogated the inhibition of splenocyte proliferation by hAEC in either MOG peptide or ConA-stimulated cultures (Fig. 5A, B). Blocking of TGF-β reduced the suppressive effects on MOG-stimulated splenocytes by nearly 75% and almost 90% on ConA-stimulated splenocytes. Similarly, blocking of PGE2 by indomethacin reduced the suppression of MOG-stimulated splenocytes and of ConA-stimulated splenocytes by approximately 20%. Thus, these data confirmed that TGF-β and PGE2 are crucial for hAEC-mediated suppression *in vitro*.

Detection of CFSE-labeled hAEC

Given the beneficial clinical outcome of hAEC infusion on EAE development, we further investigated the possible locations of these cells in their mouse recipient. We injected CFSE-labeled hAEC and tracked them in peripheral organs and CNS of hAEC-treated mice, and detected CFSE positive cells in lung tissues 7 days after cell administration (Fig. 6). On average, 2 5 CFSE positive cells were detected per 5 μm thick lung section. However, we failed to detect CFSE positive cells from spleen, inguinal lymph node, liver, brain and spinal cord sections (data not shown). In addition, flow cytometric analysis of spleens and inguinal lymph nodes yielded the same results as frozen sections.

Discussion

This is the first study to report the therapeutic effect of hAEC in a mouse model of multiple sclerosis and that TGF-β is utilized by hAEC for immunosuppression. hAEC transplantation has previously been reported to ameliorate fibrosis models in the liver and in the lung [20,26]. These cells have also ameliorated a Parkinson's disease model by increased production of neurotrophic factors that enhanced local repair [27]. Our study demonstrates that hAEC have potent immunosuppressive ability both *in vitro* and *in vivo*. In ConA-stimulated and MOG-stimulated hAEC-splenocyte co-culture assays, hAEC exerted suppression in both antigen non-specific and antigen-specific settings. Furthermore, hAEC infusion on day 9 after EAE induction protected mice from disease development.

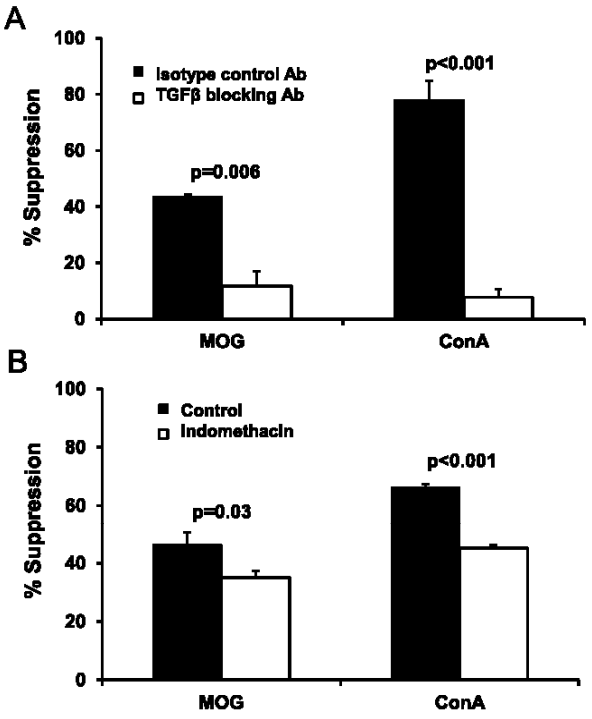

Figure 5. TGF-β blocking antibody and indomethacin reversed hAEC suppression of splenocyte proliferation stimulated by ConA and MOG peptide. hAEC:splenocyte at 1:40 ratio were co-cultured with splenocytes from EAE mice and MOG (10 μg/ml), or with splenocytes from naïve mice and ConA (5 μg/ml). Data shown is from n = 3 experiments each using pooled cells from n = 4 donors. Addition of TGF-β neutralizing antibody (**A**) or PGE2 antagonist indomethacin (**B**) significantly reduced the suppression exerted by hAEC in both settings. Data presented as percentage of suppression compared with TGF-β neutralizing antibody or PGE2 antagonist treated control groups (splenocytes+MOG/ConA), respectively. All data are means±SEM.
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We identified TGF-β and PGE2 as molecules utilized by hAEC for immunosuppression. TGF-β, a T cell growth inhibitor which inhibits T cell proliferation and DNA synthesis [28,29,30], is a powerful immunosuppressive molecule. TGF-β deficient mice suffer severe multifocal inflammatory lesions [31] while blocking TGF-β signaling in T cells causes disruption in T cell development and function [32]. TGF-β signaling in dendritic cells is also needed to control autoreactive myelin-reactive T cells [33]. TGF-β is thought to promote the resolution of inflammation with systemic treatment suppressing CNS inflammatory lesions and signs of EAE [34,35]. In a previous study, using human specific PCR primers we have shown that TGF-β mRNA is expressed in lungs of hAEC-treated mice [26], and therefore it is likely that TGF-β produced by hAEC contributed to the improvement of EAE in the current

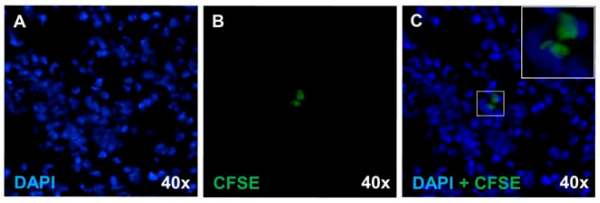

Figure 6. Tracking of CFSE-labeled hAEC. Lung section obtained from mice injected with CFSE-labeled hAEC after MOG immunization and organ collected 7 days later (5 mice for each group, 9–16 sections per organ were examined). (**A**) Frozen lung section staining with DAPI. (**B**) Same field as (A) showing CFSE positive cells. (**C**) Overlay of (A) and (B). Insert showing enlarged view of CFSE-DAPI positive cells.
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study. On the other hand, PGE2 has a variety of immunosuppressive properties including inhibition of T cell proliferation and stimulating the production of Th2 cytokines such as IL-5 and IL-10 [36,37]. The actions of these molecules are in agreement with our observations that splenocytes from hAEC-treated, EAE-protected mice, proliferated significantly less when stimulated *ex vivo* with MOG, and that supernatants from these cultures revealed a Th2 shift in their cytokine profile with significantly elevated IL-5. Upregulation of Th2 cytokines such as IL-5 have been shown to have a protective effect on EAE [38,39].

Multiple sclerosis and EAE are considered T cell-mediated diseases [1,2] because adoptive transfer of CNS antigen-activated T cells is sufficient to induce EAE [40,41]. Activation of CNS-reactive T cells initiates local microglia expansion and recruitment of blood-borne monocytic cells [42,43]. These cells secrete pro-inflammatory cytokines and participate in demyelination [44]. Monocytic infiltration into the CNS is correlated with progression of clinical disease and blocking their infiltration prevents EAE progression [22]. Thus, the significant milder EAE and reduced demyelination in hAEC-treated mice are in agreement with our observations of dramatic reduction in $CD3^+$ T cell and $F4/80^+$ monocyte/macrophage infiltration in the CNS.

Taken together, our findings suggest that by producing TGF-β and PGE2, hAEC treatment limited the expansion of MOG-reactive T cells and their infiltration into the CNS which in turn limited monocytic cell infiltration, and disease development. In addition, the reported neurotrophic factors such as brain-derived neurotrophic factor and nerve growth factor [16] produced by hAEC may also have contributed in part to the beneficial effect in hAEC-treated EAE mice.

We attempted to track the whereabouts of the systemic delivered hAEC. Seven days after the injection of hAEC into EAE-induced mice, we could only detect CFSE-labeled hAEC in the lungs without detectable cells in CNS or peripheral lymphoid organs. From the mesenchymal stem/stromal cell (MSC) literature, MSC were found to provide clinical benefits without their necessary presence in the affected organs, with most of the cells trapped within the lungs [45,46]. Instead, it is now being proposed that MSC act from a distance by producing immunomodulatory molecule(s) during their transient appearance after systemic injection. Whether a similar scenario applies to hAEC awaits further investigation.

Our study demonstrated that hAEC have potent immunosuppressive capacity in the murine model of MS both *in vitro* and *in vivo*. Given the ready availability of human placenta and the ease of hAEC isolation from this organ, we suggest that hAEC has potential for application as cell therapy for multiple sclerosis. However studies using fewer numbers of hAEC in the mouse model and more information on the immunomodulative effects of hAEC in humans should be acquired before consideration can be given for clinical application in MS.

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Author Contributions

Conceived and designed the experiments: YHL UM BHT JC PC. Performed the experiments: YHL VV JYT KT DYO PC. Analyzed the data: YHL UM BHT JC PC. Contributed reagents/materials/analysis tools: UM JC BHT PC. Wrote the paper: YHL UM BHT JC PC.

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Chapter 3. Human Amniotic Epithelial Cells Suppress Relapse of Corticosteroids-Remitted Experimental Autoimmune Disease

Following the positive results from the first chapter, my second part of study tries to design a strategic hAEC therapy for MS model to provide a more practical method for future application. In the previous study EAE mice were treated with hAEC before they showed paralytic signs, therefore the treatment was regarded as a preventive therapy. In this chapter I use a different design in which EAE mice that develop their disease signs were first treated with corticosteroids until their disease remitted completely, then we withdrew corticosteroids treatment and injected them with hAEC as an intervention therapy. The experimental design mimics what happens in human MS because patients only seek treatment after disease development and may need controlling disease symptom by corticosteroids treatment. The data showed mostly similar results compared to the preventive study, where peripheral autoimmune responses were reduced significantly. CNS pathology was partially decreased and here was evidence of enhanced peripheral regulatory T cell population. All the above mentioned data implies that hAEC can be applied for intervention therapy to a murine MS model EAE. The results from this chapter together with the previous chapter suggest that hAEC has potential to be applied as cell-based therapy for human MS.

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Human amniotic epithelial cells suppress relapse of corticosteroid-remitted experimental autoimmune disease

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Abstract

Background aims. Multiple sclerosis (MS) is considered to be a T-cell–mediated disease. Although MS remits with corticosteroid treatment, the disease relapses on discontinuation of therapy. Human amniotic epithelial cells (hAEC) from the placenta are readily accessible in large quantities and have anti-inflammatory properties. Previously we reported that hAEC given near disease onset ameliorated clinical signs and decreased myelin oligodendrocyte glycoprotein (MOG)-specific immune responses in MOG-induced experimental autoimmune encephalomyelitis (EAE), an experimental MS model. *Methods.* To examine the therapeutic effect of hAEC in a clinically relevant setting, we first treated MOG peptide–induced EAE mice with a corticosteroid, prednisolone, in drinking water to induce remission. hAEC were then infused intravenously into the remitted mice. Anti-MOG antibodies in serum were detected by enzyme-linked immunoassay. Splenocyte proliferation was assessed by ^{3}H-thymidine incorporation. Immune cell subpopulations in spleens and lymph nodes and secreted cytokines in splenocyte culture were quantified by flow cytometry. Central nervous system histology was examined with the use of hematoxylin and eosin, Luxol fast blue and immunostaining. *Results.* With cessation of prednisolone treatment, hAEC delayed EAE relapse for 7 days, and, after another 7 days, largely remitted disease in six of eight responder mice. Splenocyte proliferation was suppressed, anti-MOG_{35-55} antibodies in serum were decreased and interleukin-2 and interleukin-5 production by splenocytes were elevated after hAEC treatment. In the central nervous system, hAEC-treated mice had decreased demyelination and fewer macrophages in the inflammatory infiltrates. hAEC treatment also increased $CD4^{+}CD25^{+}FoxP3^{+}$ regulatory T cells in inguinal lymph nodes. *Conclusions.* These data demonstrate that the therapeutic effects of hAEC after corticosteroid treatment in an MS model probably are the consequence of peripheral immunoregulation. We suggest that hAEC may have potential as a cell therapy for remitted MS.

Key Words: *amniotic epithelial cells, corticosteroids, experimental autoimmune encephalomyelitis, immunosuppression, multiple sclerosis, regulatory T cells*

Introduction

Multiple sclerosis (MS) is a neurological demyelinating disease affecting one in 1000 people. Genetic factors, environmental risk factors and infections contribute to the as-yet unclear disease etiology in which autoreactive T lymphocytes are thought to mediate the destructive autoimmune responses toward the myelin sheath of the central nervous system (CNS) (1,2). Considering the difficulties of studying MS in humans, experimental autoimmune encephalomyelitis (EAE), an animal model of MS, is widely used instead. Animals with EAE display clinical and pathological characteristics similar to MS in humans such as paralytic symptoms, demyelination, perivascular mononuclear cell infiltrate in the CNS and neuroantigen-specific T-cell responses (3,4). Several approved treatments for MS have been developed in the EAE model before clinical trials (4–6).

MS can be treated with the use of immunosuppressive agents, immunomodulatory drugs and humanized monoclonal antibodies. These therapies are only partially effective and often accompanied by undesirable side effects (7). More recently, hematopoietic and mesenchymal stromal cell therapies have shown beneficial effects in clinical trials (8,9); however, the procurement of these cells is invasive, and

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cell expansion is time-consuming and expensive. Human amniotic epithelial cells (hAEC) derived from the embryonic epiblast have been considered as an alternative source to mesenchymal stromal cells and hematopoietic stem cells. hAEC have some features of stem cells and are easily acquired in large quantities with fewer ethical concerns; therefore, the cost and time involved for cell expansion is minimized (10,11). hAEC have been used in ophthalmic applications and surgery for their wound repair and anti-inflammatory properties (12–14). In the past few years, animal studies have shown encouraging results in hAEC treatment of lung fibrosis, liver fibrosis and neurological diseases. The beneficial immune regulatory effects of hAEC transplantation in these disease models were shown as reduced local inflammation, apoptosis and pro-inflammatory cytokines (15–18).

Our previous study showed that hAEC infused intravenously near the onset of EAE ameliorated paralytic symptoms, CNS inflammation and peripheral immune responses, which suggests that hAEC may have therapeutic potential for the treatment of MS (18). However, it is not clear whether hAEC therapy can be effective in established EAE that mimics MS in patients who have already developed the disease and require anti-inflammatory medication. To address this issue, we first established EAE by means of immunization of myelin oligodendrocyte glycoprotein (MOG) peptide. We then treated these mice with prednisolone, an anti-inflammatory corticosteroid, to induce temporary remission through the use of a protocol that we have described previously for methylprednisolone (19). hAEC were administered during the remission phase to determine whether the mice could be maintained in remission. Our results showed that hAEC can inhibit EAE relapse after cessation of prednisolone treatment. Therapeutic efficacy of hAEC was shown in improvement of the neurological clinical score, spinal cord pathology and peripheral immune responses in six of eight prednisolone-remitted mice. Moreover, we found that in this setting, hAEC elevated the regulatory T-cell population that may have contributed to peripheral immune suppression and disease amelioration.

Methods

Ethics statement

Human amnion tissue collection was approved by the Royal Women's Hospital Human Ethics Committee. Informed written consent was obtained before tissue collection from healthy women with a normal singleton pregnancy undergoing elective caesarean section at term (37–40 weeks of gestation; n = 4). Animal experimentation was approved by the Animal Ethics Committee, Monash Medical Centre (approval No. MMCB 2009/16).

hAEC isolation and preparation for cell therapy

As previously described, amnion membranes were diced and digested twice in trypsin (0.05% trypsin: ethylenediaminetetraacetic acid; Gibco, Grand Island, NY, USA) for 40 min at 37°C. Trypsin was then inactivated with newborn calf serum (Gibco). The dispersed cells were washed in DMEM/F12 medium (Gibco), separated on a Percoll gradient followed by hypotonic solution to lyse erythrocytes (20,21). All hAEC batches used (n = 4) displayed a cobblestone epithelial morphology in culture and were >97% positive for the epithelial markers cytokeratin-7 and 8/18 (Dako, Glostrup, Denmark) when examined by flow cytometry. hAEC from four donors were pooled and cultured in DMEM/F12 with 10% fetal calf serum (Gibco) for 6 days. Cells were lifted in TrypLE (Invitrogen, Grand Island, NY, USA) and washed repeatedly before injection.

Induction of EAE, prednisolone-induced remission followed by hAEC treatment

Female C57BL/6 mice ages 18–19 weeks were immunized intravenously with 350 ng pertussis toxin (Sigma-Aldrich, St Louis, MO, USA) in phosphate-buffered saline followed by subcutaneous injection of 200 µg of MOG_{35-55} peptide (GL Biochem, Shanghai, China) emulsified in 100 µL of complete Freud's adjuvant (Sigma-Aldrich) and 4 mg/mL of *Mycobacterium tuberculosis* (Difco Laboratories, Franklin Lakes, NJ, USA) in phosphate-buffered saline. The pertussis toxin injection was repeated 2 days later to complete EAE induction. Mice were monitored daily for their clinical status and scored with the use of a well-established scale in which 0 = no clinical signs of disease; 1 = limp tail; 2 = limp tail and hind limb weakness; 3 = severe hind limb paresis; 4 = complete hind limb paresis and fore limb weakness and 5 = moribund or death. On reaching a clinical score of ≥2, mice were treated with prednisolone at a dose of 10 mg/kg per day. To set up the oral delivery of prednisolone, 30 mg of prednisolone powder (Sigma-Aldrich) was dissolved in 1 mL of ethanol and mixed with 500 mL of drinking water. Prednisolone-treated water was changed every other day to maintain a consistent level of 60 µg/mL prednisolone for mice as reported before (22,23). Prednisolone treatment induced remission of neurological symptoms, causing the clinical score to decrease to 0. The first day of clinical

score 0 is defined as day 0 for each mouse. After 3 consecutive days of complete remission at a clinical score of 0, prednisolone was withdrawn (day 3), followed by another 2 days to allow clearance of prednisolone (19). The mice in the treatment group were given 2 million hAEC in basal media pooled from the four donors at passage 0 by intravenous injection on day 5, whereas the control mice remained untreated.

Mice were humanely euthanized by CO_2 inhalation at the end of the experiment or on reaching a clinical score of 3, as required by the animal ethics guidelines of Monash University. The hAEC-treated mice consisted of eight mice. Two of these mice were culled for severe relapse of score 3 on day 17, as required by ethics guidelines, and were excluded from further analysis. The control group consisted of 10 mice.

Splenocyte proliferation assay

The assay was performed to determine the proliferative response of splenocytes to stimulation of MOG_{35-55} peptide. Single-cell suspensions were prepared and stimulated with MOG_{35-55} peptide (1, 10 and 100 µg/mL) in complete Roswell Park Memorial Institute-1640 medium (SAFC Biosciences, St Louis, MO, USA) for 72 h. ^{3}H-thymidine (MP Biomedicals, Santa Ana, CA, USA) was then added, and cultures were maintained for another 18 h before the amount of ^{3}H-thymidine incorporation was measured with the use of a beta-counter (24). Thymidine incorporation was expressed as the stimulation index, which is the ratio of MOG-stimulated cultures to control splenocytes cultured in Roswell Park Memorial Institute-1640 medium only. Furthermore, the culture supernatants were collected just before addition of thymidine and stored at −20°C for mouse cytokine detection. Splenocytes were isolated from hAEC-treated mice (n = 6) and control mice (n = 7).

Cytokine detection in splenocyte culture supernatants

The following cytokines in MOG_{35-55} peptide-stimulated splenocyte culture media were assessed: interleukin (IL)-1α, IL-2, IL-4, IL-5, IL-6, IL-10, interferon-γ, tumor necrosis factor-α, granulocyte-macrophage colony-stimulating factor and IL-17 with the use of a mouse T-helper (Th)1/Th2/Th17 10 Plex FlowCytomix assay according to the manufacturer's instructions (eBioscience, San Diego, CA, USA). Data were analyzed with the use of FlowCytomixPro software. Splenocyte culture media from hAEC-treated mice (n = 6) and control mice (n = 10) were analyzed in duplicate.

Detection of anti-MOG antibodies

Assay for anti-MOG antibodies was carried out to determine the humoral immune response to MOG_{35-55} peptide. Autoantibodies against MOG_{35-55} peptide in mouse sera were detected as described previously (24). Briefly, sera were collected from mice by centrifugation of the whole blood at 4000 rpm for 10 min. Serum samples were tested at 1:100 and 1:300 dilutions in 96-well plates coated with 5 µg/mL of MOG_{35-55} peptide. The antigen-antibody complexes were detected by means of enzyme-linked immunoassay with the use of horseradish-peroxidase–conjugated goat anti-mouse immunoglobulin (Ig)G (Dako) and 3,3′,5,5′-tetramethylbenzidine substrate (Thermo Scientific, Waltham, MA, USA). The optical density was measured by means of the VICTORE X Multilabel Counter (PerkinElmer, Waltham, MA, USA). Samples were analyzed in triplicate, with readings taken from uncoated control wells deducted from the test samples. Sera from hAEC-treated mice (n = 6) and control mice (n = 10) were analyzed.

Flow cytometry

Flow cytometry was carried out to determine lymphocyte populations in spleen and inguinal lymph nodes of hAEC-treated mice, and results were compared with that in control mice. Single-cell suspensions prepared from spleen and inguinal lymph nodes were stained with PB-, PE- or antigen-presenting cell–conjugated monoclonal antibodies as described before (18). Fc receptors on splenocytes were blocked by anti-mouse CD16/CD32 antibodies (2.5 µg/mL; BD, Franklin Lakes, MI, USA) before the addition of test antibodies. Cells were incubated with antibodies against CD4 (1 µg/mL, BD), CD8 (1 µg/mL, BD), CD19 (0.25 µg/mL, eBioscience), CD25 (1 µg/mL, BD), Foxp3 (1 µg/mL, APC anti-mouse/rat staining kit, eBioscience) or the matched isotype control IgG (BD or eBioscience). The cells from hAEC-treated mice (n = 6) and control mice (n = 9) were analyzed with the use of the FACSCanto flow cytometer (fluorescence-activated cell sorting) (BD).

Histology

Histology of the spinal cord was assessed as described before (18,25). Inflammatory cell infiltrate and demyelination in spinal cords were evaluated with hematoxylin and eosin and Luxol fast blue (LFB) staining, respectively. Paraffin sections, 5 µm thick and 20 µm apart from each other from five sectors of each spinal cord, were stained (24,26). For random scanning of lesions along the

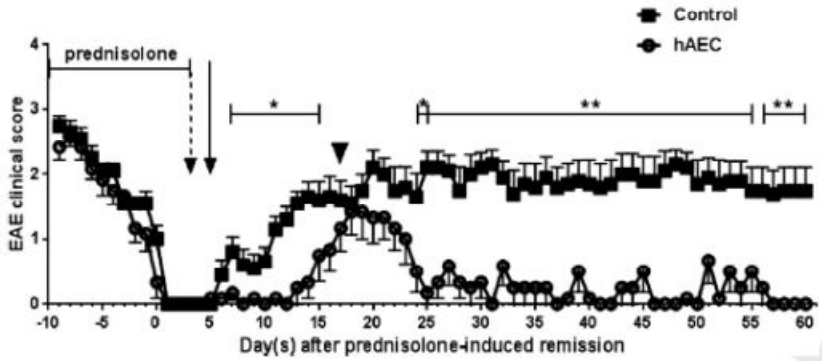

Figure 1. hAEC ameliorated EAE relapse in prednisolone-remitted mice. EAE was induced by immunization with MOG_{35-55} peptide and pertussis toxin. Clinical scores were evaluated on a scale of 0–5. Mice that developed EAE at score 2 (hind limb weakness) were treated with 60 μg/mL of prednisolone in their drinking water. With prednisolone treatment, EAE mice remitted to score 0. After 3 consecutive days at score 0, prednisolone treatment was withdrawn (day 3, dashed arrow). Two days after prednisolone withdrawal, hAEC (2×10^6) were given intravenously to the treated group (day 5, solid arrow, n = 8), whereas mice without cell infusion were kept as controls. hAEC infusion delayed EAE relapse (days 7–14). Arrowhead at day 17 indicates when the two hAEC-treated mice were culled on reaching score 3, as required by our animal ethics committee, and were excluded from the analysis. Analysis of the remaining mice, designated the responder group, showed that hAEC treatment lowered their clinical scores from day 24 onward until day 60. Data from the hAEC-treated responder group (n = 6) and control group (n = 10) are shown. Data are mean ± standard error of the mean. Solid line, *P* value for all hAEC-treated mice compared with controls. $*P < 0.05$, $**P < 0.01$.

spinal cord, a minimum of 36 sections for hematoxylin and eosin and a minimum of 31 sections for LFB were collected and examined from each mouse (n = 6 hAEC-treated mice and n = 10 control mice). For inflammatory cell infiltrate, hematoxylin and eosin–stained sections were scored as 0 = no inflammatory cell infiltrate; 1 = cellular infiltrate only in the perivascular areas and meninges; 2 = mild cellular infiltrate in parenchyma; 3 = moderate cellular infiltrate in parenchyma and 4 = severe cellular infiltrate in parenchyma. Demyelination in LFB-stained sections were scored as 0 = no demyelination; 1 = mild demyelination; 2 = moderate demyelination and 3 = severe demyelination.

Sections for immunostaining were treated with 0.01 mol/L citrate buffer for antigen retrieval. Endogenous peroxidase activity was quenched by 0.6% H_2O_2, followed by CAS block (Invitrogen) to prevent non-specific binding. Primary anti-CD3 (1:1000; Abcam, Cambridge, UK) or anti-F4/80 (1:2000, eBioscience) antibodies were applied, followed by appropriate secondary antibodies. Stained cells were detected with the use of the ABC kit (Vector Laboratories, Burlingame, CA, USA), followed by 3,3′-diaminobenzidine chromogen (Sigma-Aldrich) and counterstained with hematoxylin. A minimum of five sections for CD3 and a minimum of four sections for F4/80 were analyzed for each mouse to calculate the average amount of $CD3^+$ and $F4/80^+$ cells. Sections from six hAEC-treated mice and 10 control mice were analyzed.

Statistical analysis

All data are presented as mean ± standard error of the mean and evaluated by Student's *t* test. Significance was defined at a value of $P < 0.05$.

Results

EAE relapse after corticosteroid-induced remission is inhibited by intravenous hAEC treatment

The daily clinical score observed in mice induced with EAE reflects the level of pathology in its disease course. EAE-induced mice that developed a clinical score of ≥2 with hind limb weakness had prednisolone added to their drinking water to induce disease remission (19). The remission caused the clinical score to decrease to 0 after an average of 17 days. After 3 days of complete remission, prednisolone was withdrawn from the drinking water (dashed arrow, Figure 1). After another 2 days, remitted mice were given hAEC by intravenous injection (day 5, solid arrow, Figure 1). Control mice without hAEC infusion (n = 10) started to relapse on day 5; their clinical scores gradually increased, reaching a maximum average score of 2.1 on day 25, and maintained an average score of 1.75 until the end point of experiment on day 60 (Figure 1, filled squares). In contrast, all eight hAEC-treated mice had a delayed relapse on day 12 to develop EAE with a lower average score of 1.68. Within the hAEC-treated mice cohort, there were two non-responder mice that were like the responder group in that they remained in remission until day 12 but relapsed and

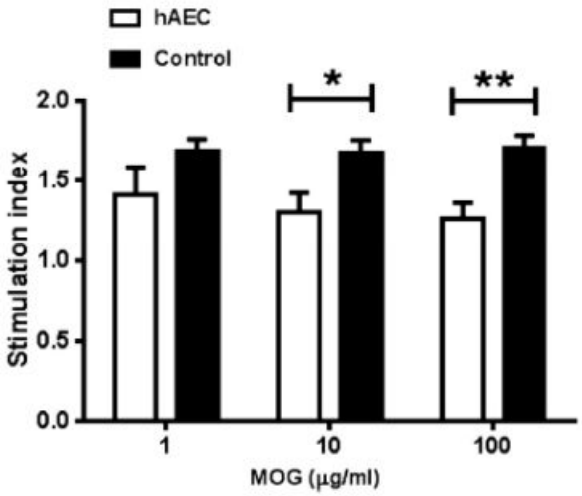

Figure 2. hAEC infusion decreased MOG-stimulated splenocyte proliferation *in vitro* in the prednisolone-treated EAE responder group. Mouse splenocytes were stimulated by MOG_{35-55} peptide (1, 10 or 100 μg/mL) for 72 hours. ^{3}H-thymidine was introduced to the culture media and left for another 18 h, and thymidine incorporation by dividing cells was determined. hAEC-treated mice (n = 6) showed lower ^{3}H-thymidine incorporation, expressed as stimulation index with that was significant with 10 and 100 μg/mL MOG_{35-55} concentrations, compared with splenocytes from control mice (n = 7). $^{*}P < 0.05$, $^{**}P < 0.01$. Data are mean ± standard error of the mean.

had to be culled on day 17 after reaching a clinical score of 3, as required by our animal ethics committee (Figure 1, arrowhead). In the remaining hAEC-treated responder mice (n = 6), clinical scores dropped between days 20–25, with almost complete remission by day 25 and complete remission (score 0) on several days after day 31 that persisted until day 60 (Figure 1, open circles). Overall, hAEC infusion significantly delayed relapse for 1 week (days 5–12), and induced remission in the responder group that persisted over the test period of 5 weeks (Figure 1, solid line indicating $P < 0.05$ on day 24 and $P < 0.01$ on days 25–54, 56–60).

Clinical improvement of responder mice is associated with decreased immune response to MOG_{35-55}, increased regulatory T cells and altered cytokines

T cells coordinate immune responses contributing to MS pathogenesis, including its own activation and subsequent responses involve B cells, macrophages and other immune cells. Proliferation and cytokine production of T cells under specific antigen challenge reveal the intensity of specific T-cell responses. In the present study, we stimulated splenocytes with MOG_{35-55} peptide, in which MOG-specific T cells proliferated and produced a combination of cytokines that were measured. MOG_{35-55} peptide at concentrations of 1, 10 and 100 μg/mL stimulated splenocyte proliferation of control mice (Figure 2, filled bars). In contrast, proliferation of splenocytes from hAEC-treated mice to 10 and 100 μg/mL concentrations of MOG_{35-55} peptide were significantly lower (Figure 2, open bars compared with filled bars). Furthermore, we examined MOG-specific IgG production by activated B cells in EAE mice at the end point of experiment. We found that MOG-specific IgG in serum was significantly reduced in hAEC-treated mice compared with controls for both serum dilutions (Figure 3, open bars versus filled bars). These data clearly showed that hAEC treatment decreased peripheral MOG-specific lymphocyte responses.

There were significant alterations to cytokines in the supernatants collected from splenocyte cultures stimulated with MOG_{35-55} peptide. IL-5 (a Th2 cytokine) and IL-2 (a Th1 cytokine) were elevated in splenocytes isolated from hAEC-treated mice (Figure 4, open bars) compared with control mice (Figure 4, filled bars). Other cytokines, including IL-1α, IL-4, IL-6, IL-10, IL-17, granulocyte-macrophage colony-stimulating factor, interferon-γ and tumor necrosis factor-α, did not change significantly after hAEC treatment (Figure 4). hAEC infusion also led to a greater percentage of regulatory T cells (Treg), as shown by flow cytometry analysis. The percentages of Treg in the draining inguinal lymph nodes from the hAEC-treated mice were significantly higher than those from the controls, as shown by increased percentage of $CD4^{+}CD25^{+}$,

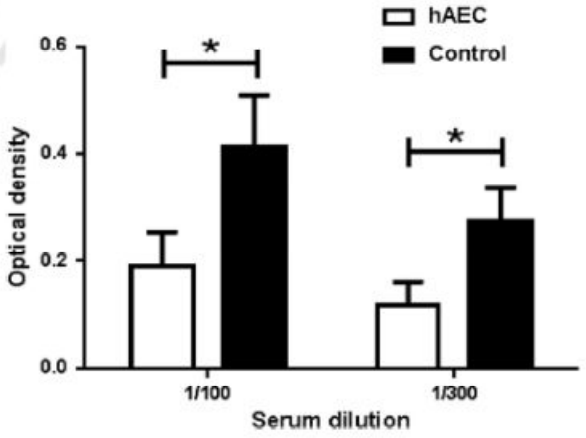

Figure 3. hAEC-treated responder mice have reduced anti-MOG antibodies in serum compared with control mice. Sera collected at the end point of each mouse experiment were examined for MOG-specific antibodies with the use of enzyme-linked immunoassay. Sera of all hAEC-treated mice (n = 6) had significantly less MOG-specific IgG compared with the control mice (n = 10). Graph shows the optical density readings taken from sera that were tested in dilutions of 1:100 and 1:300 ($^{*}P < 0.05$). Data are mean ± standard error of the mean.

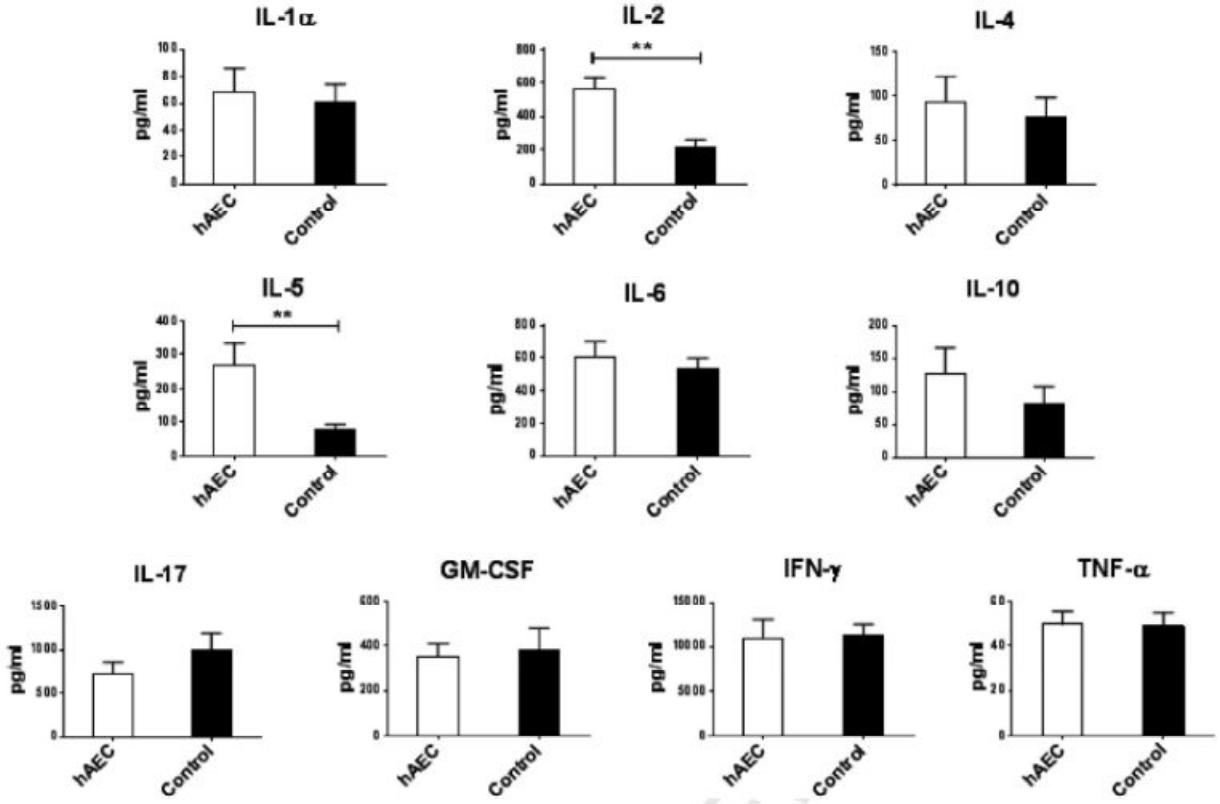

Figure 4. Cytokines in supernatants from cultured splenocytes stimulated with MOG_{35-55} peptide. Splenocytes prepared from control mice or hAEC-treated responder mice were stimulated with 10 μg/mL MOG_{35-55} for 72 h. Culture supernatants were collected and cytokines measured by flow cytometry. IL-5 and IL-2 were significantly elevated (**$P < 0.01$) in the hAEC-treated group (n = 6) compared with the control group (n = 10). Data are mean ± standard error of the mean.

$CD4^+FoxP3^+$ and $CD4^+CD25^+FoxP3^+$ cells (Figure 5A; $P < 0.05$). This difference was not observed in the spleens (Figure 5B). The main subsets of T and B lymphocytes did not alter after hAEC treatment, as $CD4^+$ T cells, $CD8^+$ T cells and $CD19^+$ B-cell populations did not change significantly in the hAEC-treated group compared with the controls, either in the inguinal lymph nodes or spleens (Figure 5C,D).

hAEC reduced inflammatory cell infiltrate and demyelination in the spinal cord

In MS and EAE, immune cells are recruited to the CNS and transmigrate through the blood-brain barrier, resulting in local inflammation and demyelination. We examined spinal cord pathology of responder mice through the use of hematoxylin and eosin and LFB staining for inflammatory cell infiltrate and demyelination, respectively. Compared with control mice, responder hAEC-treated mice had significantly reduced demyelination (Figure 6A,B; $P < 0.05$). The reduction of inflammatory cell infiltrate by hAEC treatment did not reach significance compared with control mice (Figure 6A,B) but was substantiated by the decrease in $F4/80^+$ macrophages in the hAEC-treated responder mice, as assessed by immunostaining (Figure 6C,D; $P < 0.05$). The decrease of $CD3^+$ T cells in hAEC-treated mice did not reach statistical significance compared with controls. Overall, the milder pathology in the spinal cords was observed in hAEC-treated responder mice (Figure 6A–D).

Discussion

In the present study, we found that six of eight hAEC-treated mice showed beneficial effects on the clinical symptoms and immune modulation in EAE mice that had undergone prednisolone-induced remission before hAEC delivery. We designated this group the responder group. Two mice that we designated as non-responders also exhibited delayed onset of relapsed EAE but had to be culled when they reached score 3, as required by our animal ethics committee. Whether these mice would have subsequently remitted like the rest of the responder mice remains unknown. hAEC infusion delayed the onset

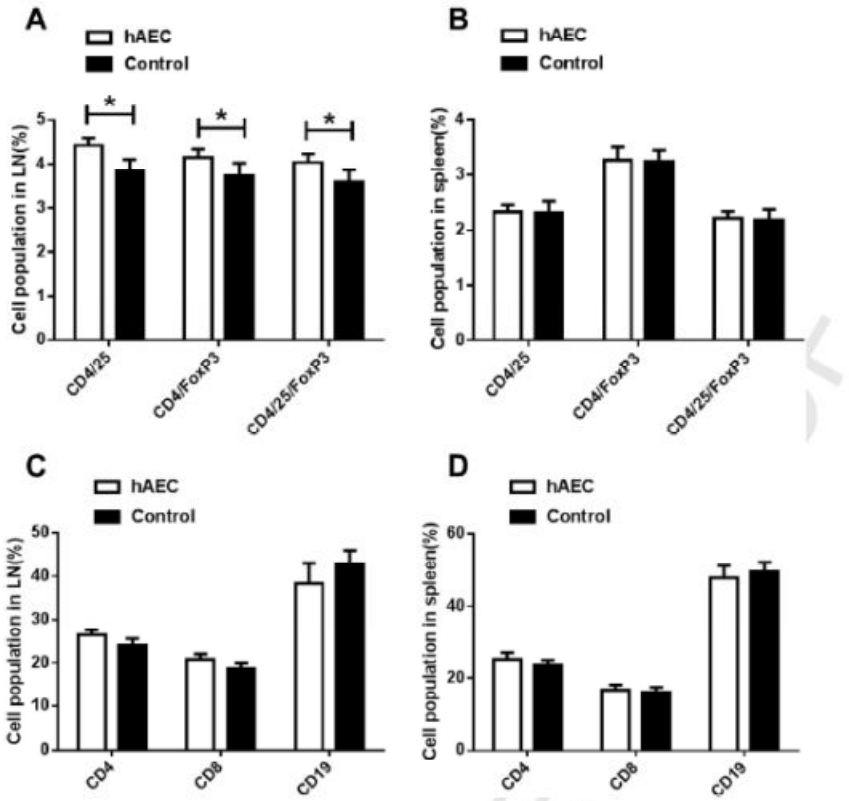

Figure 5. Elevated regulatory T cells in inguinal lymph nodes from hAEC-treated responder mice. Cells from spleen and inguinal lymph nodes were stained with the use of fluorescent-tagged antibodies to identify regulatory T cells (CD4, CD25 and FoxP3), CD4, CD8 and CD19 by fluorescence-activated cell sorting. Regulatory T cells (A,B) $CD4^+CD25^+$, $CD4^+FoxP3^+$ or $CD4^+CD25^+FoxP3^+$ combinations ($^*P < 0.05$) were significantly higher in inguinal lymph nodes but not in splenocytes in hAEC-treated mice (n = 6). $CD4^+$, $CD8^+$ or $CD19^+$ lymphocyte populations were similar between hAEC-treated mice and the control group (n = 9) in both lymph nodes and spleens (C,D). Data are mean ± standard error of the mean.

of EAE relapse and induced EAE remission in the responder group. Compared with our previous data suggesting that hAEC could serve as prevention therapy (18), our present finding showed that hAEC could also be applied as an intervention therapy after corticosteroid-induced remission. The control group from our previous prevention study developed disease with clinical scores reaching 2.71 at the end point of experiment, whereas the hAEC-treated group had a lower average score of 1.68 (18). In the present study, the relapsed control group developed clinical symptoms with a maximum average score of 2.1 (Figure 1, day 20) and later maintained an average score of 1.75 until the end point (Figure 1, day 60). Mice from the hAEC-treated responder group had milder disease and reached complete remission (score 0) on several days after day 31. These findings show that hAEC have the potential to ameliorate EAE and even reverse long-term disability in remitted EAE mice.

Compared with the control group, MOG-specific antibody production in serum was significantly reduced in the hAEC-treated responder group (Figure 3). This reduction was even more significant compared with our prevention study (18). Although autoantibody levels have been linked to MS disease severities (27), the significance of antibody titer in relation to disease severity of MS is uncertain.

EAE generated through injection of MOG antigen and complete Freund's adjuvant in mice induces a mixed Th1 and Th17 immune response (28,29). Because increased IL-5 was also seen in patients with MS after some therapies (30,31), the elevated IL-5 in hAEC-treated mice may represent a change toward a beneficial cytokine profile after hAEC treatment. The result was also similar to that in our previous prevention study, with same trends of elevated IL-5 and IL-2 (18); however, the role of elevated IL-2 remains unclear in the pathogenesis of these EAE models.

Neuroantigen-specific autoreactive T lymphocytes have a central role in the pathogenesis of MS. We report that splenocytes from responder mice given hAEC had a lower MOG-specific response

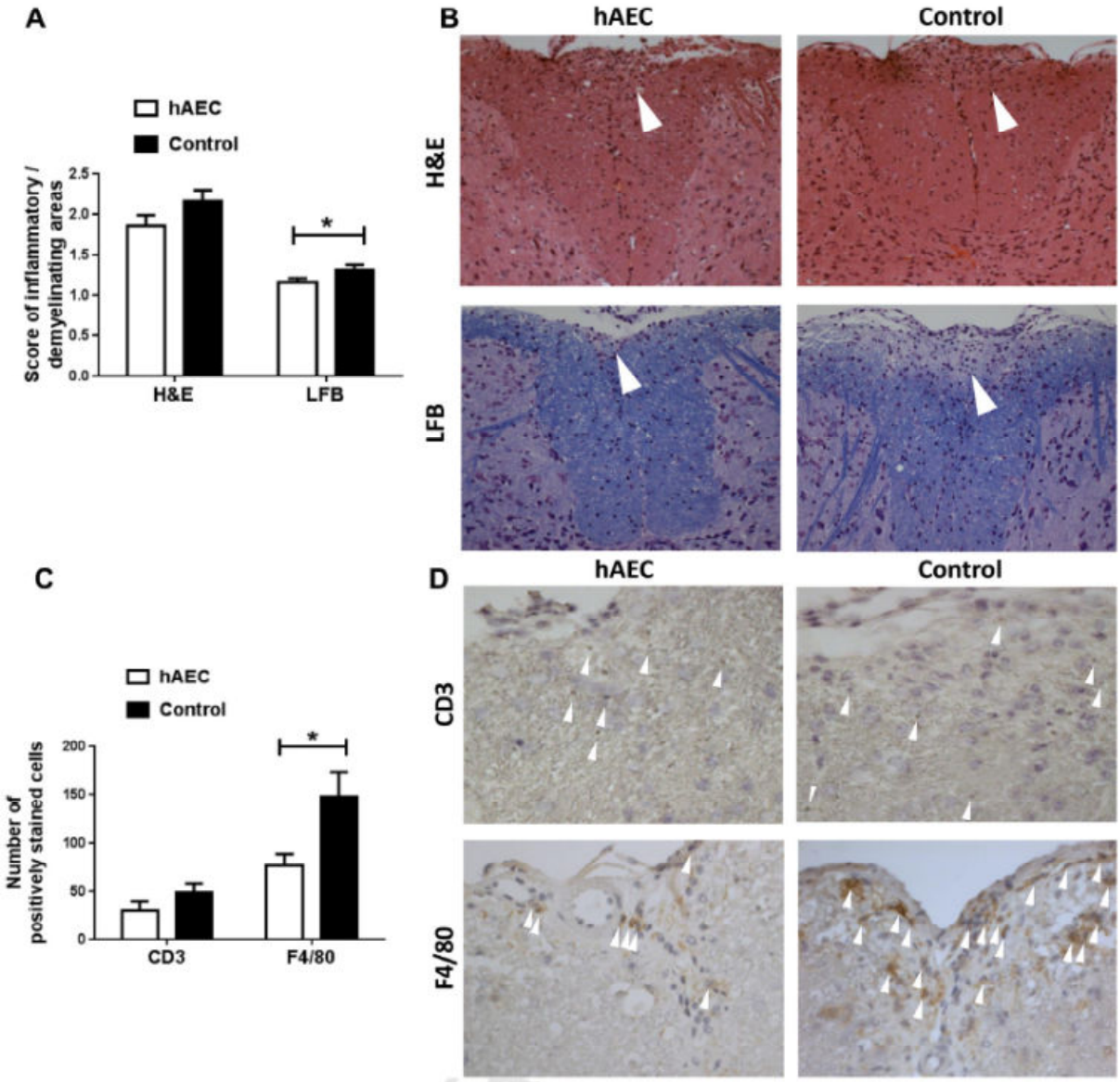

Figure 6. Spinal cord histopathology in hAEC-treated responder mice and control mice. Severity of spinal cord histopathology was determined by scanning discontinuous mouse spinal cord sections. (A) Inflammatory infiltrate assessed by hematoxylin and eosin was lower in the hAEC-treated mice (n = 6), but the difference did not reach statistical significance. Axonal demyelination examined by LFB staining was significantly reduced in hAEC-treated mice compared with the control group (n = 10). (B) Representative photos of hematoxylin and eosin and LFB staining from hAEC-treated responder and control groups are shown (original magnification ×40). Arrows indicate inflammatory cell infiltrate and demyelination (pale area). (C) Immunostaining of $CD3^+$ T cells and $F4/80^+$ macrophages showed a reduction in numbers of T cells and macrophages in the hAEC-treated mice that reached statistical significance with macrophages. (D) Representative photos of immunostaining from hAEC-treated responder and control groups are shown (original magnification ×40). Arrows indicate $CD3^+$ T cells and $F4/80^+$ macrophages. Data are mean ± standard error of the mean. $^*P < 0.05$.

compared with the controls (Figure 2). Besides the reduced proliferative reaction from conventional T cells, an enhanced Treg population was also noted. Higher Treg numbers in the periphery may help to ameliorate MS because reduced numbers of peripheral Treg have been reported in some patients with relapsing-remitting MS, and transfer of $CD4^+CD25^+$ Treg protects mice from EAE (32,33). In the present study, we found that the Treg population was increased in the draining inguinal lymph nodes near the injection sites of MOG emulsion in hAEC-treated mice. An elevated Treg population was also observed in our previous study at 2 weeks after EAE induction (unpublished data) but was not

sustained until the end of assessment (18). The elevation in hAEC-treated mice almost 8 weeks after hAEC administration suggests that hAEC may have promoted the homing of Treg from blood to lymph nodes. It is not clear why increased Treg were only seen in inguinal lymph nodes. It is tempting to speculate that the increase in Treg in inguinal lymph nodes draining the sites of MOG immunization may have had a role in decreasing the immune response to MOG.

Additionally, these $CD4^+$ Treg express CD25 and the transcription factor FoxP3 (Figure 5A). Loss of FoxP3 in Treg has been reported in some patients with MS who have similar numbers of Treg compared with healthy individuals (34). The enhanced $CD4^+CD25^+FoxP3^+$ Treg population in draining inguinal lymph nodes together with lower splenocyte proliferation to MOG peptide suggest that hAEC may have a therapeutic role in modulating peripheral T-cell functions that might contribute to the prevention of relapse in corticosteroid-remitted EAE.

On the basis of our present findings, we propose that combinational therapy of hAEC and an immunosuppressive drug such as prednisolone has the potential to improve the outcomes for patients with MS.

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Chapter 4. Effects of Culture and Differentiation of Human Amniotic Epithelial Cells for Potential Clinical Applications

As an alternative cell source for stem cell-based therapy, one should consider the changes made to the cell after the necessary propagation and/or differentiation. Here I address these issues *via* two studies in hAEC: the first study named "Changes in culture expanded human amniotic epithelial cells: Implications for potential therapeutic applications" discusses the influence of serial expansion to hAEC when using different culture formula. The second study, named "Immunogenicity and immunomodulatory properties of hepatocyte-like cells derived from human amniotic epithelial cells", address the effects of differentiation process on hAEC.

Compared with the currently well-applied mesenchymal stem cells (MSC) in many clinical trials, hAEC have the advantage of higher cell number obtained from a procedure which is neither invasive nor costly. The ideal cell type for cell-based therapy should be able to provide abundant cells which contain enough cells from a single donor, to prevent micro-chimerism or immune responses caused by pooling cells from different hAEC donors. Even though there are about 150-200 million of hAEC that can be obtained from each term amnion membrane, the number may not meet the

requirement for clinical use yet. Researchers have suggested that hAEC cultured in animal serum-supplemented media after a few passages are more like mesenchymal cells because of the transition in gene expression and surface antigen expression profiles and their mesenchymal-like phenotype. In the first part of this chapter, hAEC cultured in xenobiotic-free media were compared with hAEC cultured from animal serum-supplemented media and with the primary hAEC to understand the differences of surface marker expression, differentiation capacity, production of immunomodulatory molecules and ability to suppress splenic T cell proliferation in three different cell types.

Despite using undifferentiated hAEC for specific diseases, applying terminal-differentiated hAEC has also shown their potential in allogeneic organ transplantation. Stimulation-induced differentiation induces some additional features of hepatocyte to hAEC. In the latter part of this chapter, hAEC-derived hepatocyte-like cells were compared with primary hAEC with their expression of human leucocyte antigen (HLA) and costimulatory molecules, as well as their immunogenicity and immunomodulatory properties. The results showed hAEC-derived hepatocyte-like cells have higher immunogenicity but retain their immunomodulatory properties to allogeneic peripheral blood mononuclear cells (PBMC) possibly via several identified immunomodulatory molecules.

The two studies represented in this chapter have explored more aspects for potential future clinical application of hAEC, including cell number issue, culture procedure and terminal differentiation. Primary hAEC seems to be the best and the least risky choice considering their potent immunosuppressive properties, higher expressions of immunomodulatory molecules, and lower immunogenicity. It is a consistent

characteristic from xenogeneic to allogeniec T cell proliferation that TGFβ and/or PGE2 as well as other molecules are important in hAEC-mediated suppression, of which I have similar results mentioned in earlier chapters. But for cultured hAEC or hAEC differentiated hepatocyte-like cells, retention of some primary hAEC features possibly enable them to be used for different therapeutic purposes.

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Changes in Culture Expanded Human Amniotic Epithelial Cells: Implications for Potential Therapeutic Applications

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Abstract

Human amniotic epithelial cells (hAEC) isolated from term placenta have stem cell-like properties, differentiate into tissue specific cells and reduce lung and liver inflammation and fibrosis following transplantation into disease models established in mice. These features together with their low immunogenicity and immunosuppressive properties make hAEC an attractive source of cells for potential therapeutic applications. However, generation of large cell numbers required for therapies through serial expansion in xenobiotic-free media may be a limiting factor. We investigated if hAEC could be expanded in xenobiotic-free media and if expansion altered their differentiation capacity, immunophenotype, immunosuppressive properties and production of immunomodulatory factors. Serial expansion in xenobiotic-free media was limited with cumulative cell numbers and population doubling times significantly lower than controls maintained in fetal calf serum. The epithelial morphology of primary hAEC changed into mesenchymal-stromal like cells by passage 4–5 (P4–P5) with down regulation of epithelial markers CK7, CD49f, EpCAM and E-cadherin and elevation of mesenchymal-stromal markers CD44, CD105, CD146 and vimentin. The P5 hAEC expanded in xenobiotic-free medium differentiated into osteocyte and alveolar epithelium-like cells, but not chondrocyte, hepatocyte, α- and β-pancreatic-like cells. Expression of HLA Class IA, Class II and co-stimulatory molecules CD80, CD86 and CD40 remained unaltered. The P5 hAEC suppressed mitogen stimulated T cell proliferation, but were less suppressive compared with primary hAEC at higher splenocyte ratios. Primary and P5 hAEC did not secrete the immunosuppressive factors IL-10 and HGF, whereas TGF-β1 and HLA-G were reduced and IL-6 elevated in P5 hAEC. These findings suggest that primary and expanded hAEC may be suitable for different cellular therapeutic applications.

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Introduction

Human amniotic epithelial cells (hAEC) line the inner of two fetal derived membranes attached to the placenta. hAEC arise from pluripotent epiblast cells of the embryo and are among the first cells to differentiate in the conceptus [1]. Studies have shown that even at term pregnancy, primary hAEC isolated from amnion membranes retain some of the features of their founder cells, expressing pluripotency associated genes and differentiating into lineages derived from each of the three primary embryonic germ layers *in vitro* [2,3]. Primary hAEC also display similarities to multipotent mesenchymal stromal/stem cells (MSC) expressing some of the surface antigens defining MSC, and like MSC lack hematopoietic and monocytic lineage markers [4,5,6].

Importantly, primary hAEC have several features that make them most attractive for cellular therapies. Compared with adult tissue derived stem cells, hAEC are plentiful and obtained without invasive and expensive procedures from term placenta, a widely accepted non-controversial source of stem cells. Replacement of cells damaged by disease, injury and aging remains a key goal in many therapeutic applications. In this context, hAEC have been shown to differentiate into functional neurons in spinal cord injury models [7,8], insulin secreting pancreatic β-islet like-cells that normalized blood glucose in diabetic mice [9] and recently into surfactant producing alveolar epithelial cells in the lung [6]. Therapies aimed at reducing tissue inflammation and scarring to promote host tissue repair are another important potential application of stem cells. Studies in murine models of lung and liver fibrosis have shown that primary hAEC reduce inflammation and fibrosis and induce tissue remodeling and repair [6,10,11]. Further, hAEC transplantation appears to be safe and tumour or teratoma formation has not been demonstrated in spite of Oct-4, Sox-2 and Nanog expression that are linked to teratoma formation by embryonic and induced pluripotent stem cells [2,3,6,11].

Another key feature is that primary hAEC appear to be amenable to allogeneic transplantation and indeed have been successfully transplanted into non-related human recipients during trials for lysosomal storage diseases [12]. Successful transplantation across histocompatibility barriers is probably facilitated by low HLA Class IA antigen expression and absence of HLA Class II antigens [2,4,13,14]. Primary hAEC have also been shown to exert potent immunosuppressive properties inhibiting T cell proliferation [13,14,15], although the mechanisms remain unclear.

Approximately 50 100 million hAEC can be harvested from each term amnion membrane [3,16]. However, cellular therapies would require several billion cells from each cell line for multiple dosing regimens and, importantly, to prevent micro-chimerism and potential immune responses arising from cells that have been pooled from several unrelated donors. For clinical applications, large numbers of MSC are generated by serial expansion under xenobiotic-free conditions to comply with good manufacturing practices (GMP) [17]. hAEC do not appear to be amenable to extensive expansion in animal serum supplemented media. Expression of pluripotency genes was suppressed during expansion in fetal calf serum (FCS) accompanied by changes in phenotype and surface antigen expression suggestive of an epithelial to mesenchymal transition [4,5]. While hAEC expanded in FCS differentiated into osteocytes *in vitro* [5], whether expanded cells retain the ability to differentiate into lineages having therapeutic potential, such as hepatocytes and pancreatic β-islet cells, remains unknown. Importantly, the immunogenicity, immunosuppressive and secretory properties of expanded hAEC are unknown. We investigated if hAEC could be expanded in xenobiotic-free media and compared the differentiation, immunophenotype and immunosuppressive properties of cells expanded in xenobiotic-free medium with FCS supplemented medium and primary cells. The findings showed that expansion led to significant differences in the expression of markers, capacity to differentiate, ability to suppress T cell proliferation and the secretion of immunosuppressive factors by hAEC.

Materials and Methods

Ethics Statement

The study was approved by Southern Health and Royal Women's Hospital Human Research Ethics Committees and Institutional Review Boards of Monash University and University of Melbourne. Informed, written consent was obtained from each patient prior to amnion tissue collection. Amnion membranes were retrieved from placentae delivered by healthy women with a normal singleton pregnancy undergoing elective cesarean section at term (37 40 weeks gestation) for breech presentation or prior section (n = 30). Membranes were collected in DMEM/F12 medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B and 2 mM L-glutamine (Gibco, Grand Island, NY). Isolation of splenocytes from C57BL/6 mice was approved by the Animal Ethics Committee, Monash University (approval number MMCB 2009/16).

Isolation and Characterization of hAEC

Cells were isolated using a method described previously [2]. Briefly, tissue was digested twice in 0.25% trypsin containing 0.5 mM EDTA in Hanks Balanced Salt Solution (HBSS) for 15 min at 37°C with gentle shaking. Trypsin was inactivated with newborn calf serum, solution filtered and centrifuged at 175×*g*. The cells were washed in DMEM/F12 and contaminating erythrocytes lysed in hypotonic solution (8% ammonium chloride, 0.84% sodium bicarbonate and 0.37% EDTA) for 10 min at 37°C. Media and reagents were purchased from Gibco. Purity of the isolates was determined by flow analysis for the epithelial marker cytokeratin-7 (CK7; Dako, Carpentaria, CA), as described earlier [2]. Isolates that were >99% positive for CK7 and exhibiting a cobblestone appearance in primary culture were used in the experiments described below. Each of the following studies were carried out on hAEC isolated from (n = 4 6) amnion membranes.

hAEC Expansion

To determine if hAEC could be expanded under xenobiotic-free conditions, commercially available serum free media and human serum were tested. Freshly isolated hAEC (1.5×10^6) were plated in 25 cm^2 flasks and cultured in the following: 1) Epilife medium with xenobiotic-free S7 supplement (Cascade Biologics, Portland, OR); 2) PC-1 medium (Lonza, Walkersville, MD); 3) Stempro MSC medium (Gibco); 4) CnT22 medium (Millipore, Billerica, MA); and 5) 2 10% heat inactivated human serum (Gibco) in DMEM/F12. Comparisons were made against hAEC grown in DMEM/F12 with 10% FCS. Preliminary studies demonstrated better growth with addition of recombinant human epidermal growth factor (rhEGF). Therefore, all culture media were supplemented with 10 ng/ml rhEGF (Invitrogen, Carlsbad, CA). Primary cultures were designated as passage 0 (P0). Media were changed thrice weekly and cells passaged at ~80% confluence using a split ratio of 1:2. Cells were lifted using TrypLE (Gibco) and counted (Countess™ Automated Cell Counter, Invitrogen). Cumulative cell numbers (CCN) [4] and cumulative population doublings (CPD) were determined. CPD was calculated using the formula: CPD = [ln (cumulative cell number)]/ ln 2 [18]. Where possible, cells were maintained until P7.

To monitor changes in morphology with expansion, P2 hAEC with cobblestone epithelial appearance were labeled with CFSE (Invitrogen) following manufacturer's instructions and then expanded. Cultures were passaged twice after reaching ~80% confluence and photographed under an inverted fluorescence microscope (Olympus IX71, Melville, NY).

Flow Cytometry

hAEC (approximately 1×10^5 cells) suspended in 100 μl of PBS/ 2% FCS/0.01% sodium azide were incubated with directly conjugated or unconjugated anti-human primary antibodies or matched-isotype control IgG (Table 1) for 45 min at 4°C. After several washes cells were incubated with phycoerythrin (PE)-conjugated goat anti-mouse Ig $F(ab')_2$ fragments (10 μl/ml; Chemicon, Melbourne, Australia), Alexa Fluor (AF) 647-conjugated goat anti-mouse IgG (10 μg/ml; Molecular Probes, Eugene, OR) or AF 488-conjugated chicken anti-rat IgG (10 μg/ml) for 30 min at 4°C except for CD31, CD45 and CD90. Blocking serum (5 μl chicken serum for CD29 and CD49f; 5 μl goat serum for the balance) was also included during incubation with primary and secondary antibodies. Cells were analyzed by flow cytometry using Cyclops SUMMIT software (Version 5.0; Dako Cytomation, Fort Collins, CO).

Immunocytochemistry

hAEC cultured in 8-well chamber slides (2×10^4 cells/well) were fixed in 4% paraformaldehyde for 20 min. Endogenous peroxidase activity was quenched in 0.3% H_2O_2 (Orion Laboratories, Balcatta, Australia) in methanol and non-specific binding blocked in PBA solution (Shandon, Pittsburgh, PA) for 15 min. Cells were incubated with antibodies against HLA-G (1:100; BD Biosciences, San Jose, CA) or CK7 (1:100; Dako) in DPBS containing 0.2% Triton X-100 for 1 h at 37°C. Negative controls were incubated with mouse $IgG_{1,k}$ (BD Biosciences). Cells were washed and incubated with anti-mouse-biotinylated secondary antibody

Table 1. Antibodies Used to Phenotype hAEC by Flow Cytometry.

Primary Antibodies/Fluorochrome	Isotype	Working Concentration	Source of primary antibodies
E-cadherin	Mouse IgG1	(supernatant)	Hans-Jorg Buhring, Tbingen, Germany
CD49f	Rat IgG2a	5 μg/ml	BD Biosciences, San Jose, CA
EpCAM	Mouse IgG1	11.8 μg/ml	Dako, Glostrup, Denmark
CD44	Mouse IgG2b	1 μg/ml	BD Biosciences, San Jose, CA
CD90/APC	Mouse IgG1	1 μg/ml	BD Biosciences, San Jose, CA
CD105	Mouse IgG1	10 μg/ml	BD Biosciences, San Jose, CA
CD146	Mouse IgG2a	(supernatant)	Stem Cell Centre, Melbourne, Australia
Vimentin	Mouse IgG1	0.28 μg/ml	Invitrogen, Camarillo, CA
PDGFR-β	Mouse IgG1	20 μg/ml	R&D Systems, Minneapolis, MN
CD29	Rat IgG2a	1 μg/ml	BD Biosciences, San Jose, CA
CD45/FITC	Mouse IgG1	10 μg/ml	Caltag, Burlingame, CA
CD31/PE	Mouse IgG1	4 μg/ml	Dako, Glostrup, Denmark
HLA-A-B-C	Mouse IgG1	0.5 μg/ml	BD Biosciences, San Jose, CA
HLA-DR-DP-DQ	Mouse IgG2a	2 μg/ml	BD Biosciences, San Jose, CA
CD40	Mouse IgG2a	0.4 μg/ml	Abcam, Cambridge, UK
CD80	Mouse IgG1	4 μg/ml	Abcam, Cambridge, UK
CD86	Mouse IgG1	0.4 μg/ml	Abcam, Cambridge, UK

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(1:200; Vector Laboratories, Burlingame, CA) for 30 min followed by ABC kit reagents (Vector Laboratories). Immunostaining was visualized using DAB chromogen (Sigma-Aldrich, St. Louis, MO).

Karyotype Analysis

Chromosomal analysis using G-banding was performed by Southern Cross Pathology, Monash Medical Centre. After 4 6 h treatment in colchicine, cells were harvested and fixed in methanol/acetic acid. G-Bands were visualized by 0.025% trypsin (BD Difco, Sparks, MD) treatment for 5 20 sec, followed by incubation in 0.04% Leishman's stain (Sigma-Aldrich, Castle Hill, Australia) for 5 min.

Clonal Culture

P0 hAEC have been shown to form clonal colonies [2]. To determine if P5 hAEC were clonogenic, cells were seeded at low density (~30 50 cells/cm^2 in 100 mm diameter petri dishes). Cultures were maintained in DMEM/F12 with 10% FCS or Epilife with 10 ng/ml rhEGF for up to 21 d with media replaced once weekly. Clusters containing more than five cells were considered to be colonies. Cloning efficiency was calculated using the formula: cloning efficiency (%) = (number of colonies/number of cells seeded)×100 [2].

Alkaline Phosphatase Activity

hAEC were seeded in 8-well chamber slides (2×10^4/well) and fixed in 4% paraformaldehyde for 1 2 min at room temperature (RT). Alkaline phosphatase activity was detected using a kit (Millipore), following manufacturer's instruction and then counter-stained with haematoxylin for 10 15 sec. A human embryonal carcinoma (hEC) cell line (provided by Prof. Martin Pera, University of Southern California, USA), served as a positive control [19].

Transmission Electron Microscopy (TEM)

Cultures were fixed in 2.5% glutaraldehyde for 2 h at RT, washed in 0.1 M cacodylate buffer and post-fixed in 1% osmium tetroxide for 2 h. Cells were dehydrated through ethanol, infiltrated, embedded in resin-araldite mixture and polymerized at 60°C for 24 h. Ultrathin sections (90 nm) were stained with uranyl acetate for 10 min and Reynolds lead for 2 min. Sections were viewed on Hitachi H-7500 (Tokyo, Japan) transmission electron microscope and images acquired digitally.

Differentiation and Characterization

P0 and P5 hAEC were plated in 8-well chamber slides (2×10^4 cells/well) or 6-well plates (2.5×10^5 cells/well). Cells were cultured in Small Airway Growth Medium (SAGM; Lonza, Walkersville, MD; Table 2) to induce differentiation into alveolar epithelium-like cells [6]. Supplements listed in Table 2 were added to basal medium to differentiate cells into mesodermal and endodermal derived lineages. Chondrocytic differentiation was induced by pelleting hAEC (3×10^5 cells) and adding supplements (Table 2). Treated cultures and non-stimulated controls in basal medium were maintained for up to four weeks with media changes thrice weekly.

Secreted insulin was measured by Southern Cross Pathology, Monash Medical Centre using the Access/DXI Ultrasensitive Insulin assay (Beckmann-Coulter, Sydney, Australia). Immunocytochemistry was carried out to identify the hormone glucagon (GCG), produced by pancreatic α-cells. hAEC were fixed with 4% paraformaldehyde, endogenous peroxidase activity quenched in methanol and non-specific binding blocked in PBA. Cells were incubated anti-GCG (1:50; R&D Systems; Minneapolis, MN) in PBS containing 0.2% Triton-X, overnight at 4°C. Mouse IgG2a (Dako) was applied to negative controls. Antibody binding was detected with DAB.

Differentiation into alveolar epithelium-like cells was also tested by immunocytochemistry for prosurfactant protein-C (proSP-C; Millipore). hAEC were fixed in ethanol and incubated with antibody against proSP-C (1:200) in PBS containing 0.01% Tween 20, overnight at 4°C. Rabbit IgG (Dako) was applied to negative controls.

Table 2. Differentiation media and supplements.

Lineage	Differentiation medium/supplements/duration/references	Characterized by
Endodermal		
Alveolar Epithelium	SAGM medium containing hydrocortisone, BSA-fatty acid free serum, bovine pituitary extract, rhEGF, epinephrine, transferrin, insulin, retinoic acid and tri-iodothyronine. Four weeks [6].	Prosurfactant Protein-C
Pancreatic	Nicotinamide (10 mM), retinoic acid (1 mM), N2 supplement, rhEGF (10 ng/ml), exendin-4 (10 nM). Two weeks [42].	Insulin/glucagon (GCG)
Hepatic	rhEGF (10 ng/ml, 5 days), then dexamethasone (0.1 μM), insulin (0.1 μM) for 3 weeks [2,3].	Hepatocyte Nuclear Factor-4α, Albumin
Mesodermal		
Osteocytic	1,25-Dihydroxyvitamin D3 (0.01 μM), ascorbic acid (50 μM), b-glycerophosphate (10 mM). Four weeks [2].	Alizarin Red Stain
Chondrocytic	Insulin (6.25 μg/ml), ascorbic acid-2-phosphate (50 μM), transforming growth factor-β1 (10 ng/ml). Four weeks [43].	Alcian Blue

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Differentiation into hepatocyte-like cells was tested by immunofluorescence for hepatocyte nuclear factor-4α (HNF-4α; Cell Signaling Technology, Danvers, MA) and albumin (R&D Systems). hAEC were fixed in ethanol and incubated with antibodies against HNF-4α (1:6000) in PBS containing 0.01% Tween 20, overnight at 4°C. Controls were incubated with corresponding concentration of rabbit IgG. Antibody binding was detected using AF 488-conjugated goat anti-rabbit secondary antibody (1:1000; Molecular Probes). Albumin antibody (1:10 in 0.1% triton X-100) was applied overnight at 4°C. Mouse IgG2a was added to controls. Binding was detected using goat anti-mouse AF-568 (1:1000; Molecular Probes). Cells were washed and mounted in Vectorshield containing DAPI nuclear stain (Vector Laboratories).

Osteocytic differentiation was assessed by identifying calcium deposition using Alizarin red staining. Cells were fixed in 10% neutral buffered formalin for 15 min at RT, washed twice in distilled water and incubated in 1 ml of 1% of Alizarin red (pH 4.1). Cultures were washed with distilled water and dried. Chondrocytic differentiation was assessed by Alcian blue staining. Cell pellets were fixed in 10% formalin for 1 h and embedded in paraffin. Sections were incubated in 1% Alcian blue in 0.1 M hydrochloric acid for 30 min, dehydrated and mounted in DPX.

T cell Proliferation Assay

To compare the immunosuppressive properties of P0 and P5 hAEC, T cell proliferation assays were carried out as described previously [20]. In brief, splenocytes from C57BL/6 mice were seeded in 96-well plates (5.0×10^5 cells/well) in complete RPMI 1640 medium (Gibco) supplemented with 10% FCS. P0 and P5 hAEC were irradiated (20 Gy) and added to splenocytes in different hAEC stimulator : splenocyte responder cell ratios. Then, 10 μg/ml Concanavelin A (Con A) was added to each well. Con A stimulated splenocytes minus hAEC served as a positive control. After 72 h incubation at 37°C in 10% CO_2, 2 μCi [^{3}H]-thymidine in a volume of 20 μl was added to each well and incubated for a further 24 h. Cells were harvested using a Packard Micromate 196-cell harvester (Packard Biosciences, Meriden, CT) and incorporated [^{3}H]-thymidine measured using a Packard Tri-Carb 1900TR liquid scintillation analyzer. Measurements were taken from triplicate wells from each sample tested. Data were expressed as the percentage suppression of [^{3}H]-thymidine uptake relative to Con A stimulated splenocytes.

Measurement of Cytokines and Growth Factors

The production of cytokines and growth factors associated with immunosuppression [interleukin-(IL)-6, IL-10, transforming growth factor (TGF)-β and hepatocyte growth factor (HGF)] by P0 and P5 hAEC were measured using ELISAs (R&D Systems) following manufacturer's instructions. Samples were assayed in duplicate. The co-efficients of variation between sample duplicates was <8%.

Migration Assay

To compare the migratory properties of P0 and P5 cells, hAEC were seeded in 6-well plates (2.5×10^5 cells/well) and maintained in DMEM/F12+10% FCS or Epilife until confluent. Cross shaped scratch wounds were made using plastic pasteur pipettes and cultures washed several times to remove the dislodged cells. Cell migration over the wound areas was observed using phase contrast microscopy and images captured at regular intervals.

To investigate if CXCR4 played a role in migration, P0 and P5 hAEC were plated in 8-well chamber slides (2.0×10^4 cells/well), fixed in methanol for 5 min and incubated with Image-iTTM FX signal enhancer (Molecular Probes) for 30 min at RT. Primary antibody against CXCR4 (1:200; Abcam, Cambridge, UK) was applied and left for 1 h at RT. Corresponding concentration of goat serum was added to the negative controls. AF 488-conjugated rabbit anti-goat secondary antibody (1:1000) was applied for 1 h at RT. Cells were washed and mounted in Vectorshield containing DAPI nuclear stain.

Statistical Analysis

Data are shown as mean±SEM and analyzed using ANOVA followed by Tukey's post hoc and paired comparisons by the Student's t test (GraphPad Prism software, v5.02, San Diego, CA). Significance was accorded when $p<0.05$.

Results

hAEC Expansion in Xenobiotic Free Media

To investigate whether hAEC could be expanded under xenobiotic supplement free conditions, the cells were cultured in commercially available serum free media and human serum supplemented with rhEGF. hAEC were readily expanded until P5 and plateaued thereafter in Epilife culture medium supplemented with S7 additive (Fig. 1A). A similar trend was seen in control

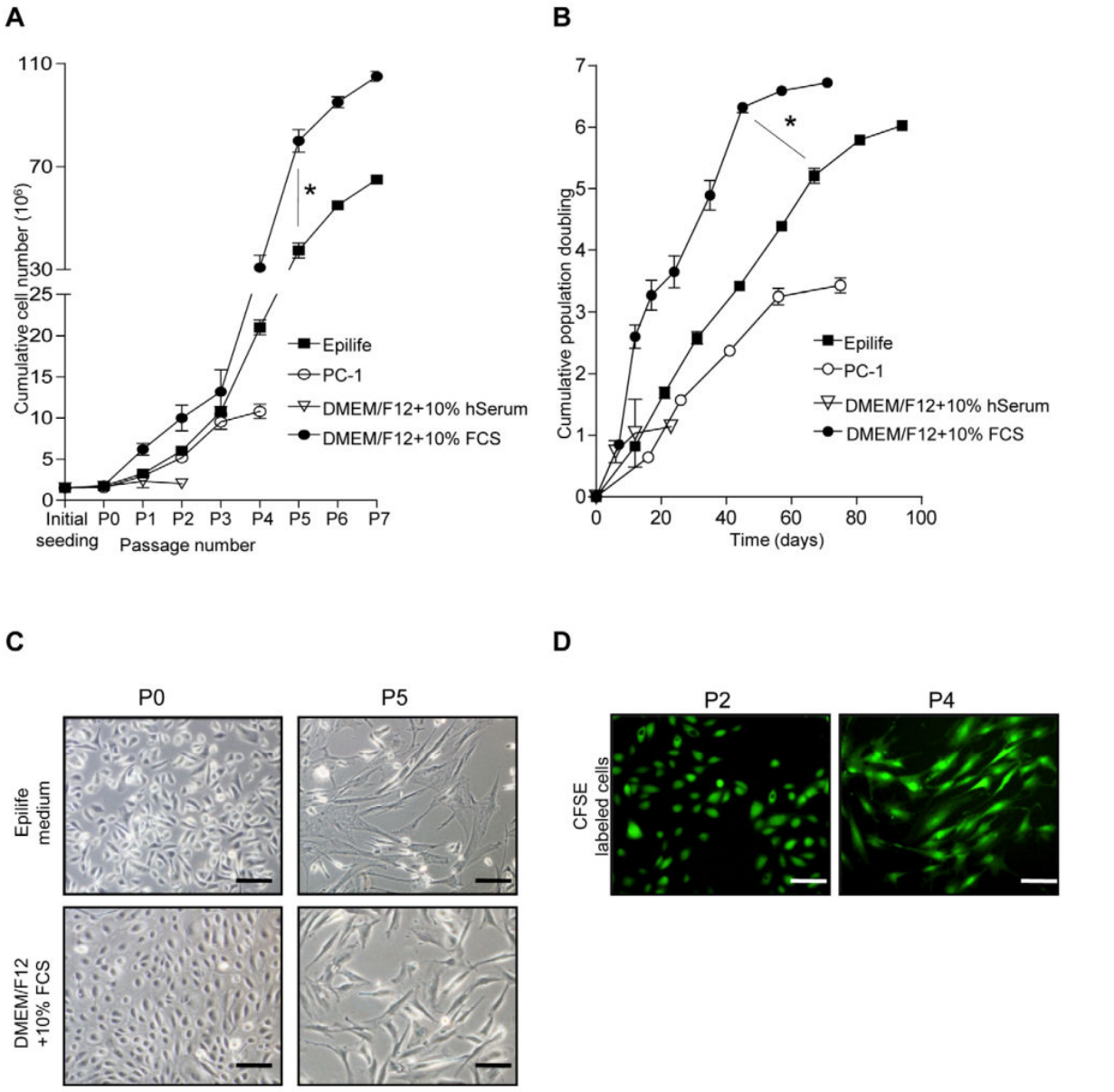

Figure 1. Expansion of hAEC. The hAEC grew well in the xenobiotic-free media Epilife until passage 5 (P5) and plateaued thereafter. A similar trend was seen in control cultures grown in DMEM/F12+10% FCS but the cumulative cell number in Epilife was lower than controls (*$p = 0.002$; **A**). Cumulative population doubling of hAEC cultured in Epilife media was lower than DMEM/F12+10% FCS (*$p = 0.0026$; **B**). hAEC appeared stromal-like at P5 in Epilife and DMEM/F12+10% FCS (**C**). Stromal-like cells at P4 retained the CFSE label suggesting the stromal cells arose from the labeled P2 epithelial cells (**D**). Scale bars = 100 μm.
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cultures expanded in DMEM/F12 with 10% FCS and rhEGF. Cultures expanded in PC-1 media showed signs of senescence by P4. The cells failed to grow in serum free DMEM/F12, CnT22 and Stempro MSC media. hAEC maintained in DMEM/F12 containing 2 10% human serum could not be expanded beyond P2.

After an initial seeding density of 1.5×10^6 cells, the CCN in Epilife reached $37.44\pm2.95\times10^6$ cells by P5 but was significantly lower than cultures grown in DMEM/F12 containing 10% FCS ($80\pm4.33\times10^6$ cells; $p = 0.002$; Fig. 1A). In PC-1 and 10% human serum the CCN reached $10.80\pm0.88\times10^6$ and $2.04\pm0.12\times10^6$ cells by P4 and P2, respectively. The time needed for hAEC to reach P5 in Epilife was 64.75 ± 1.6 d compared with 45.50 ± 2.02 d in DMEM/F12 containing 10% FCS ($p<0.0001$). The CPD of 5.21 ± 0.12 for hAEC cultured in Epilife by P5 was also significantly lower than in DMEM/F12 with 10% FCS (6.32 ± 0.08; $p = 0.0026$; Fig. 1B).

During expansion, the cells changed their phenotype from a typical epithelial morphology in P0 P2 to transitional epithelial-stromal cells in P3 P4 and completely stromal-like cells by P5 in Epilife and DMEM/F12 containing 10% FCS (Fig. 1C). Cells

cultured in PC-1 were stromal like at P1 (data not shown). We labeled P2 hAEC growing in Epilife and DMEM/F12+10% FCS showing typical epithelial cobblestone morphology with the intracellular dye CFSE to investigate whether the stromal-like cells at P4 would retain the dye. At P4, the stromal-like cells were labeled with CFSE (Fig. 1D), suggesting that these cells arose from the epithelial cells.

We compared features of P5 cells expanded in Epilife and DMEM/F12+10% FCS (DF) with the P0 cells. Analyses of P0 cells cultured in Epilife and DF showed no significant differences for any of the parameters tested; hence data for cultures grown in DF are shown in Figs. 2 6.

Properties of Expanded hAEC

Karyotype analyses were carried out to determine if chromosomal abnormalities arose during expansion. P5 cells expanded in Epilife and DF were found to retain the normal autosomal and XX or XY sex chromosome complement of the P0 cells (Fig. 2A).

We also compared the ultrastructural features of the P0 and expanded cells by TEM. P0 and P5 hAEC had a high nuclear : cytoplasmic ratio and prominent nucleoli (Fig. 2B). P0 hAEC were typically roundish cells with surface microvilli and cytoplasmic blebs. A multiloculated peripheral appearance with well developed intercellular junctions, particularly desmosomes and small amounts of rough endoplasmic reticulum (rER) were also seen in the P0 cells. In contrast, P5 hAEC expanded in Epilife and DF had extensive rER and associated Golgi complexes and very few surface villi compared to P0 cells.

Investigating clonal colony formation, small clusters containing >5 cells were observed in P0 cultures seeded at low density within two weeks (Fig. 2C). Large clonal colonies were evident by three weeks. The percentage cloning efficiency of P0 cells was 1.89±0.17. Cells from the primary epithelial colonies were subcloned 3 4 times but changed into stromal-like cells (data not shown). The expanded P5 hAEC failed to form clonal colonies.

Next, we tested for alkaline phosphatase activity. The P0 and P5 hAEC lacked alkaline phosphatase activity measured using a commercial assay, unlike the hEC cell line used as a positive control (Fig. 2D).

Expression of Epithelial and Mesenchymal Markers with hAEC Expansion

Given the phenotypic changes observed with expansion, we ascertained if there were changes in epithelial (E-cadherin, CD49f, CK7, EpCAM), stromal (vimentin) and MSC associated markers (CD44, CD90, CD105, CD146, PDGFR-β, CD29). P5 DF-hAEC had reduced numbers of cells with epithelial markers compared to P0 (E-cadherin, CK7 and CD49f; $P<0.001$; Fig. 3A), and more cells with stromal/MSC markers (vimentin, CD90, CD146, CD44 and CD105; $P<0.001$). P5 Epilife cells also expressed stromal

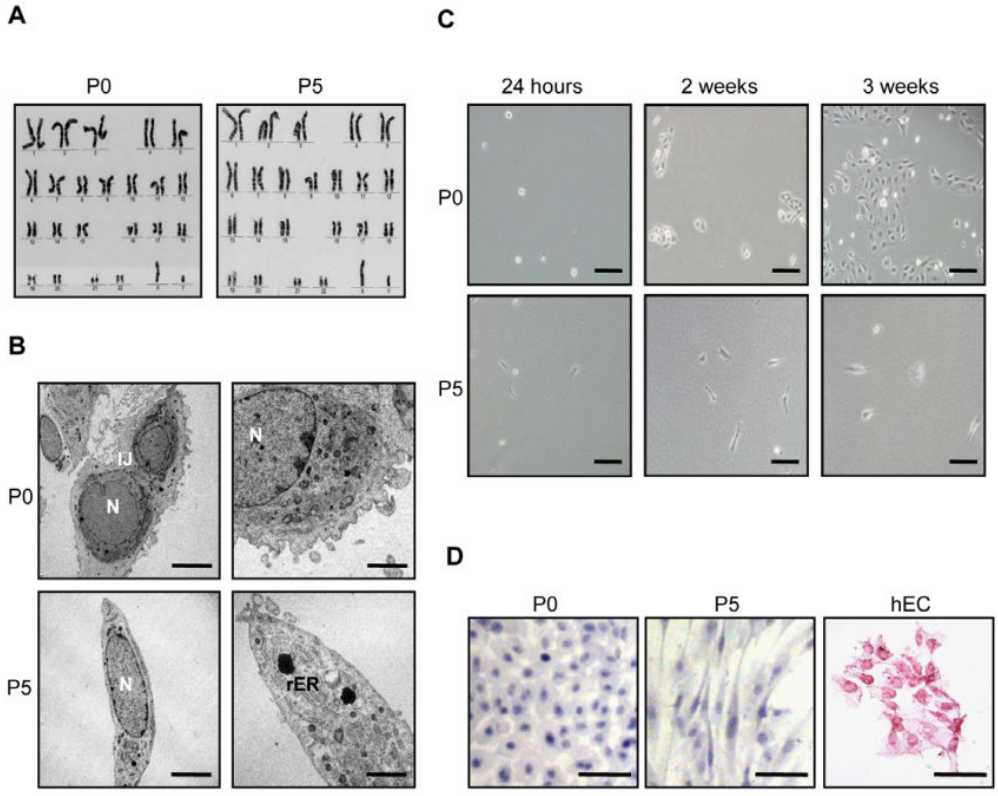

Figure 2. Features of cultured primary (P0) and cells expanded in Epilife medium to passage 5 (P5). Normal karyotype of P0 hAEC was retained at P5 (**A**). Transmission electron micrograph of P0 hAEC showed intercellular junctions (IJ), a multiloculated peripheral appearance compared to P5 hAEC. P5 cells had extensive rough endoplasmic reticulum (rER) and fewer cell surface projections. P0 and P5 cells showed high nuclear (N) to cytoplasmic ratio (**B**). P0 hAEC seeded at low density formed clonal colonies unlike P5 hAEC (**C**). hAEC lacked alkaline phosphatase activity unlike human embryonal carcinoma (hEC) cell line used as a positive control (**D**). Scale bars = 100 μm (**A–B**); 5 μm and 1 μm (**C**).
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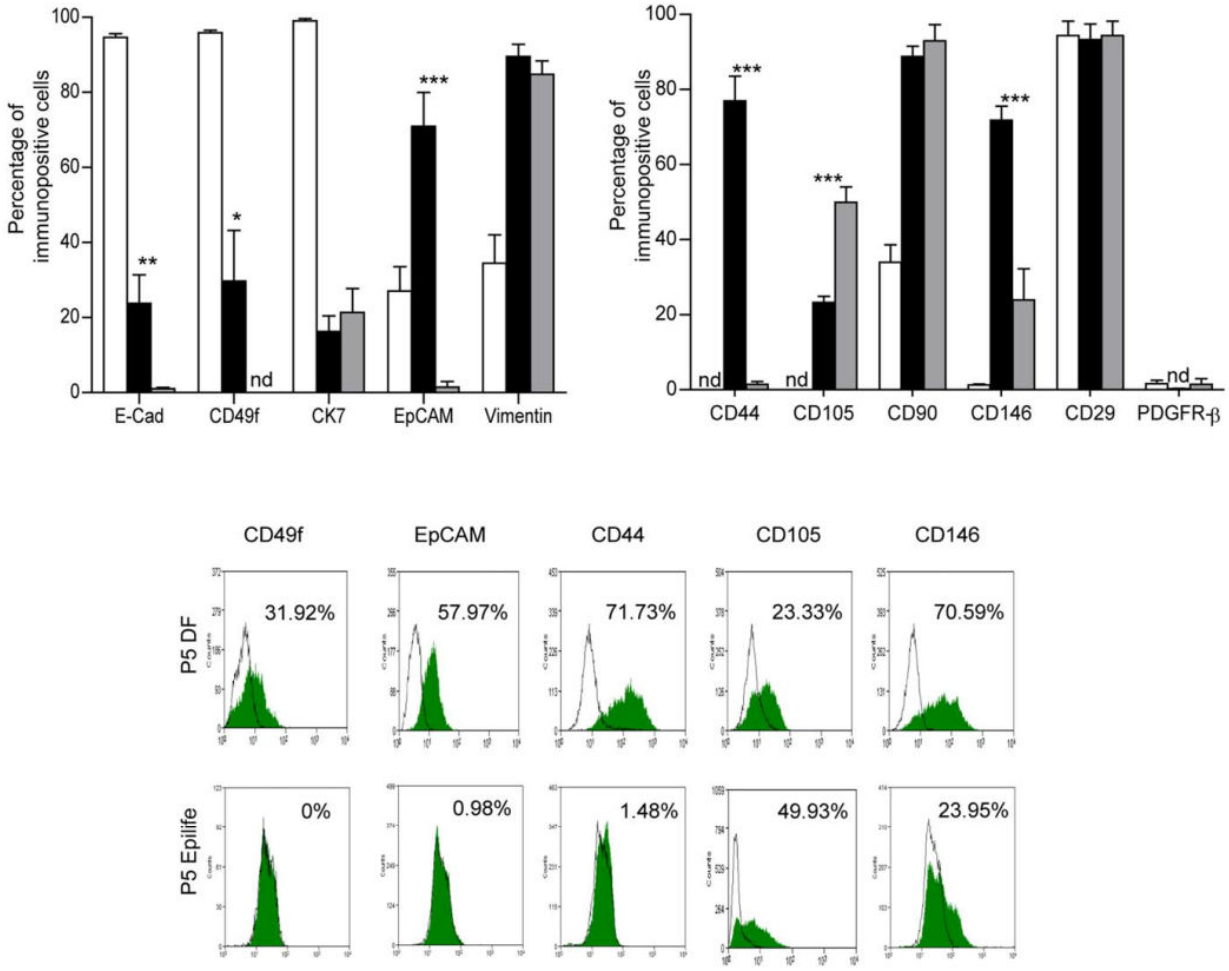

Figure 3. Phenotype of primary (P0) and passage 5 (P5) hAEC. Cells expressing epithelial markers E-cadherin, CD49f and CK7 declined with expansion while percentage of cells with MSC associated markers CD90, CD146 and the stromal marker vimentin were elevated in P5 cells grown in Epilife and DMEM/F12+10% FCS (DF). However, notable differences were also found between Epilife and DF expanded cells. Representative flow cytometry plots of markers that differed are shown. Open bars = P0, black shaded bars = P5 DF and grey bars = P5 Epilife. *$p<0.05$; **$p<0.01$; ***$p<0.0001$ by ANOVA and Tukey's post hoc test.
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markers but were notably different to P5 DF-hAEC with lower percentage of CD44 and CD146 ($P<0.0001$) and higher numbers of CD105 positive cells ($p<0.0001$). Epithelial markers E-cadherin, CD49f and EpCAM were also reduced in P5 Epilife hAEC ($P<0.01$ compared to P5 DF-hAEC).

The percentage of CD29 positive cells was unaltered with expansion while PDGFR-β was low in P0 and P5 cells (Fig. 3A). Further, endothelial (CD31) and hematopoietic (CD45) markers were absent in P0 and expanded cells.

Differentiation of hAEC and Characterization

We compared the differentiation potential of P0 hAEC and cells expanded in Epilife and DF into endodermal (alveolar epithelial cells, pancreatic cells, hepatocytes) and mesodermal (osteocytes, chondrocytes) lineages. P0 and P5 Epilife hAEC grown in SAGM medium (Table 2) produced proSP-C, a protein specific to type 2 alveolar epithelial cells (Fig. 4). However, the DF-P5 cells failed to grow in SAGM medium. P0 hAEC induced to differentiate into pancreatic-like cells secreted insulin (75.8±38.mIU/L). Some of the cells also stained positively for the hormone glucagon (GCG, Fig. 4). Neither insulin secretion nor GCG staining were detected in P5 cultures. Nuclear staining of the hepatocyte specific transcription factor HNF-4α and albumin was used to assess the differentiation of hAEC into hepatocyte-like cells. P0 hAEC stimulated with EGF followed by insulin and dexamethasone showed nuclear HNF-4α and albumin staining (Table 2; Fig. 4). However, expanded cells did not show evidence of differentiation into hepatocyte-like cells. In contrast, P0 and P5 hAEC maintained in osteocytic differentiation medium showed Alizarin red stained calcium deposits suggesting differentiation into osteocyte-like cells (Fig. 4). P0 and P5 Epilife hAEC failed to aggregate and did not differentiate into chondrocyte-like cells, whereas DF-P5 cells produced cartilage proteoglycans that were detected by Alcian blue staining (Fig. 4). Staining was absent in control cultures that were maintained in basal media minus the supplements shown in Table 2.

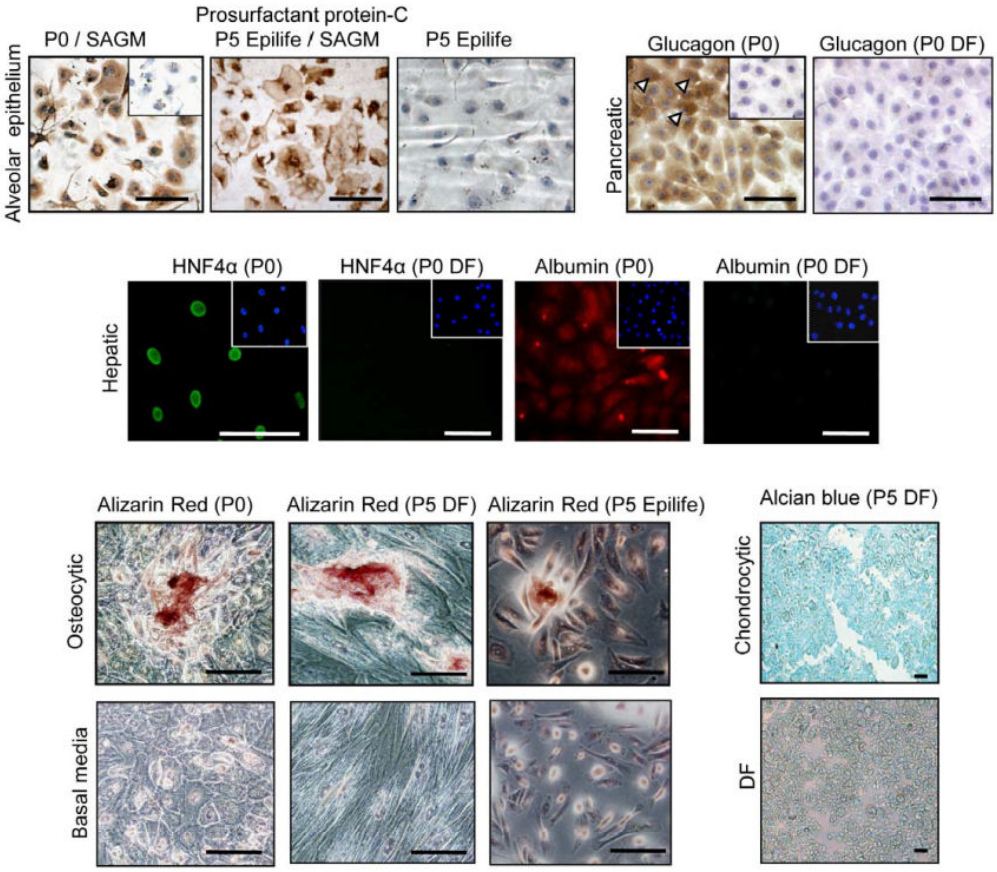

Figure 4. Differentiation of hAEC and their characterization. Primary (P0) and Epilife expanded passage 5 (P5) hAEC grown in SAGM produced prosurfactant protein-C characteristic of type 2 alveolar epithelial cells. Control P5 cultures maintained in Epilife lacked staining. Stimulated P0 hAEC contained glucagon (GCG), found in alpha pancreatic cells (arrow heads). Glucagon was absent in control cultures grown in DMEM/F12 medium with 10% FCS (DF). Inserts within panels show isotype controls (upper panel). P0 hAEC induced with supplements expressed hepatocyte nuclear factor-4α (HNF-4α) and albumin, unlike control cultures grown in DF. Cell nuclei stained with DAPI are shown in the inserts (middle panel). Alizarin red staining indicating calcium deposition characteristic of osteocytes in stimulated P0 and P5 cultures. Cartilage proteoglycans stained with Alcian blue in stimulated P5 DF expanded hAEC. Non-stimulated control cultures maintained in basal medium lacked evidence of differentiation into osteocyte and chondrocyte-like cells (lower panel). Scale bars = 100 μm.
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Immunophenotype and Immunosuppressive Properties

We investigated if expansion induced changes in the expression of HLA Class I and II antigens and the co-stimulatory molecules. In excess of 90% of P0 and P5 cells expressed low to moderate levels of HLA-A-B-C (Fig. 5A). HLA-DP-DQ-DR was absent in primary and expanded cells. The co-stimulatory molecule CD40 was expressed at low levels by the majority of P0 and P5 hAEC tested (Fig. 5A). Very low levels of CD80 and CD86 were detected in $<10\%$ of P0 and P5 hAEC (Fig. 5A).

We also compared the immunosuppressive properties of the primary and expanded cells. P0 and P5 hAEC suppressed the proliferation of splenocytes from C57BL/6 mice stimulated with Con A (Fig. 5B). However, the P0 cells were highly suppressive over a much wider range of hAEC stimulator : splenocyte responder cell ratios, unlike the P5 hAEC that had significantly reduced ability to inhibit T cell proliferation at higher splenocyte ratios ($p<0.01$; Fig. 5B).

Production of Immunosuppressive Factors

Secretion of factors (TGF-β1, IL-6, IL-10 and HGF) that have been shown to suppress T cell proliferation was measured. Primary and expanded hAEC secreted TGF-β1 and IL-6, but not IL-10 and HGF. However, significant differences were seen between P0, DF-hAEC and Epilife expanded hAEC in TGF-β1 and IL-6 production (Fig. 5C). HLA-G, a non classical Class IB antigen with restricted expression is also known to exert anti-inflammatory properties by suppressing T and Natural Killer cell activity. While P0 hAEC were HLA-G positive, the number of HLA-G producing cells decreased significantly with expansion in DF and were notably absent in cells expanded in Epilife ($p<0.0001$). MCP-1 is known to regulate monocyte chemotaxis. P0 and Epilife expanded hAEC did not secrete MCP-1 whereas P5 DF hAEC secreted substantial amounts of MCP-1 (mean±^{m} sem = 866.5±80.42 pg/ml).

Migratory Properties

Cell migration to inflamed and damaged tissue sites is also an important feature of MSC. We investigated the migratory capacities of the P0 and expanded cells using a standard scratch wound assay. P0 hAEC migrated into the wound within 24 h and completely obliterated the scratch wound by 72 h. The DF and

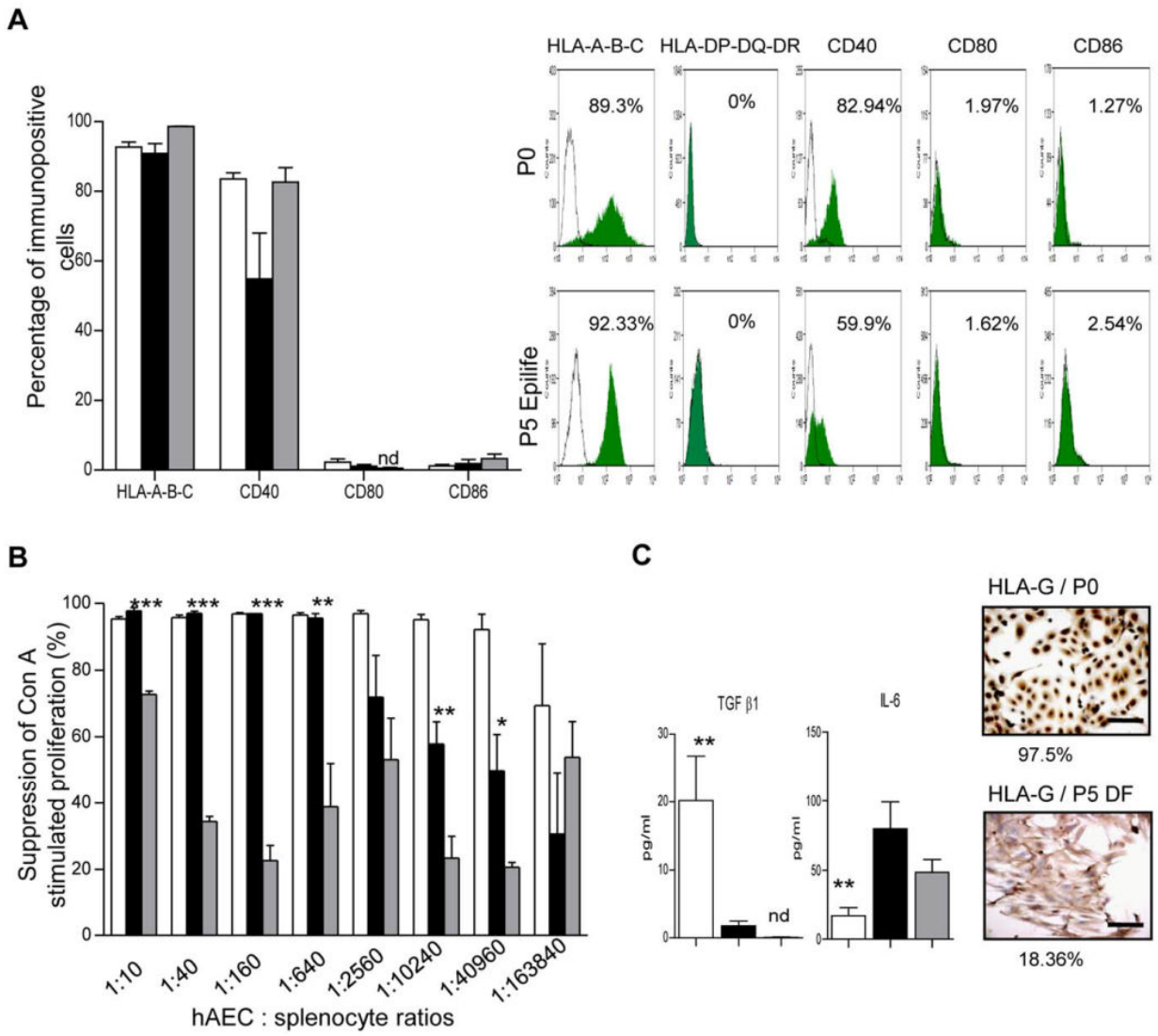

Figure 5. Immunogenicity of hAEC and effects on T cell proliferation. Primary (P0) and passage 5 (P5) hAEC expressed HLA-A-B-C but lacked HLA-DP-DQ-DR. Co-stimulatory molecules CD40, CD80 and CD86 remained unaltered at P5. Representative flow plots for HLA antigens and co-stimulatory molecules are shown (**A**). P0 and P5 hAEC suppressed Concanavelin A stimulated splenocytes from C57/BL6 mice, however P5 cells showed reduced suppression at higher splenocyte ratios (**B**). P0 and expanded cells produced the immunosuppressive factors TGF-β and IL-6. HLA-G that was abundant in P0 cells declined significantly in P5 grown in DMEM/F12+10%FCS (DF), while hAEC expanded in Epilife lacked HLA-G. Open bars = P0, black shaded bars = P5 cells expanded in DF and grey bars = P5 cells expanded in Epilife. Scale bars = 100 μm. *$p<0.05$; **$p<0.01$ and ***$p<0.001$.

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Epilife expanded hAEC showed reduced migration with the scratch wound still visible after 72 h (Fig. 6A). The chemokine receptor CXCR4 has been widely implicated in regulating the migration of MSC in response to CXCL12 [21]. Immunolocalization studies showed that CXCR4 was absent in the cultured P0 and expanded P5 cells. However, hAEC lining amnion membrane stained positively for CXCR4 (Fig. 6B).

Discussion

We showed that hAEC can be expanded in xenobiotic-free media, but that cell expansion was limited and that the hAEC underwent phenotypic changes consistent with an epithelial-mesenchymal transition (EMT). Further, we found notable differences in differentiation capacity, migration, immunosuppressive properties and secretion of immunomodulatory factors between the P0 and P5 Epilife expanded hAEC. These changes would need to be taken into account as they would have a marked impact on the potential therapeutic applications of the expanded hAEC.

Bilic et al. [4], reported that hAEC from term fetal membranes showed limited expansion in FCS supplemented DMEM/F12 medium. Since stem cells need to be expanded in xenobiotic-free media to comply with GMP for therapeutic applications [17], we expanded hAEC in commercially available xenobiotic-free and human serum containing media with rhEGF supplementation as

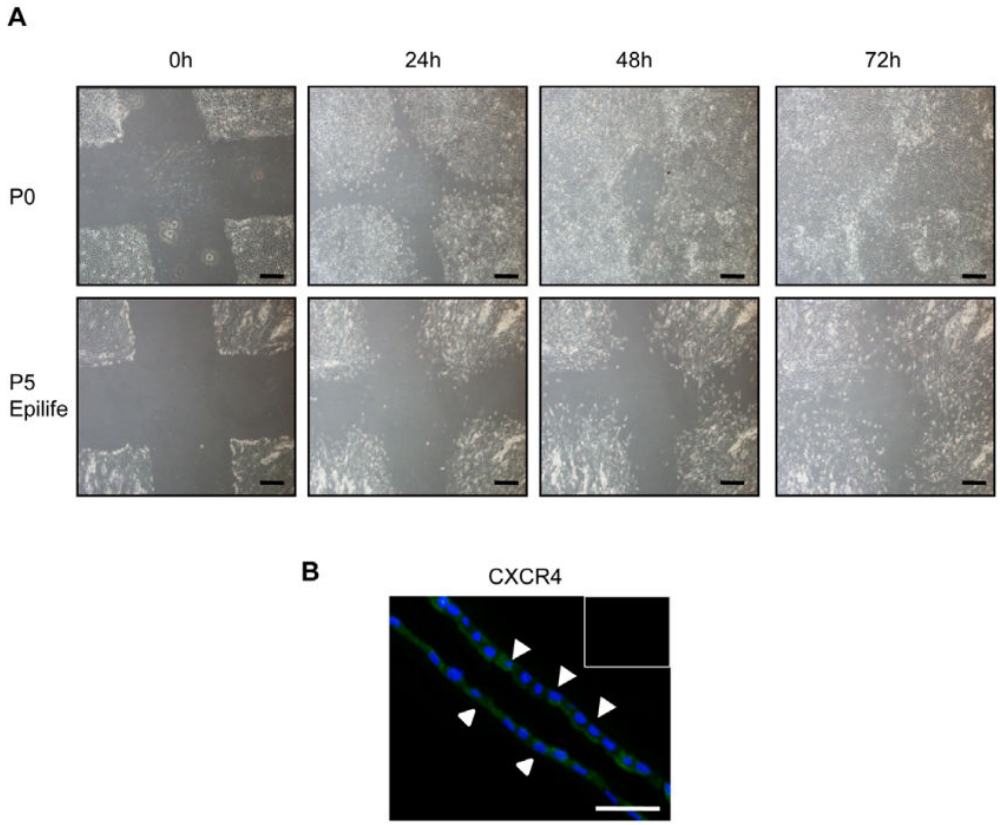

Figure 6. Cell migration assay. Primary, passage 0 (P0) hAEC migrated more rapidly compared with expanded passage 5 (P5) cells in a scratch wound assay (**A**). Cultured P0 and P5 cells lacked the chemokine receptor CXCR4 that has been implicated in cell migration (data not shown), however hAEC lining term delivered amnion membranes were immunopositive for CXCR4 (white arrows head = hAEC; **B**). Insert within panel shows staining of isotype control. Scale bars = 500 μm (**A**) and 100 μm (**B**).
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this growth factor has been shown to induce proliferation [2,3,22]. Among the media tested, hAEC grew well in Epilife yielding approximately 3.7×10^7 viable cells at P5 with a CPD of 5.2 after an initial seeding density of 1.5×10^6 cells. Given that 50 100 million hAEC are routinely harvested from each membrane [3,16], approximately 1.2 2.5×10^9 cells could potentially be generated from each amnion membrane by P5. The CPD of hAEC in Epilife was low compared with human bone marrow derived MSC that have been reported to have CPD of 10 12 by P4 without losing their differentiation capacity [23]. The CCN and CPD of hAEC in Epilife was significantly lower than controls, suggesting that supplementation of cultures grown in Epilife with serum derived factors may be beneficial. Apart from EGF, hepatocyte growth factor (HGF), TGF-β, basic fibroblast growth factor (bFGF), insulin, transferrin and triiodothyronine have also been shown to promote hAEC proliferation [24]. These factors may need to be tested alone and in combination in xenobiotic-free media formulations. Since growth factors such as bFGF can induce differentiation into neuronal lineages [25] such differentiation would also need to be monitored. Human platelet lysate has also been shown to be very effective in expanding MSC while retaining their differentiation and immunosuppressive properties [26,27]. Platelet lysate is prepared soon after blood collection and obtaining sufficient volumes for large scale culture may limit its usage.

We observed gradual morphological changes during expansion with the P0 epithelial cells changing into stromal-like cells by P3 4 in Epilife and FCS supplemented media. hAEC with an epithelial morphology were labeled with CFSE and the stromal-like cells were shown to retain the dye label. These changes were consistent with an EMT as shown by decrease in the epithelial markers E-cadherin, CD49f (integrin α6) and CK7. In contrast, MSC associated markers CD90, CD105 and CD146 increased significantly at P5 hAEC, in agreement with a previous report where hAEC were expanded in FCS containing medium [5]. We also showed that the stromal marker vimentin increased at P5. Interestingly, human embryonic stem (hES) cells grown without feeder layers have been found to change into stromal-like cells with down-regulation of E-cadherin and up-regulation of vimentin [28]. Primary hAEC display some of the pluripotency features of hES cells and it would be worthwhile investigating if culture on feeder layers could delay or prevent the changes observed in hAEC. TGF-β has been shown to induce EMT during cancer cell metastases and in chronic fibrotic diseases [29,30]. TGF-β signaling induces Slug and Snail transcription factors that suppress E-cadherin expression [29]. The P0 hAEC secrete TGF-β and the effect of inhibiting TGF-β signaling and/or other factors linked to EMT such as tyrosine kinase receptor signaling, small GTPases, ZEB transcription factor induced by miRNA-200 family [31,32], should be examined to determine which factor(s) play a role in

changes observed during expansion of hAEC. Further, EGF also has been reported to induce EMT in some cancer cell lines [33,34], but enhanced expression of MSC-related antigens in hAEC occur in cultures without addition of EGF [5]. In preliminary experiments addition of EGF to P0 P2 cultures did not stimulate EMT (data not shown), suggesting that EGF does not play a role.

Mature, polarized epithelial cells that undergo EMT display migratory properties [29,35]. Interestingly, using a scratch wound assay we found that not only the stromal-like P5 hAEC were able to migrate, but that the P0 hAEC had a higher migratory capacity. hAEC lining the amnion membrane have no known migratory properties and hence factors that could regulate the migration of hAEC have not been investigated. Interactions between chemokines and their receptors, in particular CXCR4, are believed to play important roles in the migration of MSC [21]. We localized CXCR4 to hAEC lining the amnion membranes but neither P0 nor P5 hAEC expressed CXCR4. Expression of CXCR4 has been shown to decline in MSC during culture [21] and this may account for the loss of CXCR4 in the P0 cells. Since chemotactic and adhesion factors play important roles in regulating migration of stem cells to target sites of tissue inflammation and damage, it would be important to identify these factors in assessing the therapeutic applications of hAEC of the primary and expanded hAEC.

Changes in morphology and reduced differentiation capacity due to senescence have been reported in porcine and human MSC expanded in long term culture [23,36,37]. We found that unlike the P0 cells, P5 hAEC failed to differentiate into important endodermal lineages such hepatocytes and pancreatic cells and would limit derivation of these lineages for potential cell replacement therapies to primary hAEC and cells from early passages. However, as P5 Epilife expanded cells differentiated into osteocyte and surfactant producing alveolar epithelial-like cells, it suggests a functional alteration rather than senescence being responsible for changes in differentiation, and it would be important to determine if the expanded cells can undergo tissue specific differentiation *in vivo* as has been demonstrated for P0 hAEC [6,9,11]. Indeed, the TEM studies showed that the expanded hAEC had well developed rER and Golgi complexes consistent with maturation and a well developed secretory profile and not senescence. Down regulation of ES markers TRA1-60 and TRA1-81 has been reported during expansion [5] and it is possible that expression of lineage specification and differentiation pathways also alter during hAEC expansion. Expansion may also lead to selection of sub-populations within the primary isolates as notable differences in both marker expression, secretory profile and differentiation was found between FCS supplemented and Epilife expanded hAEC.

The low immunogenicity exhibited by expanded MSC from bone marrow and gestational tissue have enabled clinical trials involving allogeneic transplantation. We showed that P0 hAEC expressed low to moderate levels of HLA class IA and lack HLA class II antigens, consistent with previous reports [4,13,14]. Expression of HLA and the co-stimulatory molecules CD80, CD86 and CD40 is required to activate T cells and subsequent immune rejection of the transplanted cells. We found CD40 expressed by P0 cells, while both CD80 and CD86 were negligible. There were no significant differences in the expression of these antigens in the P5 hAEC. These findings may explain the survival of P0 hAEC following xeno-transplantation into immune-competent animals over prolonged periods [8,11] and also suggest that P0 and P5 hAEC are unlikely to be rejected following xeno-transplantation.

We also examined the immunosuppressive properties of the P0 and expanded hAEC. Consistent with previous reports, P0 hAEC suppressed T cell proliferation [13,14,15]. The P5 hAEC also suppressed T cell proliferation, however the P0 cells were more effective at higher splenocyte ratios. The immunosuppressive properties of MSC are well established and HLA-G, IL-6 and TGF-β [38,39,40] among the factors known to play a role. Djouad et al [38] proposed that IL-6 secreted by MSC inhibits dendritic cell maturation and subsequently impairs T cell proliferation and induces tolerance. TGF-β1 has also been shown to inhibit T cell proliferation [39]. We found that IL-6 and TGF-β1 were secreted by P0 and expanded hAEC and these factors may partly contribute towards the suppression of T cell proliferation. On the other hand, a high percentage of P0 cells were HLA-G positive compared with P5 hAEC with cells cultured in Epilife lacking this non-polymorphic Class IB antigen. HLA-G has been shown to inhibit proliferation by binding to killer immunoglobulin-like receptors and/or immunoglobulin-like transcript on $CD4^+$ and $CD8^+$ T cells [41]. HLA-G is also known to modulate the cytotoxic activity of Natural Killer cells. MSC secrete other anti-inflammatory factors such as IL-10 and HGF. Interestingly, neither the P0 nor expanded hAEC secreted IL-10 or HGF.

Recent studies show that transplantation of P0 hAEC reduces tissue inflammation and fibrosis in murine liver and lungs [6,11], although the mechanisms remain largely unknown. P5-DF expanded hAEC secreted significant amounts of MCP-1 that could induce the recruitment of monocytes and promote fibrogenesis. In addition to the immuno-modulatory effects, TGF-β1 and IL-6 play an important role in promoting fibrogenesis. Therefore, the effects of expanded P5 hAEC on tissue inflammation, monocyte chemotaxis and fibrosis would need to be tested in animal models.

In conclusion, we have shown that expanded hAEC have different properties to the primary cells. P0 hAEC may be useful for generating hepatocyte and pancreatic like cells for therapeutic applications and expanded cells for mending bone fractures and contributing towards the alveolar epithelial cell population damaged in lung diseases. Further, the P0 cells may be more useful in suppressing tissue inflammation and fibrosis and as a treatment for autoimmune diseases and graft vs host disease where it would be important to limit T cell activation. Characterization of the transitional hAEC at passages 2 3 and testing expanded hAEC *in vivo* models would be beneficial in assessing the suitability of the expanded hAEC for cellular therapeutic applications.

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Author Contributions

Conceived and designed the experiments: GP VV CG UM. Performed the experiments: GP VV JYT YHL. Analyzed the data: GP VV JC UM. Contributed reagents/materials/analysis tools: JYT YHL CT PM UM. Wrote the paper: GP VV UM.

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Immunogenicity and Immunomodulatory Properties of Hepatocyte-like Cells Derived from Human Amniotic Epithelial Cells

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Abstract: Hepatocyte transplantation is being trialled as an alternative to whole organ transplant for patients with acute liver failure and liver specific metabolic diseases. Due to the scarcity of human hepatocytes, hepatocyte-like cells (HLC) generated from stem cells may become a viable alternative to hepatocyte transplantation. Human amniotic epithelial cells (hAEC) from the placenta have stem cell-like properties and can be differentiated into HLC. Naïve hAEC have low immunogenicity and exert immunomodulatory effects that may facilitate allogeneic transplantation. However, whether the immunogenicity and immunomodulatory properties alter with differentiation into HLC are unknown. We further characterized HLC generated from hAEC, examined changes in human leucocyte antigens (HLA) and co-stimulatory molecules and effects exerted by the HLC on human peripheral blood mononuclear cells (PBMC). HLC derived from hAEC expressed proteins found in hepatocytes, had CYP3A4 drug metabolizing enzyme activity and secreted urea. IFN-γ treatment increased HLA Class IA, Class II and co-stimulatory molecule CD40 expression in the HLC. IFN-γ treated HLC stimulated proliferation of PBMC in one-way mixed lymphocyte reactions and were more immunogenic than undifferentiated hAEC. However, the HLC showed immunomodulatory properties and inhibited mitogen induced PBMC proliferation *in vitro*. PBMC proliferation may have been inhibited by IL-6, TGF-β1, PGE2 and HLA-G secreted by the HLC. The retention of immunomodulatory properties may enable HLC grafts to survive for longer periods despite the immunogenicity of the HLC.

Keywords: Amniotic epithelial cells, anti-inflammatory cytokines, fetal membranes, hepatocyte-like cells, immunogenicity, immunomodulation.

INTRODUCTION

Hepatocyte transplantation is becoming an increasingly important alternative to orthotropic or auxiliary liver transplantation for patients with acute or chronic liver failure and metabolic diseases such as hypercholesterolemia, ornithine transcarbamoylase deficiency and glycogen storage disease [1-3]. While human hepatocytes are the preferred choice for transplantation the scarcity of suitable donor tissue available for cell isolation, short life span of hepatocytes in culture and difficulties with cryopreservation have precipitated the search for alternative sources of cells [1-3]. Transformed human hepatocyte cell lines, fetal hepatocytes, liver progenitors and hepatocyte-like cells (HLC) derived from embryonic, induced pluripotent and adult stem cells are potential alternatives [4, 5]. However, difficulties in sourcing fetal tissue, cell isolation and expansion, multistep differentiation protocols, inefficient differentiation and tumour formation are among the shortcomings identified with some of these cell types [6]. Human amniotic epithelial cells (hAEC) form a monolayer lining the amniotic membrane attached to the placenta. hAEC are derived from pluripotent embryonic epiblast cells prior to gastrulation and exhibit some of the properties of their founder cells even at the end of pregnancy [7]. hAEC have been shown to differentiate into lineages derived from each of the three primary germ layers *in vitro*, but are described as stem cell-like cells as hAEC have a limited proliferative capacity and low clonogenicity [8, 9]. However, unlike embryonic and adult tissue derived stem cells, hAEC possess some features of hepatocytes and express several genes present in hepatocytes [9, 10]. Following stimulation with factors that have been shown to induce differentiation of adult stem cells into HLC, hAEC displayed some additional features of mature hepatocytes [8, 10-12]. The stimulated hAEC had ultra-structural features of hepatocytes [11] and enhanced gene expression of drug metabolizing CYP enzymes [10, 13, 14]. These features together with easy access to amniotic membranes and yields exceeding 150-200 million cells per membrane make hAEC a potentially important source of HLC [8, 15].

Fetal derived cells of the villous placenta and chorionic membrane have low immunogenicity which in part serves to

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prevent maternal immune cell mediated rejection of the placenta. Even though hAEC are not exposed to maternal immune cells, the amnion cells exhibit low immunogenicity [9, 16]. hAEC fail to elicit an appreciable proliferative response in human PBMC *in vitro* and have been transplanted into allogeneic recipients during clinical trials for lysosomal storage diseases without adverse consequences attributed to the transplanted cells [16-18]. Low levels of HLA Class IA expression, absence of HLA Class II and co-stimulatory molecules CD40/CD80 by hAEC may be partly responsible [8, 9, 16]. However, with differentiation stem cells can increase HLA and co-stimulatory molecule expression and elicit immune responses leading to graft rejection in allogeneic recipients [19-21]. It is therefore important to examine changes in immunogenicity as hAEC differentiate into HLC to gauge their potential for clinical transplantation.

Another significant feature of hAEC is their immunomodulatory properties. Like mesenchymal stromal/stem cells (MSC), hAEC suppress mitogen or antigen stimulated splenocyte or PBMC proliferation [16, 17, 22, 23]. hAEC secrete PGE2 and other immunomodulatory factors [22, 23] and recently we showed that PGE2 and TGF-β1 from hAEC suppress T cell proliferation [23]. However, little is known about changes to immunomodulatory properties following differentiation. This is important given that the retention of immunomodulatory properties by HLC could facilitate potential transplantation into allogeneic recipients. In this study, we further characterized HLC derived from hAEC, investigated the immunogenicity and immunomodulatory properties of HLC and potential mechanisms involved.

MATERIALS AND METHODS

hAEC Isolation and Differentiation

Amnion membranes were collected with the approval of Southern Health and Royal Women's Hospital Human Research Ethics Committees from women with a normal singleton pregnancy following caesarean section at term (37-40 weeks gestation; n=16). hAEC were released by serial digestion of amnion membranes in 0.05% trypsin:EDTA at 37 °C for 40 min [24]. Batches with >98% cytokeratin-7, 8/18 (Dako, Glostrup, Denmark) positive cells by FACS and cobblestone epithelial morphology in culture [9, 11] were used for experiments described below. Cells were plated in DMEM/F12 with 20% FCS and 1% penicillin, streptomycin and L-glutamine (reagents from Gibco, Grand Island, NY). Differentiation into HLC was induced in DMEM/F12 with 10% FCS containing 10 ng/ml EGF (Invitrogen, Carlsbad, CA), insulin and dexamethasone (0.1 μM each) for 3 weeks [22]. Control cultures were maintained in DMEM/F12 with 10% FCS.

Characterisation of Stimulated hAEC

Cultures were fixed in 4% paraformaldehyde for 20 min or ice cold ethanol for 7 min (HNF-4α) followed by serum-free protein block (Dako) for 15 min to minimise non-specific staining. The following anti-human antibodies were diluted in DPBS containing 0.01% Tween 20 (Sigma-Aldrich, St Louis, MO) and applied overnight at 4 °C: GATA-4 and HNF-3β (1:100; Santa Cruz Biotechnology, Santa Cruz, CA); albumin (1:20; R&D Systems, Minneapolis, MN); OTC (1:500; Lifespan Biosciences, Seattle, WA); α-FP (1:500; Sigma-Aldrich) and HNF-4α (1:6000, Cell Signalling Technology, Danvers, MA). After several rinses, cells were incubated with Alexa Fluor488 or Alexa Fluor568 secondary antibodies (1:1000; Invitrogen) for 30 min at room temperature in the dark. Cell nuclei were counterstained with DAPI (Vector Laboratories, Burlingame, CA). Negative controls received rabbit IgG (HNF-4α, OTC); mouse IgG2a (albumin, GATA-4, α-FP) or goat serum (HNF-3β), in lieu of primary antibodies.

Glycogen stored in cells was detected by Periodic Acid Schiff staining. Cells were treated with periodic acid for 5 min followed by Schiff's stain for 15 min, rinsed thoroughly and counterstained in haematoxylin.

TDO expression was analysed by RT-PCR. Total RNA was isolated using RNeasy columns (Qiagen, Hilden, Germany), contaminating DNA removed (Life Technologies, Mulgrave, Victoria, Australia) and converted to cDNA (Superscript III; Life Technologies). Primer sequences were: (F) 5'-AGGTCAATGATAGCATCTGCC-3' and (R) 5'-TGTCATCGTCTCCAGAATGG-3'. cDNA diluted 1:20 was amplified for 35 cycles with anneal/extension at 56°C for 60 sec.

Activity of the drug metabolising enzyme CYP3A4 was measured using a commercial kit (Promega, Madison, WI) after stimulation with 10 μg/ml rifampicin for 48 h.

Cells were treated with 10 mM NH_4Cl for 48 h and secreted urea was measured by the Standard Enzymatic Assay using a Beckmann Coulter LX20 Pro analyser at Southern Cross Pathology, Monash Medical Centre. Samples were concentrated 10x prior to analysis.

Flow Cytometry

HLA and co-stimulatory molecules expressed by ±IFN-γ stimulated cultures (100 ng/ml; R&D Systems) were analysed by flow cytometry. Cells ($2.5x10^5$) were mixed with anti-human HLA-A,B,C (0.5 μg/ml), HLA-DP,DR,DQ (2 μg/ml; both from BD Biosciences, San Jose, CA), co-stimulatory molecules CD40 (0.4 μg/ml), CD80 (4 μg/ml) and CD86 (0.4 μg/ml; all from Abcam, Cambridge, UK), or isotype-matched IgG for 1 h at 4°C (n=6). After several washes, Alexa Fluor488 conjugated goat anti-mouse secondary antibody (10 μg/ml; Invitrogen) was added and incubated for 30 min at 4°C in the dark. Non-specific staining was blocked with 5 μl goat serum during incubation with primary and secondary antibodies. Cells were washed and analysed by flow cytometry and data analysed using Summit Software (v5.0.1.3804, Dako Cytomation, Fort Collins, CO).

Flow cytometry was also used to determine whether anti-human antibodies were generated against the HLC. Healthy C57BL/6 mice (n=4) were injected with HLC ($2x10^6$; derived from n=4 hAEC cultures) and blood collected two or four weeks later (single and double HLC dose, respectively). Murine serum was diluted 1:100 and mixed with suspensions of the same batches of HLC that had been injected previously. HLC and serum were incubated for 30 min to enable anti-human antibodies in the murine serum to bind to the HLC. Following three washes, HLC were incubated for 30

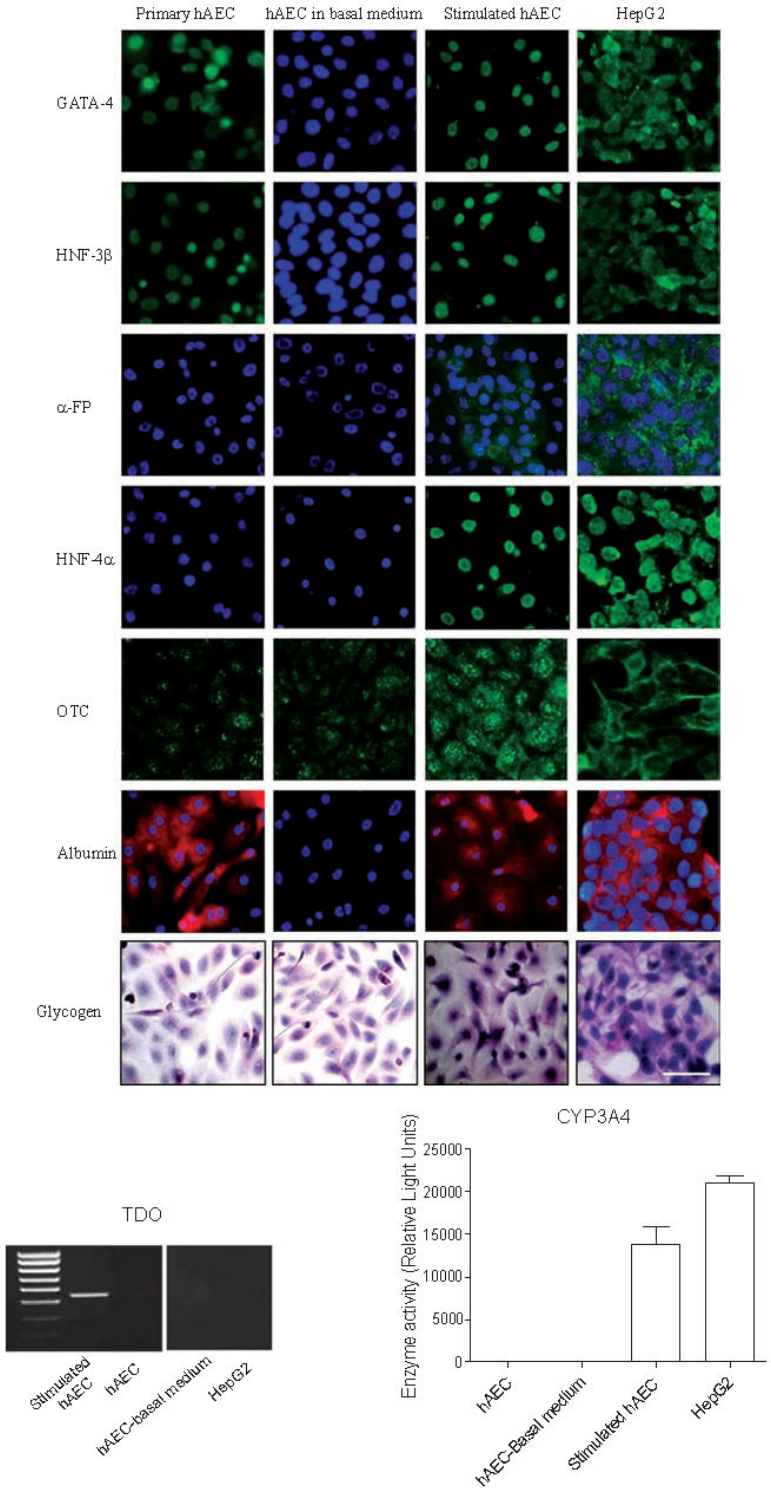

Fig. (1). Characterisation of the hepatocyte-like cells (HLC) derived from human amniotic epithelial cells (hAEC). hAEC were stimulated with EGF followed by dexamethasone and insulin. Stimulated hAEC expressed endodermal lineage / hepatic specification markers, proteins present in human hepatocytes, stored glycogen, expressed TDO and showed CYP3A4 enzyme activity measured after stimulating cells with rifampicin. Primary hAEC and controls maintained in DMEM/F12 + 10% FCS also expressed some of these proteins. Data obtained from the human hepatocyte cell line HepG2 are shown for comparison. Scale bar = 100 μM.

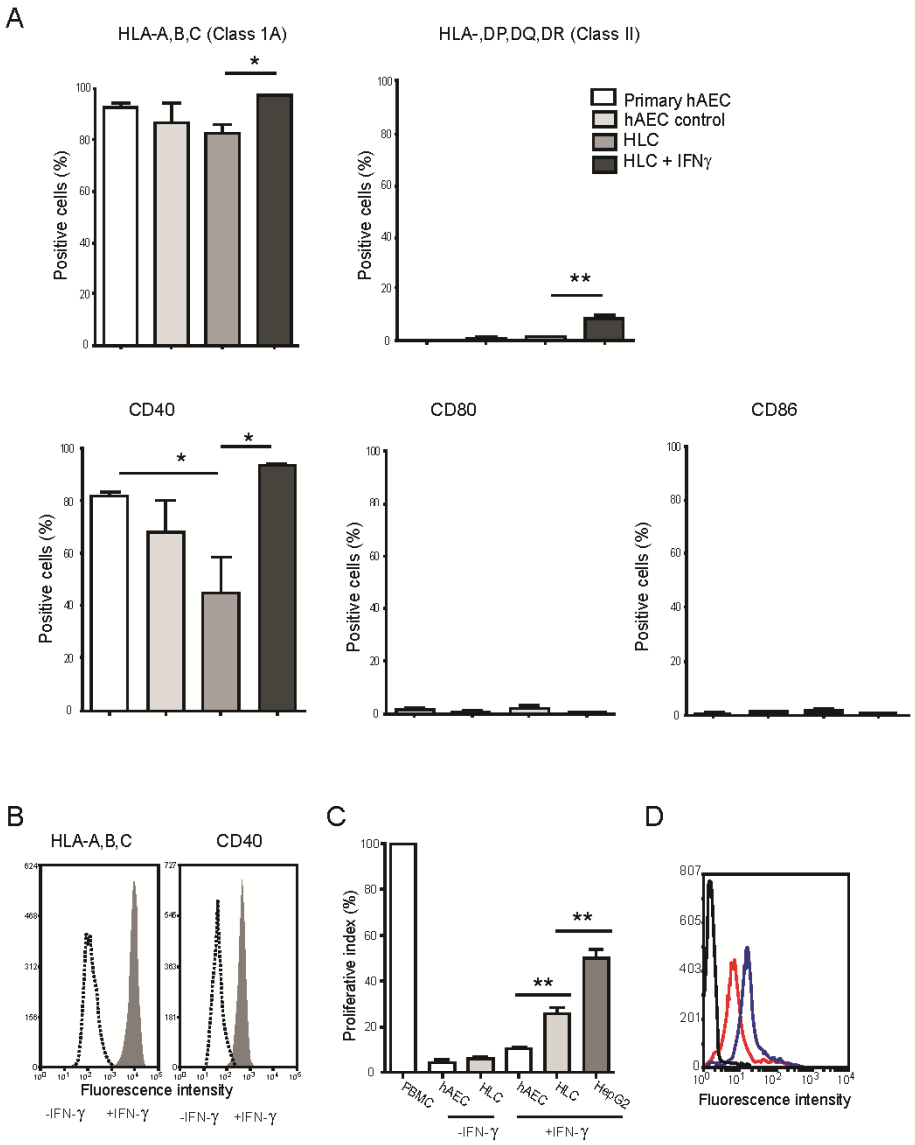

Fig. (2). Immunogenicity of hepatocyte-like cells (HLC). Cells were analysed by flow cytometry and numbers of positive cells shown graphically **(A)**. The HLC and human amniotic epithelial cells (hAEC) expressed HLA Class IA and CD40. Interferon gamma (IFN-γ) treatment led to increased numbers of CD40 and Class IA and to the expression of Class II antigens. Representative flow plots showing shifts in fluorescence intensities indicating increased expression levels of Class IA and CD40 with IFN-γ **(B)**. To assess immunogenicity, +/-IFN-γ treated cells were co-cultured with human PBMC in one-way lymphocyte reactions and ^{3}H-thymidine uptake measured. IFN-γ treated HLC induced PBMC proliferation compared with primary hAEC. PBMC from two non-related donors and HepG2 cells served as controls **(C)**. C57BL/6 mice were injected with HLC. Anti-human antibodies in the murine serum that bound to the HLC were detected by flow cytometry. Red and blue lines represent serum of mice receiving single and dual HLC injections **(D)**. *P<0.05 and **P<0.01. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper).

min with rabbit anti-mouse AlexaFluro-488 secondary antibody (1:100; Molecular Probes, Eugene, OR). Cells were washed thoroughly and analysed by flow cytometry.

One-way Mixed Lymphocyte Reaction

To determine if the differentiated hAEC were immunogenic and would stimulate lymphocyte proliferation, human PBMC (2x10^{5}; Seracare Lifesciences, Milford, MA) were co-cultured with 20 Gy γ-irradiated hAEC or HLC cells at a 1:1 ratio for 4 d (n=7, with each batch tested in quadruplicate wells). Cells were then pulsed with ^{3}H-thymidine (10 μCi) for 18 h, harvested and thymidine incorporation measured (Tri-Carb 1900TR Packard Biosciences, Meriden, CT). A 1:1 mixture of non-irradiated : irradiated PBMC from two separate donors served as a positive control. Comparisons

were made with non-stimulated hAEC and the transformed human hepatocyte cell line HepG2.

PBMC Proliferation Assay

To examine the immunomodulatory properties, the capacity of differentiated hAEC to suppress PBMC proliferation was assessed. Cells were γ-irradiated (20 Gy) and co-cultured with human PBMC ($1x10^5$) stimulated with the mitogen PHA (5 μg/ml; Sigma-Aldrich). Cell : PBMC ratios of 1:40, 1:160, 1:640 and 1:2560 were tested. Co-cultures were maintained for 72 h (quadruplicate wells for each ratio using cells from n=6 amnion membranes). The positive control consisted of PHA stimulated PBMC alone. Cultures were pulsed with 10 μCi ^{3}H-thymidine and incorporation measured. Data is shown as percentage suppression of PHA control = [1 – (Cells + PBMC + PHA / PBMC + PHA) x 100]. Comparisons were made with non-stimulated hAEC.

ELISA and EIA

Immunomodulatory factors secreted by cells were measured by ELISA or EIA. Cells were washed and grown in serum free medium for 48 h, conditioned media collected and stored at -80 °C. Soluble HLA-G1/5 (US Biologicals, Swampscott, MA), TGF-β1, IL-10, HGF and IL-6 (all from R&D Systems) were measured following protocols supplied by the manufacturers. PGE2 was measured using an EIA kit (Cayman Chemicals, Ann Arbor, MI) according to instructions provided. Media from (n=6-8) cultures were analysed.

Blocking Assays

To determine if soluble HLA-G, TGF-β1 and PGE2 played a role in suppressing PHA induced PBMC proliferation, neutralizing antibodies or indomethacin (to block PGE2) were incorporated to cell-PBMC co-cultures at the following concentrations: HLA-G (20 μg/ml; ExBio, Prague, Czech Republic); TGF-β1 (1 μg/ml; R&D Systems) and indomethacin (1 μM; Sigma-Aldrich). ^{3}H-thymidine was added and incorporation measured as described above.

Statistical Analysis

Data are expressed as mean±SEM. Paired comparisons were made by Student's T test and multiple comparisons by ANOVA followed by Tukey's post hoc test (Graph Pad Prism, v5.03, San Diego, CA). $P<0.05$ was considered to be significant.

RESULTS

HLC Derived from hAEC

Cultures that were stimulated with EGF, dexamethasone and insulin expressed the transcription factors GATA-4 and HNF-3β that have been shown to initiate liver development from early/definitive endoderm. However, GATA-4 and HNF-3β were also expressed by primary hAEC Fig. (**1**). In contrast, stimulated hAEC produced α-FP present in hepatic progenitors and hepatocytes, HNF-4α, a key transcription factor and TDO, an enzyme that degrades excess tryptophan in mature hepatocytes. Stimulated hAEC had abundant amounts of OTC, a mitochondrial enzyme that catalyses citrulline formation during urea synthesis. Further, the stimulated hAEC secreted urea following NH_4Cl treatment (mean±SEM = 0.72±0.95 mM). Stimulated cells treated with rifampicin showed functional CYP3A4 enzyme activity; CYP3A4 being one of the principle drug metabolizing enzymes in mature hepatocytes. Control hAEC cultures did not express TDO, HNF-4α, secrete urea or show CYP3A4 activity. Mature hepatocytes store glycogen and secrete albumin; abundant glycogen and albumin were present in treated cultures and lower amounts present in the control hAEC cultures Fig. (**1**). These findings suggest that the stimulated hAEC were hepatocyte-like and had adopted several features of mature hepatocytes.

Immunogenicity of HLC Derived from hAEC

Next, we investigated whether there were changes in the expression of allo-recognition HLA Class IA and II antigens and the co-stimulatory molecules CD40, CD80 and CD86 following differentiation into HLC using flow cytometry. Consistent with previous reports, primary hAEC and cells maintained in basal medium were HLA-A,B,C+ (Class IA) and CD40+ and lacked HLA-DR,DP,DQ (Class II), CD80 and CD86 [22]. The numbers of positive cells and expression levels of Class IA, Class II, CD80 and CD86 did not alter with differentiation into HLC Fig. (**2A**). However, the HLC cultures had fewer numbers of CD40+ cells ($P<0.05$). IFN-γ is known to stimulate HLA and co-stimulatory molecule expression [16, 25], and is highly elevated in the circulation and liver of patients who are potential candidates for HLC transplantation [26, 27]. Therefore, we examined the effects of IFN-γ and found that the numbers of HLA-A,B,C+ and CD40+ HLC increased with IFN-γ stimulation ($P=0.023$ and 0.036, respectively vs untreated HLC; Fig. (**2A**) and that HLA-DR,DP,DQ was induced with approximately 10% of HLC becoming Class II+ ($P=0.012$). In addition, the mean expression levels of Class IA increased with IFN-γ treatment (88.2 fold; $P=0.001$) and CD40 (24.6 fold; $P=0.028$; Fig. (**2B**).

As the IFN-γ treated HLC expressed HLA Class IA, Class II and CD40 that could interact with T cell receptors, we tested the immunogenicity of the HLC in one way mixed lymphocyte reactions using human PBMC. IFN-γ stimulated HLC induced PBMC proliferation and were significantly more immunogenic than IFN-γ treated hAEC ($P<0.01$), but less than HepG2 cells, a transformed human hepatocyte cell line ($P<0.01$; Fig. (**2C**). In contrast, the HLC and hAEC that had not been stimulated with IFN-γ induced little to no proliferative response in the PBMC.

To determine if antibodies were generated against the HLC, we infused the HLC into healthy immune competent C57BL/6 mice and analysed murine serum 2 and 4 weeks after one and two HLC infusions, respectively. The murine serum contained anti-human antibodies that bound to the HLC when the serum and HLC were mixed together *in vitro*. The bound anti-human antibodies were detected by flow cytometry Fig. (**2D**).

Immunosuppression by HLC

Patients with acute and chronic liver disease have increased numbers of activated lymphocytes in the peripheral circulation and liver and these cells are thought to contribute to disease onset and progression [28-30]. hAEC have been

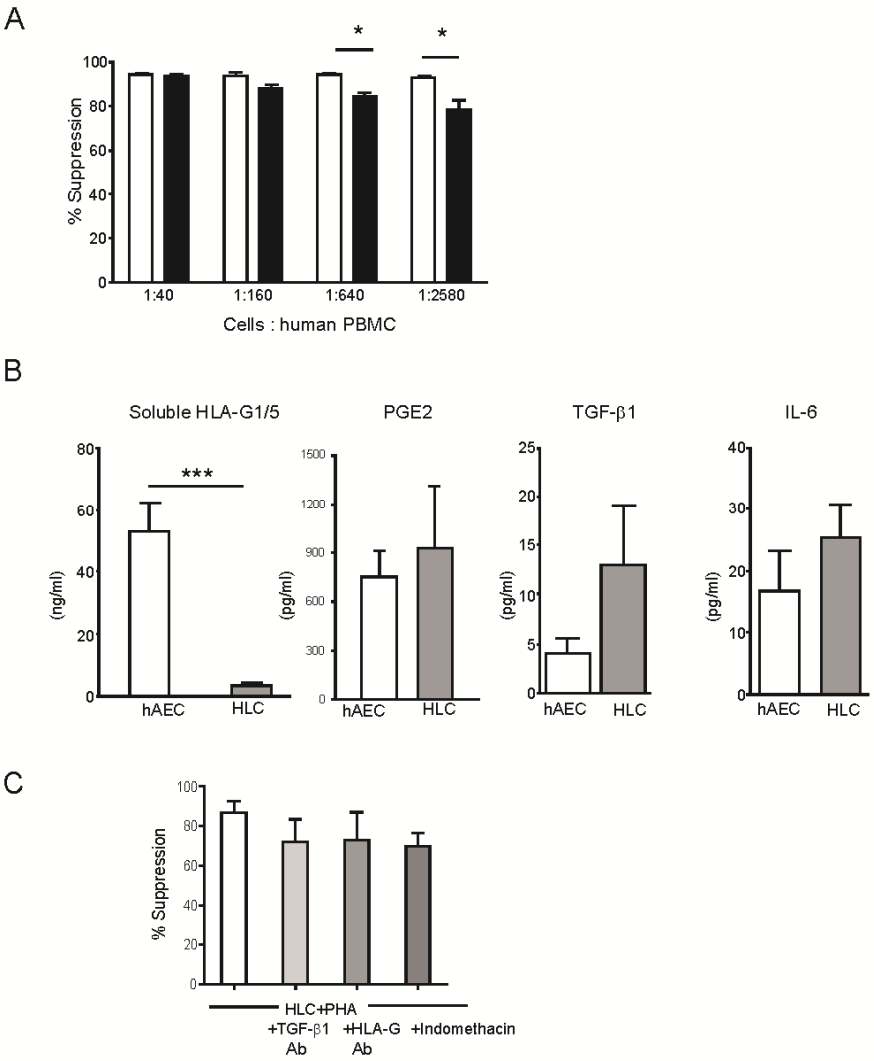

Fig. (3). Immunomodulation by hepatocyte-like cells (HLC). The HLC (solid bars) inhibited the proliferation of PBMC induced by the mitogen PHA, but to a lesser extent compared with human amniotic epithelial cells (hAEC; open bars) as the number of PBMC increased relative to the HLC **(A)**. Immunomodulatory factors secreted by the HLC and hAEC **(B)**. Addition of TGFβ-1 and HLA-G blocking antibodies and indomethacin to HLC : PBMC co-cultures of 1:40 lowered the suppressive effects on PBMC proliferation, but did not reach significance **(C)**. *, *** = P<0.05 and 0.001, respectively.

found to inhibit the proliferation of PBMC *in vitro* [17, 22, 23]. We investigated whether the HLC had similar properties. Like hAEC, the HLC suppressed PHA induced PBMC proliferation by >90% at HLC : PBMC ratios of 1:40 and 1:160, but the inhibition declined as the HLC numbers declined relative to the PBMC (P<0.05 vs hAEC at 1:640 and 1:2560; Fig. (**3A**). HepG2 cells failed to suppress PMBC proliferation (data not shown).

To explore if changes to immunomodulatory factors secreted by HLC may have led to altered PBMC proliferation, we compared the production of several cytokines, soluble HLA-G1/5 and PGE2 by primary hAEC and HLC. IL-10 and HGF were absent in media conditioned by primary hAEC and HLC. While there were no changes in IL-6, TGF-β1 and PGE2, HLA-G secretion declined significantly, although substantial amounts of HLA-G were still secreted by the HLC (P=0.0012 vs hAEC; Fig. (**3B**). Next, we examined whether TGF-β1, PGE2 and HLA-G from the HLC contributed to the inhibition of PBMC proliferation. Addition of TGF-β1 and HLA-G neutralizing antibodies and indomethacin to HLC : PBMC co-cultures, increased PBMC proliferation (i.e. reduced suppression), but not significantly (Fig. (**3C**).

DISCUSSION

We have shown that hAEC differentiate into cells with key features and functions of HLC. The HLC had low immunogenicity, but the immunogenicity was enhanced with IFN-γ stimulation. The HLC also secreted immunomodula-

tory factors that could inhibit PBMC proliferation. The transcription factors GATA-4 and HNF-3β that have been shown to promote differentiation of endodermal and hepatic progenitors were present in the HLC. In mature hepatocytes, GATA-4 has been shown to regulate the expression of hepcidin, a hormone which controls iron availability, the drug metabolizing enzymes CYP2C9 and CYP2C19 and erythropoietin [31-33]. HNF-3β can influence bile acid and glucose metabolism in hepatocytes, but it has also been reported that few genes are regulated by HNF-3β in the adult liver [34, 35]. Following differentiation hAEC expressed HNF-4α, a key transcriptional regulator of genes involved in xenobiotic, fat and cholesterol metabolism in mature hepatocytes. The enzymes IDO and TDO convert excess tryptophan from the diet into kynurenine. Unlike IDO, TDO is expressed predominantly in hepatocytes and TDO was found to be expressed in the HLC but not in hAEC and HepG2 cells. OTC enzyme converts ornithine to citrulline which is subsequently converted to urea. OTC was localised in the HLC and showed the typical punctate staining characteristic of OTC. Following stimulation with NH_4Cl the HLC secreted urea showing that OTC and other enzymes of the urea cycle were functional. Drug metabolising activity of CYP enzymes is a key function of hepatocytes. Consistent with a recent report [13], we also found that HLC derived from hAEC responded to rifampicin, a specific inducer of CYP3A4, and demonstrated functional activity. Collectively, these findings suggest that the stimulated hAEC had differentiated into HLC.

Primary hAEC have been shown to express low levels of HLA Class IA antigens and to elicit minimal proliferative responses in PBMC in one way mixed lymphocyte reactions *in vitro* [17, 22]. hAEC have even been grafted into healthy human volunteers and patients with lysosomal storage diseases without adverse immune responses being reported except for macrophage infiltration into the grafts [18, 36]. However, following differentiation of stem cells increased expression of allo-recognition molecules has been reported. Differentiation of embryonic stem cells into cardiomyocytes enhanced Class IA antigens through epigenetic mechanisms and, beta2-microglobulin and the transporter associated antigen processing proteins tapasin and tapasin components [37]. Thus, the differentiated cardiomyocytes were more immunogenic compared with undifferentiated embryonic stem cells. Increased immunogenicity has also been reported with differentiation of MSC into cardiomyocytes, chondrocytes and osteocytes [20, 38, 39]. However, we did not find increased immunogenicity following differentiation of hAEC into HLC. There were no significant changes in HLA Class IA, Class II, CD80 and CD86 while the number of CD40 positive cells declined. The low immunogenicity of the HLC was demonstrated in one way mixed lymphocyte reactions where the proliferative response by PBMC to HLC was minimal. A recent study showed that HLA-DR was not expressed in HLC derived from umbilical cord MSC and these HLC were described as cells with low immunogenicity, but whether HLA Class IA and the co-stimulatory molecules were expressed by these HLC was not reported [40]. The increased immunogenicity found in cardiomyocytes and other lineages may have been induced by the growth factors, additives and substrates used during differentiation. Importantly however, we found that when the HLC were exposed to IFN-γ, a pro-inflammatory cytokine, HLA Class IA and the co-stimulatory molecule CD40 became highly elevated and that HLA Class II was induced in some HLC. Indeed, the proliferative response by PBMC to IFN-γ stimulated HLC indicated that the HLC were immunogenic. IFN-γ is known to induce Class IA and II antigens and studies have shown an elevation in CD40 in human placental umbilical cord HUVEC and amniotic MSC with IFN-γ [16, 25, 41, 42]. HLA Class IA levels in human embryonic stem cells were also reported to increase with IFN-γ, but unlike the HLC, Class II was not detected [43]. In allogeneic cell and tissue transplants, the presence of HLA Class IA antigens on the surfaces of donor cells is thought to be the predominant reason for graft rejection by the recipient. Intracellular proteins of the donor are processed and presented by Class IA molecules. The donor peptide-HLA complex can bind to cytotoxic CD8+ T cells of the recipient and elicit an immune response [44, 45]. Class II molecules have been shown to be induced in many cells following transplantation in the presence of elevated levels of IFN-γ [44, 45]. Our findings suggest that HLA Class II antigen expression is likely to increase further if the HLC were to be grafted into recipients with acute or chronic liver disease where IFN-γ levels are highly elevated. Extra-cellular donor proteins are processed and presented by Class II antigens to elicit a response by the recipients CD4+ T-helper cells. In addition to direct presentation of donor antigens, antigen presenting cells of the recipient can process and present allogeneic donor HLA peptides on Class II antigens and elicit an immune response by CD4+ T cells *via* indirect presentation [44, 45]. Thus, increased levels of HLA and co-stimulatory molecules need to be carefully evaluated and the immunogenicity of the HLC tested in animal disease models to assess the safety of the differentiated HLC as a cellular therapy. We also found that anti-human antibodies were generated against the HLC in healthy mice. This may have been due to T cell initiated antibody generation by B cells or due to the many secreted, donor derived minor histocompatibility proteins. The existence of pre-formed antibodies can lead to a hyper acute rejection with repeated cell transplants derived from the same donor. However, the immunomodulatory properties of the HLC could potentially dampen the immunogenicity of these cells.

Primary hAEC have been shown to inhibit mitogen and antigen stimulated PBMC proliferation *via* cell : cell contact and through secreted factors [16, 17, 22]. Secreted factors from hAEC that have been shown to inhibit PBMC proliferation include PGE2, TGF-β1 and FasL [23, 46]. hAEC also secrete other immunomodulatory factors such as IL-6 and HLA-G, but not IL-10 and HGF [22). We found that HLC were able to suppress mitogen induced PBMC proliferation and secreted IL-6, TGF-β1 and PGE2, but that HLA-G secretion was reduced compared with hAEC. The secretion of PGE2 by human hepatocytes has not been reported. However, dexamethasone can stimulate PGE2 [47], and PGE2 from the HLC may result from dexamethasone present in the induction medium. In contrast, the large decrease in HLA-G output may have been due to the long term *in vitro* culture and consistent with a previous report [48]. HLA-G is reported to be absent in human hepatocytes. Interestingly how-

ever, membrane bound and secreted isoforms of HLA-G are present in hepatocytes in patients with hepatitis and may exert an anti-inflammatory role [49]. Marongiu *et al.*, have shown that in addition to differentiation *in vitro*, hAEC can differentiate into HLC in the murine liver [13]. In a recent study we found that the hAEC engrafted in the murine liver became HNF-4α positive and retained HLA-G suggesting that HLA-G expression is maintained by the HLC derived from hAEC [50]. We assessed the contribution made by HLA-G, TGF-β1 and PGE2 from HLC and found that by blocking these factors the inhibitory effects on PBMC proliferation was reversed but that significance was not reached. These findings suggest that HLA-G, TGF-β1, PGE2 and potentially other factors from HLC act in concert to suppress PBMC proliferation. In summary while HLC were derived from hAEC, further studies would be required to assess the functionality, immunomodulatory effects and immune responses by the recipient to the HLC in animal models of acute and chronic liver failure to explore the utility of HLC for allogeneic transplantation.

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Chapter 5. Integrated Discussion, Conclusions and Future Directions

Current therapies for multiple sclerosis (MS) exhibit partial efficacies and side effects with no complete cure. Researchers aim to explore potential new tools, or to improve the effects of existing therapies by combining different therapies or switching between them along the disease course (Steinman and Zamvil 2006; Caon 2009). Stem cell therapies for MS are being investigated with ongoing clinical trials using hemaetopoeitic stem cells and mesenchymal stem cells. One of the major shortcomings for stem cells is the limitation of primary cell numbers that can be obtained from the tissue. Human amniotic epithelial cell (hAEC) does not suffer from this limitation in that they are readily acquired from human placenta in large numbers. Also, hAEC have stem cell-like properties but with less undesirable side effects or risk factors, such as telomerase expression and teratoma formation (Miki, Lehmann et al. 2005; Ilancheran, Michalska et al. 2007). Application of these cells for the treatment of ocular surface disorders decades ago has led to the interest of hAEC in xenogeneic/allogeneic tissue transplantation (He, Alizadeh et al. 1999). Because hAEC express little or no HLA class I/II antigens, successful allogeneic or even xenogeneic transplantation can be achieved without rejection in a number of organs, such as the ocular site and spinal cord (Sankar and Muthusamy 2003; Parmar, Alizadeh et al. 2006).

Furthermore, recent studies have revealed their potential in several diseases, including models for Parkinson's disease (Kakishita, Nakao et al. 2003; Yang, Xue et al. 2009;

Yang, Song et al. 2010). Because MS and Parkinson's disease are both characterized by neurodegeneration, it is possible that hAEC may have similar therapeutic efficacy in MS models. MS is considered to be a T cell-mediated disease in which T cells initiate the pathological immune responses targeting CNS myelin, and the consequential tissue inflammation (Stinissen, Raus et al. 1997). I therefore performed co-culture experiments to examine whether hAEC are capable of reducing T cell responses, especially in suppressing T cells from EAE mice which is an animal model of human MS. My results showed that hAEC are able of suppressing mitogen-induced proliferation of splenic T cells from naïve mice, and importantly inhibit MOG-specific proliferation of splenic T cells from EAE mice in an antigen-specific and dose-dependent manner.

I then applied hAEC for the treatment of EAE mice in which EAE was induced by MOG_{35-55} peptide. I injected hAEC before disease symptoms occurred and showed improvement both in clinical symptoms and regulation of peripheral immune functions. In this prevention model hAEC treatment delayed the onset of EAE represented by lower clinical scores. CNS pathology was relieved which was shown by better histological results of reduced demyelination and less inflammatory cell infiltration of macrophages and T cells. Nevertheless, improvement in clinical scores only showed in the short-term and exacerbated later. This partial effect may be improved if hAEC are given in earlier time points, but it may cause higher mortality rate since EAE mice are weaker right after EAE induction, shown by reduced weight loss. Instead of injecting hAEC earlier, a boost injection may be more appropriate. The dose of cells delivered may change the outcome too, but it will raise another issue in that similar application of cell number/body weight ratio is nearly impossible in humans. However, one should not exclude the possibilities of improvement occurring after fine-tuning these delivery

strategies of hAEC in animal models.

My study investigates the use of hAEC in a mouse model of multiple sclerosis not only as a preventive therapy as mentioned above, but also as an interventional therapy after corticosteroids treatment-induced remission of EAE. hAEC in both prevention and intervention models show apparent relief of clinical signs and evidences of peripheral immune regulation. I explored the possible mechanism by which hAEC alleviate the disease signs and found transforming growth factor-β (TGF-β) and prostaglandin E2 (PGE2) are two essential molecules for the inhibition of splenic T cell proliferation. Both of these molecules exert potent immunosuppressive properties. TGF-β is expressed by most human tissues and cultured cells, including hAEC (Taipale, Lohi et al. 1995). It is a growth inhibitor for T cells that inhibits T cell proliferation, and is able to control destructive T cell-mediated responses in EAE through signaling to dendritic cells (Kehrl, Wakefield et al. 1986; Li, Wan et al. 2006; Laouar, Town et al. 2008). Administration of TGF-β in EAE mice reduce CNS inflammation and signs of disease (Racke, Cannella et al. 1992). Although there is no evidence in my study that hAEC migrate to CNS, a previous hAEC study has shown TGF-β mRNA expression in the lung (Moodley, Ilancheran et al. 2010) and brings the possibilities that hAEC may utilizes TGF-β to regulate T cell proliferation in our EAE models. The other immunosuppressive molecule, PGE2, has shown its efficacy in previous hAEC study. Similarly, PGE2 has the ability to inhibit T cell proliferation and promote the production of Th2 cytokines (Harris, Padilla et al. 2002; Woolard, Wilson et al. 2007). My findings that TGF-β and PGE2 are essential for hAEC-induced inhibition of splenic T cell proliferation supports the above literatures and also point out that TGF-β has a more prominent role than PGE2 in the inhibition (Liu, Vaghjiani et al. 2012).

In searching of other secreted molecules which are important to suppress splenic T cell proliferation, I found that some previously reported candidate molecules may not be crucial in regulating splenic T cells. Hepatocyte growth factor (HGF), nitric oxide (NO) and interleukin-10 (IL-10) are among these candidates which suppress T cell growth, proliferation or induce regulatory T cells (Benkhoucha, Santiago-Raber et al. 2010; Soleymaninejadian, Pramanik et al. 2012). However, none of these molecules were detected in my ELISA test of primary hAEC culture media. Beside these negative results, other human-specific immunomodulatory molecules may be further examined in culture with human cells even though they were excluded in my xenogeneic model. On the other hand, the TGF-β blocking antibody that I used can crossreact with mouse TGF-β and therefore is also able to block TGF-β secreted by murine cells. Further examination of the source of TGF-β utilized by hAEC for splenic T cell inhibition is necessary to elucidate the detailed mechanism.

My data from the prevention model indicate that most of the infused hAEC are located in the lungs of EAE mice after 7 days of cell injection, with absence of detectable hAEC in CNS or peripheral lymphoid organs (Liu, Vaghjiani et al. 2012). There were similar findings in EAE mice treated with human MSC which suggested these cells, while mostly being trapped in the lungs, exert their beneficial functions from a distance *via* secreting some immunomodulatory molecules (Roddy, Oh et al. 2011; Prockop and Oh 2012). Besides this assumption, Odoardi *et al* also proposed that the lung is an important organ for "licensing" T cells, based on the behaviour of myelin basic protein (MBP)-specific T cells transferred to induce a rat model of EAE (Odoardi, Sie et al. 2012). According to their data, most of the intravenously transferred T cells which are specific to MBP home to the lungs and stay in bronchus–associated lymphoid tissue (BALT) and draining lymph nodes before they re-enter the blood circulation. These T

cells will have their gene expression profile reprogrammed in the lung tissue which allows them for further migration to CNS and reactivated there (Odoardi, Sie et al. 2012). It might be possible that hAEC fit with this hypothesis. While hAEC were trapped to the lungs in EAE mice, hAEC might have served the role to interact with these T cells in BALT or draining lymph nodes and to modulate their behavior during the licensing process, and therefore affected the disease course. Future work should search for the exact compartment in the lung where hAEC are trapped and knowing what kind of interaction they had with the cells in the lymphoid tissues. These data could help us understand whether hAEC exert their function similar to other cells used for cell-based therapies.

Both murine and human $CD4^+$ T cells can be categorized to different subtypes according to their cytokine profile and effector functions (Mosmann, Cherwinski et al. 1986). Th1 and Th17 cells are considered pathogenic T cell subsets, while Th2 cells antagonize the pathogenic effects in EAE (Cua, Hinton et al. 1995; El-behi, Rostami et al. 2010). I therefore determined cytokine profiles in the culture supernatant of splenic cells from hAEC-treated EAE mice and compared with untreated EAE mice. Among 10 Th1/Th2/Th17 cytokines I found elevated Th2 cytokine IL-5 in the hAEC prevention model, and elevated IL-5 and IL-2 in the hAEC intervention model. These cytokine changes partly reflect the shift from proinflammatory Th1 profile to anti-inflammatory Th2 profile, because Th2 cytokine IL-5 were upregulated after hAEC treatment in both models. However, the reason for the change of IL-2 is still unknown. It is possible that elevated IL-2 represents resistance response to hAEC inhibition. MS patients treated with MSC have shown increased IL-2 production from peripheral lymphocytes, which accounts for weaker response to MSC inhibition. It was postulated that higher IL-2 is responsible to the relative resistance to MSC regulation

(Ben-Ami, Miller et al. 2013). Beside IL-5 and IL-2, there was no change in other Th1/Th2/Th17 cytokines. The results suggest that hAEC modulate peripheral immune responses in concordance with my other findings.

I found elevated peripheral $CD4^+CD25^+FoxP3^+$ Treg populations both in the prevention model and the intervention model. A similar study showed that after cessation of immunosuppressant, cell transplantation increased peripheral $CD4^+CD25^+FoxP3^+$ Treg population and may be a contributory mechanism to EAE remission (Meng, Ouyang et al. 2011). Meng *et al* found that elevated Treg population on day 80 after EAE induction, which is similar to the time point (day 84) that I found elevated Treg population in the intervention model ("day 84 after EAE induction" equals to "day 60 after corticosteroids-induced remission"). According to their result, the increased Treg population was not detected earlier (day 40). The clinical importance of abnormal Treg population can be seen on studies in MS patients. Two aspects of abnormal Treg population are often discussed in MS patients: First, the reduced cell numbers (Venken, Hellings et al. 2008); and the second, a similar amount but functionally impaired Treg population (Viglietta, Baecher-Allan et al. 2004; Haas, Hug et al. 2005; Venken, Hellings et al. 2006). However, these Tregs from some MS patients exhibited different tropism because they showed higher expression of adhesion molecules CD103 and CD49d, and distributed more in the cerebrospinal fluid than in the peripheral blood (Venken, Hellings et al. 2008). As Treg may contribute as part of suppressor cell mechanisms in the therapeutics of hAEC, more studies especially in MS patients providing direct evidence of mechanisms of action are needed.

The importance of combinational therapies for MS is addressed both in basic studies and in clinical trials. Novel therapies that combine approved drugs such as IFN-β with

dexamethasone, or with glatiramer acetate are either being applied in clinic or in trials which may provide better outcomes than therapies that utilize single medication (Tullman and Lublin 2005). My study using hAEC after corticosteroids-induced remission has shown lower clinical scores, also anti-inflammatory effects in the CNS and peripheral immune organs, as compared to corticosteroids-only treated mice in my experiment. It may be a practical way to provide hAEC transplantation after first-line medicines of MS in the future, similar to my study design. However, studies testing the effects of hAEC therapy either with altered delivery method or combined with other available drugs should be done to give more information to refine this potential therapeutics.

Splenocyte proliferation assay stimulated by mitogen is broadly used to understand T cell function. Assessment by ^{3}H-thymidine incorporation is a standard technique used in EAE and other mouse models (Strong, Ahmed et al. 1973; Lehmann, Forsthuber et al. 1992). My study broadly use splenocyte proliferation assay to investigate T cell function and the culture supernatants collected to determine the production of various cytokines by splenic T cells. The lower proliferation of splenocytes and shifted cytokine profile show that hAEC has modulated peripheral T cell function. Because I could not detect hAEC in organs other than lung, the possibility that hAEC migrate to CNS to change the local T cell response is less likely.

On the other hand, B cell activation will normally occur after T cell activation, and followed by subsequent antibody production. Therefore, early speculation of B cell response occurring in early phase should be avoided (Batoulis, Recks et al. 2011). I examined both T cell responses (proliferation and cytokine production) and B cell response (autoantibody production) in the end-points of EAE mouse experiments, thus

the potential problem of early speculation was not an issue in my study.

It will be even better if there were larger number of animals in each experiment so that the same immunoassay can be performed in different time points to give more information about disease dynamics. Difficulties to get large number of mice for my intervention study come from mouse-to-mouse differences in response to corticosteroids treatment, some mice took longer than other to have their paralytic signs relieved. In my intervention EAE mouse experiment, although in the beginning there were 60 mice induced with EAE and most of the mice were remitted after corticosteroids treatment, only those mice which remitted completely at the same day can be used for hAEC infusion in order to provide hAEC from the same batch at the same time. The follow-up animal experiment may consider using an even bigger number of mice to start in order to include more mice and obtain experimental results with lesser variation.

Application of stem cells to patients largely depends on the initial number of primary cells that can be acquired from the tissue. As mentioned before, hAEC has the advantage in cell transplantation compared to other immune-privileged cells because the huge cell numbers from amnion can be easily acquired. Primary hAEC are different from mesenchymal stem cells that need further propagation and careful examination of phenotypes and surface molecule profiles to ensure their stemness remained. However, if cell propagation is still needed, the xenobiotic-free medium must be used for human allogeneic transplantation to prevent potential xenogeneic effect induced by components of culture medium. In hAEC, cell passage after 4 generations has shown reduced suppressive properties and lower production of TGF-β and HLA-G, and also elevated production of IL-6 (Pratama, Vaghjiani et al. 2011). These altered properties

should be taken into consideration when applying hAEC to a specific disease model. Also, the changes after cell propagation of hAEC also include differentiation to various cell lineages and the morphological changes. However, the more differentiated phenotype may provide beneficial effect to the relevant disorders and organ type that receive cell transplantation (Tee, Vaghjiani et al. 2013).

The most cumbersome issue of allogeneic transplantation is the immunogenicity of transplanted cell type. hAEC has the advantage in that they express low or no HLA type I and type II antigens, thus preventing the chance of being rejected. Even in my xenogeneic study where hAEC have been applied to mouse, the cells did not elicit any apparent side effect of rejection. This may be the main advantage of hAEC to be used for human diseases, which is also similar to human MSC but with more advantages in cell numbers and ease of obtain cells.

Most of our knowledge about CNS inflammation in MS patients has been gathered from EAE studies (Gold, Linington et al. 2006). There is a significant number of MS therapies that were originally effective in EAE, including glatiramer acetate, mitoxantrone, and natalizumab. They subsequently gained approval for their clinical applications to MS patients. These facts indicate that animal model of MS, especially EAE, has been useful to decipher the yet not clear pathogenesis and to explore possible treatments (Steinman and Zamvil 2006). While EAE is a good MS model for studying immunomodulation, one needs to be cautious in interpreting results arising from EAE studies. EAE may not be appropriate for studying neuron repair or chronic disease courses since it is not similar to these aspects of MS (Baker and Amor 2012). Among the commonly used EAE models, my chosen one using MOG_{35-55} peptide which induced chronic EAE is more suitable to study demyelination mediated by T cells and

macrophages (Gold, Linington et al. 2006). According to these guidelines, in my studies I interpret my results by suggesting that hAEC therapy skewed peripheral immune system of EAE mice, and may further influence the behaviors of CNS-infiltrating T cells and macrophages. Neuron repair issue were untouched in my experimental model but could be studied further if the success of hAEC therapy in EAE continues in MS patients.

In conclusion, my studies has first shown the nature of hAEC as cellular therapy to murine EAE, a model of human MS. hAEC therapy is helpful either before the disease onset, or when given after cessation of anti-inflammatory corticosteroids treatment. Intravenously delivered hAEC may reside in the lung to alleviate peripheral immune responses and paralytic signs in EAE mice, as well as decrease CNS inflammation and induce regulatory T cell population *in vivo*. I propose that hAEC utilize secreted molecules TGF-β and PGE2 in controlling T cell proliferation. As an ideal cell therapy for neurological diseases should be able to induce immunomodulation, promote neuroprotection and regeneration of the CNS, my studies up to date reveals only part of the effects. However, future studies may pave the way to develop treatment method involving hAEC for EAE and MS to help patients with this devastating autoimmune disease which affects 2.5 million people worldwide.

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