Emerging Functions of Amphiregulin in Orchestrating Immunity, Inflammation, and Tissue Repair

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Type 2 inflammatory responses can be elicited by diverse stimuli, including toxins, venoms, allergens, and infectious agents, and play critical roles in resistance and tolerance associated with infection, wound healing, tissue repair, and tumor development. Emerging data suggest that in addition to characteristic type 2-associated cytokines, the epidermal growth factor (EGF)-like molecule Amphiregulin (AREG) might be a critical component of type 2-mediated resistance and tolerance. Notably, numerous studies demonstrate that in addition to the established role of epithelial- and mesenchymal-derived AREG, multiple leukocyte populations including mast cells, basophils, group 2 innate lymphoid cells (ILC2s), and a subset of tissue-resident regulatory CD4$^+$ T cells can express AREG. In this review, we discuss recent advances in our understanding of the AREG-EGF receptor pathway and its involvement in infection and inflammation and propose a model for the function of this pathway in the context of resistance and tissue tolerance.

Introduction

The immune system is composed of a complex network of leukocytes interacting with other physiological systems to protect the host from pathogen invasion and tumorigenesis. Host protection can manifest itself in two ways: as resistance, the ability to eradicate invading pathogens or other foreign stimuli; or as tolerance, the capacity to decrease pathogen- or tumor-induced damage without affecting pathogen or tumor burden (Medzhitov et al., 2012; Schneider and Ayres, 2008). Immune-mediated resistance is tailored to match the type of invading pathogen, allowing for the production of polarized factors that specifically target distinct pathogens. For example, protective immunity to many viruses and bacteria is coordinated by the type 1 or type 17 immune response, which can include increases in interferon-γ (IFN-γ) and interleukin-17 (IL-17), whereas multicellular parasites elicit type 2 responses, associated with increases in IL-4, IL-5, IL-9, and IL-13 (Allen and Wynn, 2011; Gause et al., 2013; Palm et al., 2012; Pulendran and Artis, 2012). In contrast, tolerance mechanisms that mitigate damage through control of harmful inflammation and enhanced tissue repair are more general, and similar mechanisms of tolerance are utilized in response to a wide array of pathogens and stimuli. As such, the pathways involved in tolerance often include components of the type 2 immune response and can be activated by diverse stimuli, including toxins, venoms, allergens, and infectious agents such as helminths, bacteria, and viruses (Allen and Wynn, 2011; Gause et al., 2013; Palm et al., 2012; Pulendran and Artis, 2012).

Although a wide array of factors contribute to the host response to pathogen infection or other foreign antigens, the epidermal growth factor (EGF)-like molecule, Amphiregulin (AREG), has recently been shown to play a central role in orchestrating both host resistance and tolerance mechanisms.

Although AREG and other EGF family members are originally described as epithelial cell-derived factors, recent data show that AREG can be expressed by multiple populations of activated immune cells in a variety of inflammatory conditions. At the current time, the immune cells described to have AREG-expressing capacity are primarily associated with type 2 responses (Table 1 and associated references). Recent studies indicate that AREG is a pivotal factor that can contribute to host resistance (Zaiss et al., 2006) but is primarily a key factor that induces tolerance by promoting the restoration of tissue integrity following damage associated with acute or chronic inflammation (Burzyn et al., 2013; Jamieson et al., 2013; Monticelli et al., 2011). In this review, we will summarize recent developments regarding the distribution and regulation of AREG expression in the steady-state and during inflammation. We will then discuss the role of AREG in resistance and tolerance to a variety of pathogens, in promotion of tissue repair and in the regulation of tumor progression.

Distribution and Regulation of Amphiregulin Expression

AREG, a member of the epidermal growth factor (EGF) family, is constitutively expressed by a number of epithelial and mesenchymal cell types during development and homeostasis (Berasain and Avila, 2014). In addition to being implicated in a variety of physiologic processes, including regulation of lung morphogenesis (Schuger et al., 1996), keratinocyte proliferation (Cook et al., 1991), and mammary gland development (Li et al., 1992), studies demonstrating that wounded keratinocytes rapidly induce robust AREG expression (Kennedy-Crispin et al., 2012) have contributed to the hypothesis that epithelial-derived AREG can act to promote tissue repair and integrity. Importantly, AREG-gene deficient mice display very few abnormalities under homeostatic conditions (Luetteke et al., 1999), but resolution of a

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Table 1. Innate and Adaptive Immune Cell Populations as Sources of Amphiregulin

Arm of Immune System	Cell Type	Species	Inflammatory Setting	Reference
Innate	Basophils	Human	Allergy	Qi et al. (2010)
		Mouse	Contact hypersensitivity	Meulenbroeks et al. (2015)
	Eosinophils	Human	Ex vivo GM-CSF stimulation	Matsumoto et al. (2009)
	Mast cells	Human	Ex vivo IgE cross-linking	Wang et al. (2005)
		Human	Ex vivo FcεRI aggregation	Okumura et al. (2005)
		Mouse	Dermatitis, T cell transfer colitis, cancer	Zaiss et al. (2013)
	Neutrophils	Human	Cystic fibrosis	Adib-Conquy et al. (2008)
	Group 2 innate lymphoid cells (ILC2s)	Human	Atopic dermatitis	Salimi et al. (2013)
		Mouse	Influenza	Monticelli et al. (2011)
	Dendritic cells	Human & mouse (in vitro)	ATP stimulation	Bles et al. (2010)
Adaptive	CD4^{+} T cells	Human	Ex vivo TCR stimulations	Qi et al. (2012)
		Mouse	*Trichuris muris*	Zaiss et al. (2006)
	Regulatory CD4^{+} T cells (subset)	Mouse	Muscle injury	Burzyn et al. (2013)
	Tumor infiltrating CD8^{+} T cells	Mouse	Chemical carcinogenesis	Kwong et al. (2010)

variety of inflammatory challenges is impaired in these mice (Berasain et al., 2005; Meulenbroeks et al., 2015; Perugorria et al., 2008; Zaiss et al., 2006), supporting the hypothesis that AREG plays a critical role in restoring tissue integrity following infection or injury.

As our appreciation for the dialog between tissue-resident immune cells and the epithelial cells they interact with grows, accumulating data suggest that the immune system might also play a critical role in AREG-epidermal growth factor receptor (EGFR) signaling. For example, in the context of various inflammatory stimuli, basophils (Qi et al., 2010), eosinophils (Matsumoto et al., 2009), mast cells (Okumura et al., 2005; Wang et al., 2005; Zaiss et al., 2013), group 2 innate lymphoid cells (ILC2s) (Monticelli et al., 2011; Salimi et al., 2013), dendritic cells (DCs) (Bles et al., 2010), and activated CD4^{+} T cells (Qi et al., 2012; Zaiss et al., 2006) have all been shown to express AREG (Table 1). In addition, AREG expression has also been reported in neutrophils in the sputum of cystic fibrosis (CF) patients (Adib-Conquy et al., 2008), in a subset of tumor-infiltrating CD8^{+} T cells (Kwong et al., 2010) and in a phenotypically and functionally distinct subtype of FoxP3-expressing CD4^{+} regulatory T cells (Tregs) that accumulates in injured muscles (Burzyn et al., 2013) or the inflamed colon of mice (Schiering et al., 2014). These Treg cell populations have a distinct T cell receptor (TCR) repertoire, express the IL-33R (Burzyn et al., 2013; Schiering et al., 2014) and are distinguished from other Treg cell populations in that they express AREG (Burzyn et al., 2013). Although it is possible that other leukocytes might express AREG in a variety of contexts, the cell types currently described to express AREG and their pattern of activation suggest that immune-derived AREG is associated with type 2 immune-mediated resistance and tolerance mechanisms.

Furthermore, multiple immune mediators including prostaglandin E$_2$ (Berasain et al., 2005; Qi et al., 2012; Shao et al., 2003), cAMP (Johansson et al., 2004), insulin-like growth factor-1 (IGF-1) (Rodland et al., 2008), and transforming growth factor-β (TGF-β) (Bennett et al., 1992; Zhou et al., 2012) can induce AREG expression, suggesting that AREG production might be regulated by the activation of multiple cell types during an immune response.

Diverse stimuli have been associated with production of AREG by leukocytes. For example, IL-33 exposure stimulates AREG expression in ILC2s (Monticelli et al., 2011; Salimi et al., 2013), but not in multipotent progenitor type 2 cells (Saenz et al., 2013). Basophils express high quantities of AREG following exposure to IL-3. However, human basophils produce substantially less AREG in response to immunoglobulin E (IgE)-cross-linking than to IL-3 exposure (Qi et al., 2010), which is consistent with evidence from mouse models suggesting that basophils are heterogeneous in their development and effector functions (Siracusa et al., 2011). In humans, prostaglandin E$_2$ has been shown to be a potent inducer of AREG expression in a variety of CD4^{+} T cell subsets (Qi et al., 2012). In murine T cells, AREG is expressed in a subset of tissue-resident CD4^{+} Treg cells (Burzyn et al., 2013) and could be induced by TCR stimulation in T helper 2 (Th2), but not Th1 cells (Zaiss et al., 2006), suggesting that AREG expression is restricted within subsets of CD4^{+} T cells. Consistent with this, although type 2 inflammation can be initiated independently of AREG in some contexts (Kajiwara et al., 2010; Yagami et al., 2010), there are a growing number of disease states linking AREG and type 2 inflammation.

Although the findings outlined above demonstrate that immune cells can be induced to express AREG, numerous open questions remain related to the biology of AREG responses. For example, further work is needed to determine how hematopoietic AREG expression and function is regulated in vivo. In addition, how the signals that direct AREG expression from distinct cell types during development differ from those that induce AREG expression in response to infection, inflammation, or injury remains poorly understood. Importantly, the distinct roles of hematopoietic- and non-hematopoietic cell-derived

AREG remain unclear. AREG is expressed as a membrane-bound protein, and activation of AREG on non-hematopoietic cells is regulated largely at the level of release from the cell membrane. However, the ability of hematopoietic cells to migrate to the site of inflammation and locally upregulate AREG expression can substantially influence local concentrations of this growth factor. Thus, hematopoietic and non-hematopoietic cells might play different roles in contributing to AREG-dependent responses. Finally, how other pathways regulate AREG responsiveness is incompletely defined. For instance, AREG requires heparin sulfate expression on target cells, which is tightly controlled during inflammation (Simon Davis and Parish, 2013), in order to efficiently signal via the EGFR (Johnson and Wong, 1994). These data suggest that a combination of mechanisms might allow for a highly regulated spatio-temporal accumulation of AREG at the site of epithelial damage and inflammation. Further investigation into the regulation of AREG expression, the significance of hematopoietic- versus non-hematopoietic-derived AREG and the mechanisms that regulate AREG responsiveness will require the development of new genetic tools that permit the manipulation of AREG expression and responsiveness on a cell lineage-specific basis.

Taken together, these studies demonstrate that AREG expression is elicited by diverse stimuli yet is primarily associated with immune cell populations activated in type 2 immune responses, wound repair, and the resolution of inflammation. Below, we will discuss data that suggest critical roles for hematopoietic-derived sources of AREG during infection, inflammation, and tissue homeostasis, with particular focus on recent developments in our understanding of how AREG contributes to the pleiotropic aspects of type 2 immunity.

Amphiregulin-Mediated Resistance and Tolerance to Infection

Helminths trigger potent type-2 immune responses in both mice and humans (Allen and Wynn, 2011; Gause et al., 2013; Pulendran and Artis, 2012), can cause tissue damage and often cannot be cleared by the host. The immune system therefore must develop strategies to tolerate the persisting pathogen and simultaneously mitigate any associated tissue injury. As a result, type 2 immunity can not only mediate clearance of pathogens but also has additional functions, such as rapid repair of tissue damage and promoting homeostasis of infected tissues, which includes the suppression of local inflammation (Allen and Wynn, 2011; Gause et al., 2013; Pulendran and Artis, 2012).

In the context of helminth infection, where parasite invasion and the resultant infection-induced inflammatory response can damage the epithelial layer, enhanced epithelial cell proliferation has a number of beneficial effects, including repair of damaged epithelial cells and helminth expulsion via the "epithelial escalator" (Cliffe et al., 2005). Because distinct helminth parasites utilize various routes to infect the host, a wide range of immune defense mechanisms have evolved to provide protection while limiting pathological inflammation (Anthony et al., 2007; Maizels et al., 2012). For the expulsion of gut-dwelling helminths, the host immune response induces several different effector mechanisms. In addition to signals that activate the "epithelial escalator," the type 2 cytokines IL-4, IL-5, IL-9, and IL-13 contribute to increased muscle contractility, diarrhea, and changes to the

mucus that lines the gastrointestinal tract (Cliffe and Grencis, 2004; Cliffe et al., 2005; Hasnain et al., 2011; Herbert et al., 2009; Patel et al., 2009). However, the signals that directly regulate epithelial cell proliferation during helminth infection remain less well understood.

AREG is a well-characterized mitogenic stimulus in epithelial cell layers, making it a potential candidate that could contribute to epithelial cell responses following helminth infection. Consistent with this, AREG gene-deficient mice exhibit delayed clearance of the intestinal parasite *Trichuris muris*, which correlated with diminished proliferation of colonic epithelial cells compared to infected control mice, suggesting a key role for AREG in promoting tissue-protective epithelial responses in the intestine (Zaiss et al., 2006). Although the cellular sources of AREG remain undefined in this setting, enhanced proliferation of the epithelial cell layer in infected wild-type mice is dependent on CD4+ T cells, further highlighting the importance of the crosstalk between immune and epithelial cells in the regulation of the AREG-EGFR pathway.

Recent data have demonstrated important roles for AREG in the homeostasis of inflamed tissues and epithelial integrity during non-helminth lung infections (Monticelli et al., 2011; Turner et al., 2013). Although type 1 cytokine responses are necessary for resistance to influenza virus infection, a variety of type 2-associated cell types populate the lungs following pathogen clearance to repair and resolve infection-induced tissue damage and inflammation. For example, following influenza infection of *Rag1*−/− mice, AREG promotes the regeneration of bronchial epithelium, which enhances tissue integrity and survival of infected mice (Monticelli et al., 2011). Similarly, in the context of bacterial-viral co-infections, mice that had been previously infected with influenza rapidly succumb to a consecutive bacterial infection in the lungs (Jamieson et al., 2013). This effect is not due to an overwhelming pathogen burden but is instead associated with impaired tissue integrity and immunopathology. Co-infected mice exhibit increased necrosis in the airway epithelial cell layer, and genes involved with tissue repair are significantly downregulated in comparison to single-infected mice. However, co-infected mice can be rescued by administration of AREG into the lungs, with no associated influence on pathogen burden. Finally, fungal infection of Dectin-1 gene deficient mice induces severe pulmonary pathology compared to wild-type controls despite similar pathogen burdens, and treatment with recombinant AREG promotes epithelial repair and increased survival of Dectin-1-deficient hosts (Branzk et al., 2014). These data indicate that AREG is an important growth factor that promotes host tolerance by sustaining lung tissue integrity and homeostasis following infection-induced tissue damage. The cellular sources and signaling pathways that regulate AREG induction and its tissue protective effects outside the context of helminth infection require further investigation.

Amphiregulin Orchestrates Tissue Repair and Homeostasis

An important component of type 2 immune responses is the limitation and repair of acute tissue damage (Chen et al., 2012). Helminths migrating through vital organs, such as the lung and liver, can cause severe, acute tissue damage to the host. In a number of different settings, type 2 immune responses support tissue
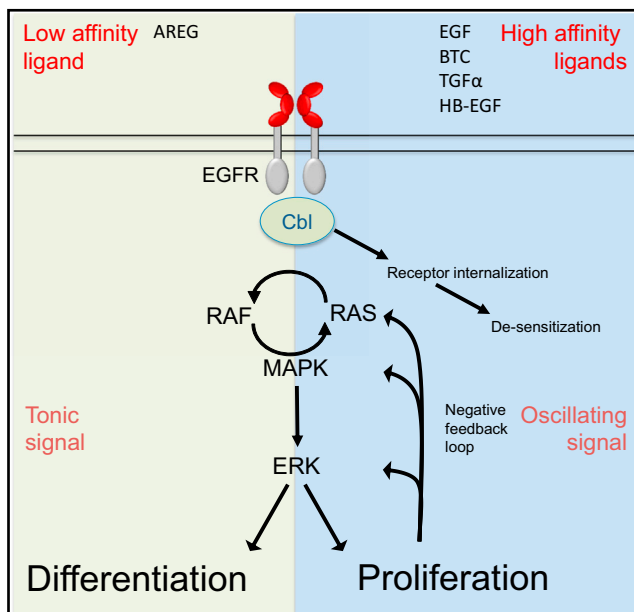
Figure 1. Amphiregulin Is a Bi-functional Growth Factor that Might Induce Either Cell Proliferation or Differentiation
The EGFR is a growth-factor receptor well known for its capacity to induce mitogenic stimuli. Nevertheless, it is also well established that the EGFR is important for the differentiation of specific cell types. In recent years, it has been revealed that binding characteristics of EGF ligands and qualitative differences in signaling downstream of the receptor dictate the dynamics of gene expression and the fate of recipient cells. Sustained signals downstream of the receptor induce stable ERK activation and leads to growth arrest and cell differentiation, while an oscillating signal downstream of the receptor induces a repetitive activation and inactivation of ERK, leading to proliferation of the target cell. AREG is distinct from most other EGF-like growth factors in that it can not only induce a mitogenic signal but can also induce cell differentiation. This is explained by a single amino acid difference in the receptor-binding domain, which decreases the stability of the AREG-EGFR. Such interaction fails to induce receptor internalization and enables AREG to induce a sustained signal downstream of the receptor. In contrast, most other EGFR ligands, such as EGF, TGF-α, or HB-EGF, bind EGFR with high affinity and thereby induce internalization and degradation of the receptor, and induce negative-feedback loops within the MAP-kinase signaling cascade. This activation and termination of an induced signal by the high-affinity ligands of the EGFR in effect lead to a ligand-specific oscillation of the MAP-kinase signaling pathway and thus a mitogenic signal in the recipient cell.

repair, such as in the lungs (Chen et al., 2012; Loke et al., 2007), the gut (Seno et al., 2009), and the skin (Lucas et al., 2010). Helminth-induced type 2 inflammation is characterized by the activation of a number of innate immune effector cell types, including ILC2s, alternatively activated (M2) macrophages and eosinophils. Although these populations have important roles in pathogen resistance, emerging data suggest that they might also play important roles in tissue repair. Importantly, tissue damage itself can provide activation signals for these cell types. Following cellular stress or tissue damage, adenosine is released at the site of inflammation, which can induce upregulation of the alarmin IL-33 and enhance the development of tissue-protective M2 macrophages and eosinophils at the site of parasite infection (Patel et al., 2014). IL-33 is a potent activator of ILC2s, an innate source of AREG, and contributes to M2 differentiation. It has been suggested that M2 macrophages, which can limit acute inflammation and directly promote tissue repair, can mitigate

acute lung injury during worm infections through expression of Arginase and the epithelial mitogen IGF-1 (Chen et al., 2012). Notably, the mitogenic activity of IGF-1 is context-dependent in that IGF-1 alone does not induce epithelial cell proliferation but can enhance EGFR signaling and proliferative responses in the presence of EGF family members (Worster et al., 2012). Furthermore, IGF-1 can induce AREG expression in keratinocytes that then contributes to the mitogenic effect of IGF-1 (Rodland et al., 2008; Vardy et al., 1995). Collectively, these data indicate the possibility that the observed potent mitogenic stimulus for epithelial cells at the site of infection might be induced by coordinated expression of AREG and IGF-1. Thus, immune cells activated by tissue damage and the type 2 immune response might influence tissue repair and homeostasis through AREG-dependent pathways. The extent to which this induced mitogenic stimulus is a direct effect of IGF-1 or in part mediated by IGF-1 induced AREG in vivo is undefined and warrants further investigation.

In the context of muscle injury, a similar reparative role of innate type 2 immune responses and AREG has recently been shown (Burzyn et al., 2013; Heredia et al., 2013). Upon muscle injury, eosinophils rapidly infiltrate the wound and eosinophil-derived IL-4 induces the proliferation of fibro/adipogenic progenitors (FAPs), thereby preventing their differentiation into adipocytes. In this way, FAPs supported myogenesis and the regeneration of skeletal muscle after injury (Heredia et al., 2013). Further, eosinophil influx is associated with accumulation of a distinct subpopulation of CD4+ Treg cells at the site of muscle injury (Burzyn et al., 2013). These muscle-resident Treg cells have a characteristic gene expression pattern and a restricted TCR repertoire that distinguishes them from circulating Treg cells. Furthermore, these muscle-resident Treg cells express AREG, which can enhance the myogenic differentiation of skeletal muscle satellite cells in vitro. In vivo, muscle healing could be enhanced by injection of recombinant AREG into the injured muscle, while healing was inhibited by Treg cell depletion (Burzyn et al., 2013). These data indicate that innate immune responses as well as Treg cell-derived AREG play an important role through various mechanisms in muscle regeneration after injury.

AREG is well-suited for these diverse roles in tissue repair, because it is distinct from most other EGF-like growth factors in that it can not only induce a mitogenic signal but can also induce cell differentiation. This unexpected activity of a "growth factor" is the basis for the name "Amphi"-regulin, as this factor induces proliferation in some cell lines, while inducing growth arrest and differentiation in others (Shoyab et al., 1988). While EGFR signaling mechanisms have been reviewed comprehensively elsewhere (Arteaga and Engelman, 2014; Avraham and Yarden, 2011), how AREG might mediate differential effects of EGFR signaling to promote tissue homeostasis is a key area of investigation. The demonstration that AREG binds the EGFR with unusually low affinity (Jones et al., 1999) and displays differential binding capacity for the EGFR compared to other EGFR ligands (Macdonald-Obermann and Pike, 2014) helps to explain how AREG can mediate distinct biological outcomes compared to other EFG-like molecules (Figure 1). Due to a single amino acid difference in its receptor-binding domain compared to other EGF-family members, AREG forms an unstable interaction with

the receptor (Adam et al., 1995) and fails to induce receptor internalization (Stern et al., 2008), leading to sustained downstream signaling. This is in contrast to other EGFR ligands, which bind EGFR with high affinity and thereby induce a rapid internalization and degradation of the EGFR, and associated negative feedback loops within the MAP-kinase signaling cascade (Avraham and Yarden, 2011).

This activation and termination of an induced signal by the high-affinity ligands of the EGFR in effect leads to a ligand-specific oscillation of the MAP-kinase signaling pathway (Shankaran and Wiley, 2010). Such a qualitative difference in signaling directly dictates the dynamics of gene expression (Purvis and Lahav, 2013). In fibroblasts, the frequency of the EGFR-induced oscillation of ERK directly correlates with the strength of the induced proliferation signal: the higher the ligand concentration, the higher the pulse frequency and therefore the higher the induced proliferation (Albeck et al., 2013). However, low-affinity binding by AREG fails to induce this oscillating signal, and sustained, stable activation of ERK is not growth-inducing, but induces growth arrest and cell differentiation (Imayoshi et al., 2013; Marshall, 1995; Pasic et al., 2011; Traverse et al., 1992). As a consequence, AREG induces differentiation in a variety of different cell lines. Neuronal PC12 cells, for instance, are induced to proliferate in the presence of EGF and to differentiate in the presence of AREG (Kimura and Schubert, 1992). Notably, kidney-derived MDCK cells (Chung et al., 2005a), mammary epithelial cells (Mukhopadhyay et al., 2013), and myoepithelial cells (Pasic et al., 2011) follow a similar pattern, suggesting conservation of this signaling pathway across multiple cell types. Because AREG can be rapidly expressed at the site of tissue damage by infiltrating leukocytes, the capacity of AREG to induce differentiation of tissue resident precursor cells, such as satellite cells in the muscle, might substantially contribute to tissue repair in a wide range of different organs.

Amphiregulin Contributes to Fibrosis

A critical aspect of wound repair is the deposition of connective tissue proteins, such as collagen and fibronectin, in and around an open wound. Several different cell types, including peripheral blood circulating fibrocytes, contribute to the deposition of connective tissue proteins into the wound (Suga et al., 2014). As a result, a layer of differentiated myofibroblasts can quickly develop at localized sites of injury where they produce collagen in order to stabilize and contract the wound, allowing for efficient healing. However, in situations of chronic inflammation, uncontrolled wound repair mechanisms, including unrestrained myofibroblast differentiation and activation and excessive collagen deposition, can lead to fibrosis and impaired organ function (Allen and Wynn, 2011; Gause et al., 2013).

Accumulating evidence suggests that dysregulation of the dialog between the immune system and EGFR signaling pathways can contribute to pathological fibrosis in the context of chronic inflammation. The immune cell-derived cytokines IL-13 and TGF-β have been demonstrated to potentiate collagen deposition and fibrosis in the lung, liver, skin, and intestine (Ask et al., 2008; Chiaramonte et al., 1999; Desmoulière et al., 1993; Hamid et al., 1994; Zhou et al., 2012), and attempts to therapeutically target these signaling pathways have revealed TGF-β-dependent and -independent mechanisms of fibrotic

disease (Kaviratne et al., 2004; Lee et al., 2001). For example, IL-13 normally signals through a heterodimeric receptor composed of the IL-4Rα and IL-13Rα1 subunits, and attempts to inhibit IL-13 signaling using a soluble high-affinity IL-13Rα2 fusion protein as a decoy receptor have achieved promising results in diminishing hepatic and pulmonary fibrosis (Chiaramonte et al., 2003; Zheng et al., 2008). However, recent studies have demonstrated that IL-13 signaling through cell-surface-expressed IL-13Rα2 on macrophages can stimulate TGF-β expression to promote myofibroblast differentiation and fibrosis (Brunner et al., 2013; Fichtner-Feigl et al., 2006). Notably, TGF-β can regulate AREG expression (Bennett et al., 1992), and AREG has been ascribed a critical role in the development of TGF-β-induced pulmonary fibrosis (Zhou et al., 2012). Whether IL-13 signaling can lead directly to AREG induction through TGF-β remains to be addressed. Collectively, these data indicate that immune pathways regulate multiple pro-fibrotic mechanisms and provoke the hypothesis that this might be achieved at least in part through AREG-EGFR-mediated effects on myofibroblasts.

Pathological fibrosis can be considered an unrestrained wound healing response and can involve activation of immune cells typically associated with type 2 immune responses including ILC2 and M2 macrophages. ILC2s have been identified as major drivers of liver, skin, and pulmonary fibrosis (Chang et al., 2011; Hams et al., 2014; McHedlidze et al., 2013; Salimi et al., 2013), and ILC2 express both IL-13 and AREG (Monticelli et al., 2011), suggesting that ILC2-derived factors might also mediate induction or progression of inflammation-associated fibrosis. Indeed, the recent demonstration that a lipid mediator, Maresin1, can limit ILC2-derived IL-13 and pulmonary inflammation supports this hypothesis (Krishnamoorthy et al., 2014). While ILC2-derived AREG might substantially contribute to protective tissue remodeling following influenza-induced damage, its role in fibrosis under chronic inflammatory conditions remains undefined. Notably, EGFR inhibitors can ameliorate symptoms of fibrotic disease in mice (Le Cras et al., 2011; Liu et al., 2012; Vargaftig and Singer, 2003). Identification of the EGFR ligands that support fibrosis, and defining the role of immune-derived AREG in this process, remains an area of ongoing investigation that might reveal new targets for therapeutic intervention.

Amphiregulin and Tregs Can Suppress Local Inflammation

Successful wound repair requires efficient resolution of local inflammation (Martin and Leibovich, 2005) as dysregulation of pathogen-elicited immune responses carries the risk of inducing collateral tissue damage. Thus, efficient resolution of local inflammation is necessary to prevent excessive wound-healing responses and tissue fibrosis. An important cell type associated with type 2 immune responses that contributes to local immune suppression is the mast cell (de Vries and Noelle, 2010). Mast-cell-derived IL-10, for example, has a prominent immune regulatory function in a number of different settings (Grimbaldeston et al., 2007; Leveson-Gower et al., 2013). Furthermore, efficient induction of immune tolerance can involve collaboration between mast cells and Treg cells (Lu et al., 2006), potentially due to the role of mast-cell-derived AREG in supporting Treg cell function (Zaiss et al., 2013). In the absence of mast-cell-
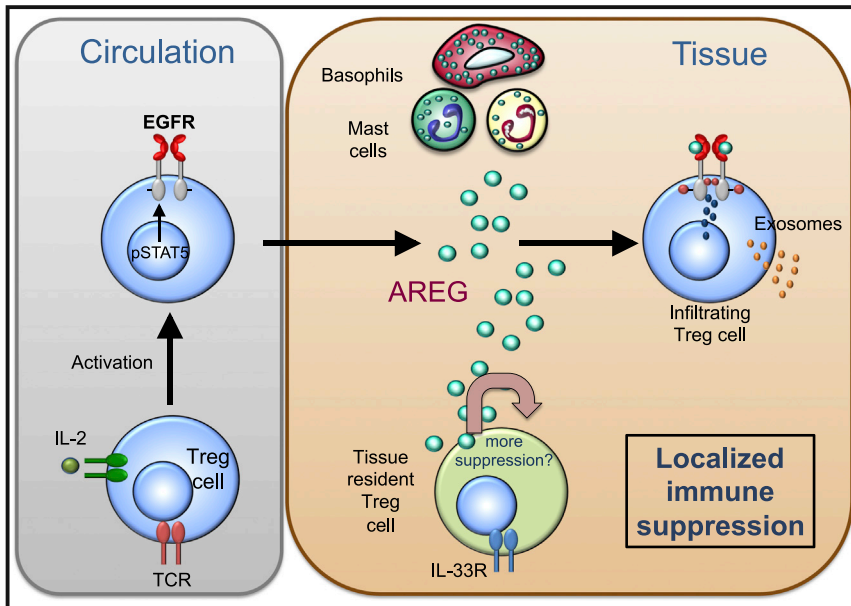
Figure 2. Amphiregulin Enhances Regulatory T Cell Function
AREG has been shown to be of critical importance for efficient CD4+ regulatory T (Treg) cell function, and in the absence of AREG, CD4+ Treg cells cannot suppress local inflammation. As CD4+ Treg cells become activated, they upregulate the EGFR, in part via STAT5-mediated signaling. These activated regulatory T cells migrate to the site of inflammation, where they are exposed to AREG, which enhances their suppressive capacity, possibly through secretion of immunosuppressive exosomes. Depending on the type and the site of inflammation, CD4+ Treg cells are dependent on different cell types for AREG; in chronic inflammation, mast-cell-derived Amphiregulin enhances CD4+ Treg cell function, whereas in an acute inflammatory response, basophils are described as the main source of AREG. In addition, some organs contain tissue-specific CD4+ Treg cells. In response to inflammation, these tissue-resident Treg cells can produce Amphiregulin that might act in an autocrine fashion to enhance their suppressive capacity. The extent that AREG derived from these different cell types contributes to immune regulation within inflamed tissues is an ongoing area of study.

derived AREG, Treg cells fail to suppress the development of colitis in a T cell transfer induced mouse colitis model, are unable to induce an immunosuppressive environment in a mouse tumor vaccination model and fail to suppress dermal inflammation (Zaiss et al., 2013). Furthermore, the development of dermatitis could be reversed by the co-transfer of bone-marrow-derived mast cells differentiated from wild-type, but not AREG-deficient bone marrow (Zaiss et al., 2013). Together, these data demonstrate that mast cells can be a critical source of AREG and can promote CD4+ Treg cell-mediated suppression of localized immune responses (Figure 2).

Recent studies have revealed a potential molecular mechanism through which AREG promotes Treg cell function (Okoye et al., 2014). Treg cells can secrete exosomes that transfer immunosuppressive micro-RNAs (miRNAs) from Treg cells to effector T cells, and AREG enhances the secretion of such exosomes (Okoye et al., 2014). The source of AREG that enhances Treg cell function appears to be dependent on the site and the type of the immune response. In addition to mast cells, basophils can also be an important source of AREG. In the context of contact hypersensitivity reactions in the skin, basophil-derived and not mast cell-derived AREG was essential for enhanced Treg cell function (Meulenbroeks et al., 2015). Taken together, these findings reveal a mechanism by which mobilized cells of the innate immune system use AREG to enhance the suppressive capacity of Treg cells to dampen local immune responses and to induce peripheral tolerance. These data also raise the possibility that the specific tissue microenvironment where inflammation is localized might determine the source of AREG and influence its function.

The recent finding that tissue-resident Treg cell populations express AREG themselves (Burzyn et al., 2013) suggests that these unique Treg cell populations might possess an autocrine positive feedback loop. Such a mechanism would release tissue-resident Treg cells from the delay associated with leukocyte-derived AREG that is dependent on the influx of cells

into the site of tissue damage. The degree to which Treg-cell-derived AREG directly contributes to wound healing and indirectly contributes to resolution of local inflammation remains undefined.

Amphiregulin Promotes Immune Suppression in the Tumor Microenvironment

Immune-mediated tolerance mechanisms such as limitation of inflammation and tissue repair extend beyond mediating the host response to pathogens. Tumor antigens also elicit immune responses that promote tolerance, which can allow tumors to grow unchecked but can also limit inflammation and collateral damage to healthy tissues surrounding the tumor microenvironment. For instance, a number of factors have been associated with the progression of epithelial-associated solid tumors, including local accumulation of CD4+ Th2 cells, M2 macrophages, and expression of IL-13 and TGF-β (Jovanovic et al., 2014; Palucka and Banchereau, 2012; Palucka et al., 2013). Epithelial-associated solid tumors also very commonly express the EGFR and are associated with local expression of EGF ligands, including AREG (Ciardiello and Tortora, 2008; Herbst et al., 2001; Shao et al., 2003; Yamada et al., 2008). While the role of AREG in anti-tumor immunity and tolerance remains unclear, the wide clinical use of EGFR antagonists for the treatment of a number of metastatic epithelial cancers suggests that AREG might play a critical role in orchestrating responses to tumors.

Despite the underlying idea that many epithelial tumors are dependent on EGFR-mediated signals for their growth, treatment of cancer patients with EGFR antagonists is associated with multiple side effects, such as skin rashes, fever, stomatitis, and diarrhea (Ciardiello and Tortora, 2008), suggesting the involvement of off-target effects. Because the EGFR is of central importance for the homeostasis and integrity of the skin, inhibition of this pathway might underlie the loss of dermal barrier function (Lichtenberger et al., 2013; Mascia
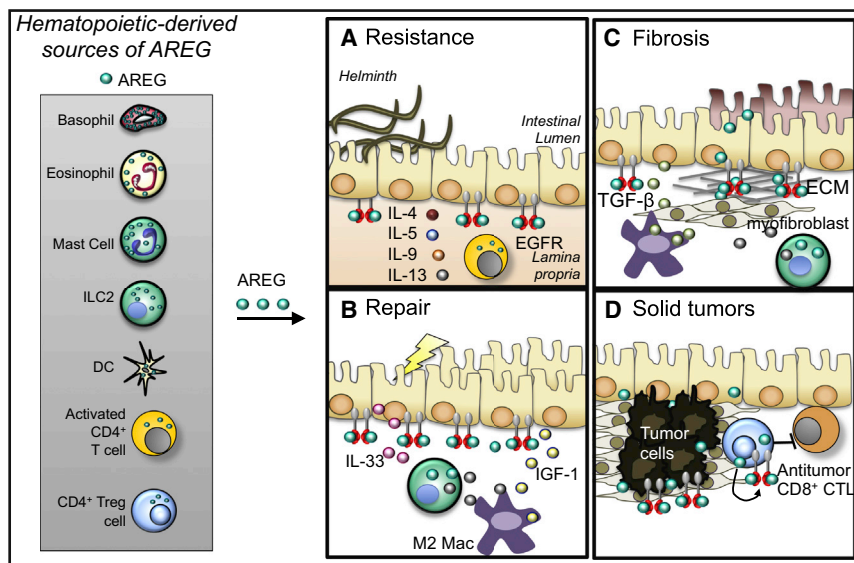
Figure 3. Leukocyte-Derived Amphiregulin in Health and Disease

Recent evidence implicates hematopoietic-derived AREG expression as an important component of pathogen resistance and tissue tolerance mechanisms. Type 2-associated immune cells of both the innate and adaptive lineages have been reported to express AREG.

(A) In the context of helminth infection, CD4+ T cells and AREG promote epithelial cell turnover to expedite helminth eradication, although the specific cellular sources of AREG in this process remain undefined.

(B) Tissue damage can cause upregulation of the alarmin IL-33, a strong positive stimulus for ILC2 accumulation and cytokine production that can support tissue-protective M2 macrophage differentiation. Recent data suggest the possibility that ILC2, M2 macrophages, AREG, and IGF-1 might collaborate to promote wound healing at mucosal barrier surfaces.

(C) Uncontrolled wound-healing responses can result in pathologic tissue fibrosis. It is possible that the same cells (ILC2 and M2 macrophage) and the same cytokines (IL-13 and AREG) that promote tissue repair can become dysregulated in the presence of chronic inflammation and an altered cytokine milieu to promote fibrotic responses, including myofibroblast differentiation, proliferation, and ECM deposition.

(D) Epithelial-associated solid tumor microenvironments can have high local concentrations of AREG and tumor infiltrating EGFR+ CD4+ Treg cells. Treatment with EGFR antagonists or Treg-cell-depleting agents can promote tumor regression. Tissue-resident CD4+ Treg cells can express and respond to AREG, suggesting the possibility that in addition to growth factor starvation of the tumor, EGFR antagonists might function in part to inhibit local CD4+ Treg cell function, thereby alleviating immunosuppression and enhancing antitumor immune responses, including the activity and function of tumor specific CD8+ cytotoxic T lymphocytes (CTLs). Much work remains to define the cellular sources of AREG and the mechanisms regulating its expression in inflamed tissues. Increased understanding of these pathways could reveal new therapeutic targets for multiple disease states.

et al., 2013). However, the severe skin reactions might also be associated with a general dysregulation of the immune system. In particular, the skin appears very sensitive to immune dysregulation (Dudda et al., 2008), and Treg cell dysfunction in IPEX children normally first manifests itself in skin rashes (Halabi-Tawil et al., 2009). Thus, it is possible that EGFR antagonists could function in part via the suppression of local Treg cell populations.

The significance of Treg cell function in downregulating immune defenses against tumors has been demonstrated in mice (Li et al., 2010). The underlying mechanism of CTLA-4 blocking antibodies in enhancing tumor immune responses in cancer patients remains controversial, as inhibition of CTLA-4 might both enhance anti-tumor effector T cell function and impair Treg cell function and thereby limit local immunosuppression. However, recent studies suggest that CTLA-4 blocking antibodies function mainly via the depletion of Treg cells within the tumor microenvironment (Simpson et al., 2013). These data indicate that in cancer patients, sustained Treg cell activity suppresses anti-tumor immunity. Thus, an effect on Treg cell function could also explain some of the inflammatory side effects reported by patients treated with EGFR antagonists. Such a mechanism might also explain a number of somewhat enigmatic clinical observations associated with EGFR antagonist treatment. A striking observation in patients treated with EGFR antagonists is that tumor regression rates did not correlate with the expression of the targeted molecule (i.e., EGFR) on the responsive tumor cells (Burtness et al., 2005; Kim et al., 2008). The EGFR is upregulated in a feedback loop, leading to the expectation that tumors overexpressing the EGFR would be particularly vulnerable to EGFR antagonism and growth factor

starvation (Herbst et al., 2001). However, in some cases, even tumors that fail to express detectable amounts of the EGFR respond in clinically objective ways to monoclonal antibody therapy (Chung et al., 2005b). Collectively, these data suggest that EGFR antagonists might restore the function of anti-tumor immune responses by interfering with local immunosuppressive EGFR-responsive Treg cells, although this hypothesis remains to be formally tested.

Recent experimental data in a murine model support the hypothesis that EGFR inhibitors can relieve local Treg cell immunosuppression and activate secondary antitumor immunity following tumor immunization (Zaiss et al., 2013). Immunization of B16 tumor-bearing mice with DCs loaded with a peptide derived from the immunogenic tumor antigen TRP2 induces a CD8+ T cell response and tumor rejection in AREG gene-deficient mice, but not in C57BL/6 wild-type mice. Treatment of tumor-bearing mice with EGFR blocking antibody following immunization with peptide-pulsed DCs also induces tumor rejection in wild-type mice. Notably, B16 melanoma cells do not express the EGFR but are dependent on Treg cell function for the induction of a tumor-intrinsic immunosuppressive environment (Sutmuller et al., 2001). Together, these data suggest that the absence of AREG-EGFR signaling enhances immunization-induced tumor specific CD8+ T cells through relief of Treg cell-mediated suppression (Zaiss et al., 2013). These experimental findings provoke the hypothesis that Treg cell function might also be diminished in cancer patients undergoing EGFR antagonist treatment. Conservation of this pathway is suggested by the finding that in a murine model of hepatitis B-virus infection, elevated hepatic AREG expression is associated with the presence of hyper-suppressive

EGFR$^+$ CD4$^+$ Treg cells that could limit antiviral CD8$^+$ T cell function (Dai et al., 2014). Further research examining the extent to which EGFR antagonists can inhibit the function of local CD4$^+$ Treg cell populations could impact therapeutic treatment of multiple disease states, including chronic viral infection and cancer.

Concluding Remarks and Future Perspectives

The immune system contributes to homeostasis by promoting host-protective mechanisms against a variety of harmful insults ranging from toxins, venoms, and allergens to cancerous cells and infectious agents. Immune-mediated resistance and tolerance mechanisms, the latter involving both the control of harmful inflammation and direct contributions to enhanced wound healing and tissue repair, are key components of the host-protective response. It is becoming clear that in many cases the same cell populations and even the same specific molecules can simultaneously contribute to the immune functions that control resistance and tolerance, as well as development or progression of fibrosis and cancer. In the context of type 2 inflammation elicited by heterogeneous stimuli, AREG is one such molecule (Figure 3). The ability of AREG to induce epithelial cell proliferation and differentiation can promote helminth expulsion in the context of a polarized type 2 response and enhance wound healing and tissue repair following infection or injury. The effects of AREG on Treg cells might also down-modulate harmful inflammation but at the same time potentiate tumor growth.

The demonstration that immune cells can express AREG and contribute to tissue homeostasis suggests that this is an important component of the crosstalk between immune and epithelial cells. Continued investigation of the signals that elicit immune-derived AREG, the mechanisms regulating EGFR-mediated ligation and signaling and development of new genetic tools to determine the cellular sources of AREG in vivo will greatly increase our understanding of the role the AREG-EGFR pathway plays in the context of resistance to infection and in tissue tolerance. Addressing these gaps in knowledge has the potential to inform development of new therapeutic interventions for immunity, chronic inflammation, and cancer.

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