Arch. Immunol. Ther. Exp. (2014) 62:41–45
DOI 10.1007/s00005-013-0255-9

REVIEW

The Reverse-Direction Method Links Mass Experimental Data to Human Diseases

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Received: 26 April 2013 / Accepted: 21 August 2013 / Published online: 31 August 2013

Abstract Genome-wide analyses such as DNA microarray, RNA sequencing and RNA interference-based high-throughput screening are prevalent to decipher a biological process of interest, and provide a large quantity of data to be processed. An ultimate goal for researchers must be extrapolation of their data to human diseases. We have conducted functional genome-wide screenings to elucidate molecular mechanisms of the inflammation amplifier, a NFκB/STAT3-dependent machinery that potently drives recruitment of immune cells to promote inflammation. Using a public database of genome-wide association studies (GWAS), we recently reported the reverse-direction method by which our mass screening data were successfully linked to many human diseases. As an example, the epiregulin–epidermal growth factor receptor pathway was identified as a regulator of the inflammation amplifier, and associated with human diseases by GWAS. In fact, serum epiregulin levels were higher in patients with chronic inflammatory disorders. The reverse-direction method can be a useful tool to narrow mass data down to focus on human disease-related genes.

Keywords Inflammation amplifier · IL-17 · IL-6 · NFκB · STAT3 · Chronic inflammation · Human diseases and disorders

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Introduction

Inflammation has traditionally been defined as symptoms with dolor, calor, rubor and tumor (Latin for pain, heat, redness and swelling, respectively), which can now be redefined as a local accumulation of immune cells and soluble mediators including cytokines and chemokines. Inflammation acts as a defense mechanism against pathogenic microorganisms and tissue injury, but it has to resolve after these incidences come to an end. Chronically prolonged inflammation has an adverse effect. Increasing evidence suggests that chronic inflammation is involved in the pathogenesis of not only autoimmune diseases, but also many other types of disorders including neurodegenerative diseases, metabolic syndromes and even psychiatric diseases (Hamdani et al. 2012; Tabas and Glass 2013). Thus, elucidation of molecular mechanisms underlying chronic inflammation is very important to develop novel therapeutic interventions to these diseases. As mentioned, inflammation can be considered as a consequence of immune cell accumulation. Chemokines, which are a soluble factor that comprises about 50 members, have a pivotal role for the immune cell recruitment (Allen et al. 2007). Dysregulated chemokine expression is therefore a potential cause of unwanted inflammation. We have discovered a cellular machinery that produces a large quantity of chemokines and cytokines, which we named the inflammation amplifier (Fig. 1) (Arima et al. 2012; Lee et al. 2012, 2013; Murakami and Hirano 2012; Murakami et al. 2011; Ogura et al. 2008). Genome-wide screenings have recently uncovered regulators and target molecules of the amplifier (Murakami et al. 2013). In order to link our screening results in an animal to human diseases, we utilized the genetic association database, a public genome-wide association studies (GWAS) database by the National Institute of Health.

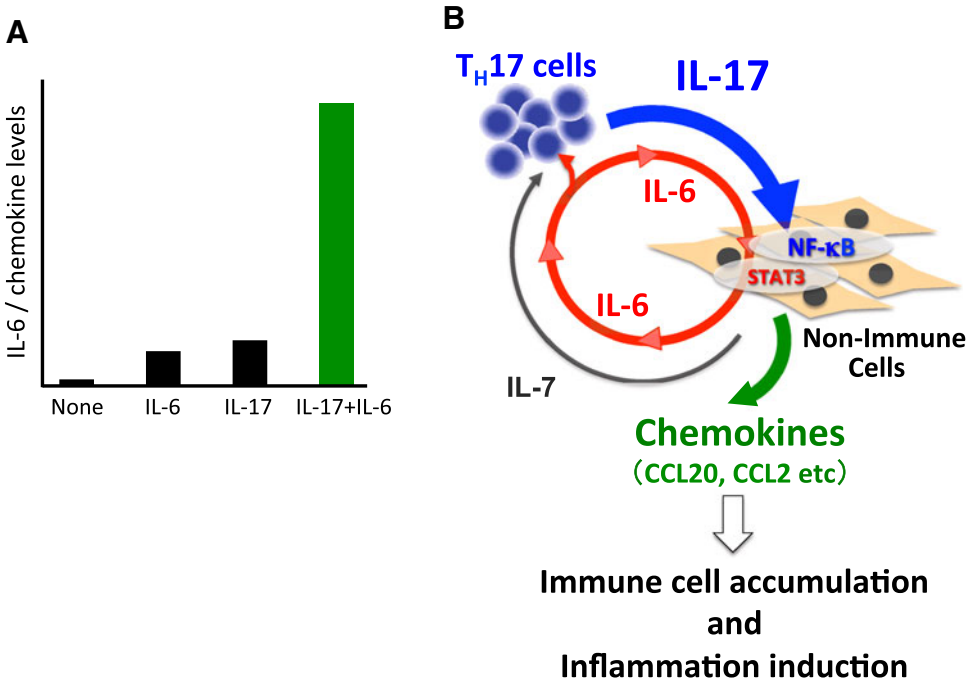

Fig. 1 The inflammation amplifier. **a** A combination of IL-17 and IL-6 produces a synergistic effect on the induction of inflammation-amplifier target chemokines in non-immune cells including fibroblasts, endothelial cells and certain synovial cells. This is a qualitative image of the inflammation amplifier targets. **b** The synergistic effect requires the concomitant activation of two transcription factors, NFκB and STAT3. In the case of autoimmune diseases, Th17-derived IL-17 induces an initial, low amount of IL-6 from non-immune cells via NFκB activation. Secreted IL-6 together with IL-17 activates the inflammation amplifier, producing an excessive amount of target genes including many inflammatory chemokines such as CCL20, CCL2, etc. Consequently, various immune cells are recruited and inflammation occurs. Persistent activation of the inflammation amplifier, as is the case in F759 mice, drives a chronic inflammation. IL-7 derived from non-immune cells aids in the proliferation and survival of Th17 cells

The Inflammation Amplifier

Development of autoimmune diseases involves a complex biological process with chronic inflammation. A suitable animal model is required for understanding the pathogenesis but it is rare. We have created a genetically modified mouse strain, F759 mice, that transmits excessive IL-6 signaling due to a lack of negative regulatory circuit, and spontaneously develop rheumatoid arthritis-like joint disease as they age (Atsumi et al. 2002). According to the genetic association database, IL-6 is linked with various human diseases, offering a rationale to study a molecular mechanism behind the F759 arthritis. In F759 mice, the number of memory/activated $CD4^+$ T cells dramatically increased with age. In addition, when F759 mice were crossed with CD4-deficient or MHC class II-deficient mice, the disease development was significantly suppressed (Sawa et al. 2006). These observations suggest that excessive IL-6 signaling in F759 mice could have a role in inducing autoimmune arthritis through activation of $CD4^+$ T cells. Because IL-6 is known to regulate the immune system, we considered at first that IL-6 directly activates immune cells for the disease development, but surprisingly found that IL-6 in fact inactivates these cells (Atsumi et al. 2009; Kitamura et al. 2005; Park et al. 2004). Subsequent bone-marrow chimera experiments demonstrated that wild-type mice transferred with F759 bone marrow did not develop arthritis, whereas F759 mice that received wild-type bone marrow did. While the necessity of $CD4^+$ T cells, it was clearly demonstrated that the F759 mutation is required in non-immune cells for F759-arthritis development. At that time, it was a paradigm shift that non-immune cells control autoimmunity. Further studies have shown that the excessive IL-6 signaling in non-immune cells promotes the production of IL-7, a cytokine important for T cell proliferation and survival, and results in excess homeostatic proliferation of $CD4^+$ T cells. These results suggest that non-immune cells actively control the status of immune cells including $CD4^+$ T cells and dysregulation of this control can cause autoimmune diseases (Sawa et al. 2006).

Th17 cells and IL-17 have been shown to be involved in many autoimmune disease models and human diseases including rheumatoid arthritis (Korn et al. 2009). Consistent with this, serum IL-17 levels and Th17 cells were unusually high in aged F759 mice. When F759 mice were bred on an IL-17-deficient background, the arthritis was significantly suppressed. In addition, the overexpression of

IL-17 in F759 mice accelerated arthritis development. Moreover, serum IL-6 levels after IL-17 overexpression was significantly higher in F759 mice than in control mice, indicating a positive interaction between IL-17 and IL-6 signaling. In vitro experiments showed that a combination of IL-17 and IL-6 stimulation synergistically induces IL-6 and inflammatory chemokines in type I collagen^{+} non-immune cells such as fibroblasts and endothelial cells (Fig. 1a). This synergistic effect of IL-17 and IL-6 depends on transcription factors activated by these ligands, namely NFκB and STAT3. This synergism, which we named the inflammation amplifier in 2008, has been found essential to the pathogenesis of autoimmune diseases including F759-arthritis and experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis (Fig. 1b) (Ogura et al. 2008). In addition to autoimmunity, the inflammation amplifier is involved in a mouse model of graft rejection, and histological evidence of the amplifier is found in the lungs from a patient with complications after lung transplantation (Lee et al. 2012, 2013). Moreover, other NFκB and STAT3 stimulators such as TNFα, norepinephrine, IFNγ, etc. also activated the inflammation amplifier in endothelial cells, fibroblasts, or epithelial cells. Thus, the inflammation amplifier is a principal machinery of inflammation and is activated by the simultaneous stimulation of NFκB and STAT3 in non-immune cells. We have recently re-defined inflammation as local activation of the inflammation amplifier, which causes an accumulation of various immune cells and dysregulation of local homeostasis after the discovery of the inflammation amplifier. Acute inflammation is induced by transient activation of the inflammation amplifier, while chronic inflammation is induced by its chronic activation. It is reasonable that the inflammation amplifier turns off negative regulators of the two pathways such as A20, Cyld, IkBa, Pten, and Pias1 for NFκB and SOCS3, PTP1B, SHP1/2, and Pias3 for STAT3 during acute inflammation. Thus, the inflammation amplifier can be applied not only to autoimmunity disorders, but also to other inflammatory disorders in vivo. These results led us to identify regulators and target genes of the inflammation amplifier to better understand the molecular mechanism.

The Reverse-Direction Method

We conducted a functional genome-wide screening and DNA microarray experiments to identify those genes related to activation of the inflammation amplifier, finding approximately 1,700 genes (Murakami et al. 2013). When these genes were analyzed using a public database of GWAS, many genes in our lists were found to be associated with human diseases and disorders. The enriched disease categories are beyond autoimmune diseases and include metabolic syndromes, such as atherosclerosis and type 2 diabetes, and neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease (Fig. 2a) (Murakami et al. 2013). These results, then, could be interpreted to show that inflammation, which is induced by the inflammation amplifier, relates to various human diseases and disorders, and that IL-6 inhibitors could have wide-range therapeutic effects. Generally, finding single

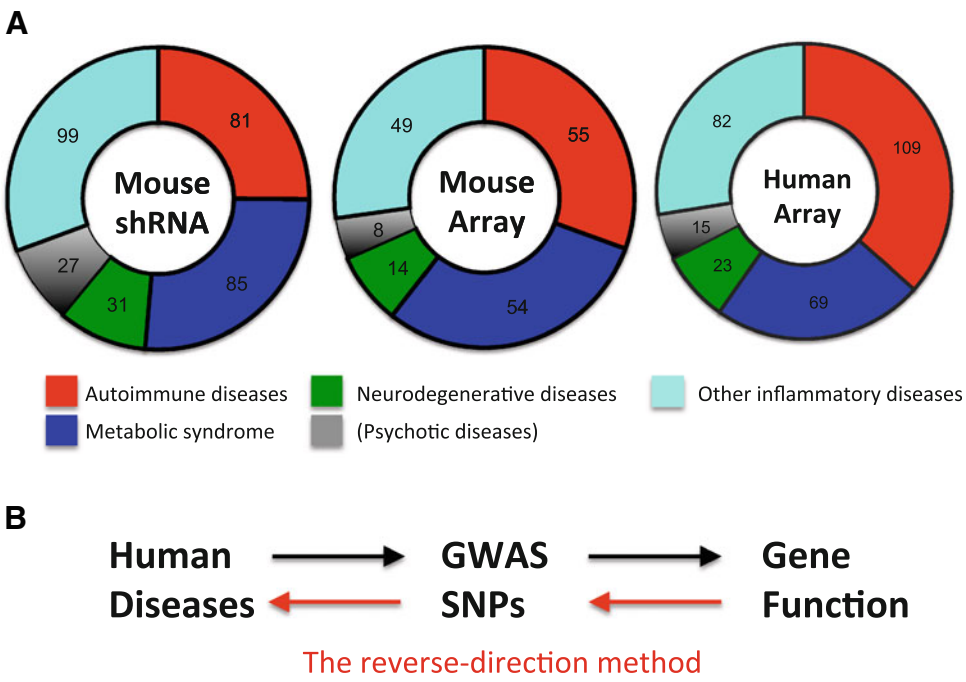

Fig. 2 The reverse-direction method. **a** Human disease association of the inflammation amplifier regulators revealed by the reverse-direction method. Genes associated with disease categories other than psychotic diseases showed statistically significant enrichment in the regulators and targets of the inflammation amplifier. Student's *t* tests (one or two tailed) were used for the statistical analyses of differences between groups. A *p* value of <0.1 was used as a cutoff for the bioinformatic results (Murakami et al. 2013). **b** A scheme of the reverse-direction method

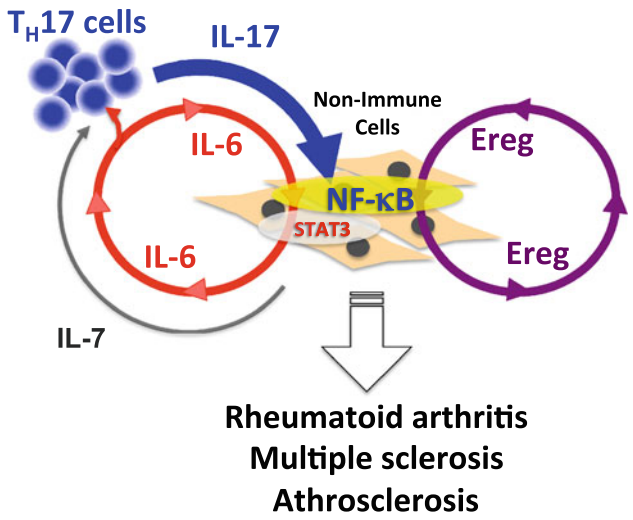

Fig. 3 Epiregulin augments the inflammation amplifier. Functional screenings revealed that epiregulin (Ereg) and EGFR (not depicted) are the positive regulators of inflammation amplifier through the enhancement of NFκB activation via PI3Kα pathway. Epiregulin is also identified as a target gene of the amplifier, suggesting a possible positive loop (*purple*). Epiregulin levels are higher in patients with rheumatoid arthritis, multiple sclerosis and atherosclerosis

nucleotide polymorphisms (SNPs) in patients and GWAS are followed by assessment of the function of genes identified. The process we took in our study was the other way around; biologically functional genes in the inflammation amplifier were identified first, followed by comparison with GWAS data (Fig. 2b). This reverse-direction method allows providing a global view of human disease association by comparing GWAS data with the large-scale screening data (Murakami et al. 2013).

The Epiregulin/EGFR Pathway as a Booster of the Inflammation Amplifier

We further investigated several genes identified in the screenings to validate the reverse-direction method. Epiregulin is a growth factor that signals through epiregulin–epidermal growth factor receptor (EGFR) (Pastore et al. 2008). Knock-down of epiregulin or EGFR gene significantly suppressed the inflammation amplifier, as expected from the first screening. In addition, exogenous epiregulin protein augmented chemokine expression driven by the amplifier in vitro. Interestingly, epiregulin was induced synergistically by the inflammation amplifier, suggesting the formation of a positive-feedback loop between the inflammation amplifier and epiregulin/EGFR pathway (Fig. 3). Furthermore, RNA interference-mediated knock-down of epiregulin or chemical inhibition of EGFR signaling at joints inhibited murine F759 arthritis in vivo. Mechanistically, epiregulin was found to enhance NFκB activity required for the inflammation amplifier through the PI3Kα pathway emanating from EGFR. Importantly, epiregulin levels in the serum from patients with rheumatoid arthritis, multiple sclerosis and atherosclerosis were significantly higher than those of healthy volunteers (Murakami et al. 2013). Moreover, the activation of EGFR was also evident in the clinical lung samples of graft rejection after lung transplantation (Lee et al. 2013).

We found several of the genes associated with human diseases in our screening lists are known to control NFκB or STAT3 activity. These include *Cckbr* (Ferrand et al. 2005), *Ets1* (Thomas et al. 1997), *Irak1* (Song et al. 2006), *Pde4* (Schmitz et al. 2007), *Pla2g1b* (Jo et al. 2004), *Pou2af1* (Wolf et al. 1998) and *Zbp1* (Kaiser et al. 2008). Furthermore, we identified many genes that had unknown function as necessary for NFκB- and/or STAT3-mediated signal transduction (data not shown). Human disease-linked NFκB and/or STAT3-driven genes such as *Mthfr* (Pickell et al. 2005), *Ptgs2* (D'Acquisto et al. 1997; Kojima et al. 2000) and *Vegfa* (Adachi et al. 2006; Rojo et al. 2004) were required for inflammation amplifier activation according to our functional screening. These results suggest a possible positive-feedback mechanism of inflammation amplifier activation through these molecules. These data strengthen the significance of the synergy between NFκB and STAT3 molecules in human diseases and highlight the amplifier as a fundamental mechanism for enhancing inflammation through synergistic activation of these two critical transcription factors in non-immune cells. Thus, the functional screening data from animal studies were successfully linked to human diseases by the reverse-direction method.

Our reverse-direction method is applicable when having identified potential molecular mechanisms from animal models to explain human disease. By considering SNP analysis (Lander 2011) and noting that recent GWASs have successfully identified SNPs that have individual susceptibilities to various pathologies, we established the reverse-direction method, which combines systemic screening and GWAS to identify which genes may potentially regulate the development of human diseases and disorders beyond the results of animal disease models. Moreover, as an example application of this new method, we have shown the results of those genes related to the inflammation amplifier activation. We found that the inflammation amplifier is indeed associated with a number of diseases and disorders beyond autoimmune ones by the reverse-direction method. The potential limitation of this new method the number of genes needed.

Conclusion

Extrapolation of animal study to human, or a translational research, is challenging but a necessary step to develop a

novel strategy for therapeutic interventions. The reverse-direction method is a useful tool to focus on promising disease-relevant genes, particularly when a large quantity of data has to be evaluated.

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