CYTOKINE NETWORKS: THE LANGUAGE OF THE IMMUNE SYSTEM

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Virtually every living creature has developed a means to fight the invasion of pathogenic microorganisms. Plants and invertebrates are endowed with a primitive, innate system of pathogen detection and eradication, whereas higher vertebrates and specifically mammals have a highly sophisticated system of cellular and humoral components dedicated to the recognition and eradication of pathogens and aberrant cells. The increasing complexity of this immune system requires that the different cell types involved in immunity have mechanisms in place to communicate with one another, in order to alert each other of potential threats. A predominant role in immune cell communication is played by cytokines. Cytokines are a family of soluble proteins, which permit cell-to-cell communication without the need for direct cell-to-cell contact. There are more than 100 known cytokines providing a basic alphabet for communication. Some of these cytokines have a very specific and dedicated function. For instance a subclass of chemotactic cytokines, broadly termed 'chemokines', are largely dedicated to the orchestration of cellular migration. Other cytokines specifically act as growth factors, activators, regulators, suppressors or mediators of cytotoxicity. Others are largely pleiotropic and induce a large range of cellular processes. This complexity is even more enhanced by the fact that different concentrations and combinations of cytokines can have synergistic or opposing effects on responder cells. Lastly, the cytokine receptors expressed by responder cells are also tightly regulated, which ultimately leads to a very complex communication system reminiscent in its breadth and combinatory depth to the human language. Over the past 16 years I have dedicated my efforts to understanding and translating this language to ultimately allow us to better comprehend the intricate functions of the immune system in health and disease. This will not be an all-encompassing review, but rather an opinion on a contemporary subject tainted with my personal views and biases stemming from my

experiences over the past few years. I will here focus primarily on a small set of molecules involved in the communication between T lymphocytes and antigen (Ag)-presenting cells in the context of cell-mediated immune responses and autoimmunity.

Evolution and Autoimmunity

As mentioned above, the immune system of primitive organisms can recognize the invasion of pathogens by the use of specific Ag-receptors. Perceived Ags are usually structures, which are 'different' to the host tissues. For example, bacteria have cell walls and specifically the cell walls of gram-negative bacteria contain a molecule called lipopolysaccaride (LPS). Eukaryotes on the other hand do not have cell walls but evolution provided even primitive eukaryotic organisms such as insects with Ag-receptors designed to recognize LPS and in turn activate phagocytic cells, which then clear invading bacteria either by ingesting them or by spraying them with toxic substances. This feature is called *innate immunity,* because these Ag-receptors, which permit the recognition of such foreign structures (cell walls, viral RNA, fungal glucans, etc.), are germ-line encoded, evolutionary conserved and usually expressed by specific phagocytic cells (mobile cells that can engulf and digest invading pathogens). We humans have inherited this innate immune system from our ancestors and also carry in our genome highly similar Ag-receptors, which also serve us in our defense against viruses and bacteria (1). But in addition, evolution provided higher vertebrates (starting with cartilaginous fish) with a versatile new system called *adaptive immunity* (2). Two additional cell types emerged and they are called B & T lymphocytes. These lymphocytes provide higher vertebrates with an amazingly versatile and diverse antigen-recognition machinery, which can provide the host with an immunological memory. Vaccination for instance is based on the ability of lymphocytes to swiftly respond to an Ag, which it has previously encountered either during an earlier infection or immunization with a vaccine. This is achieved through the rearrangement of the Ag receptor complex within the genomic DNA, generating up to 10^{16} different receptors and thus enabling lymphocytes to specifically recognize virtually every existing

constellation of protein structure. This marvelous adaptive immune system is the ultimate weapon against a pathogenic insult and even senses the emergence of cancer cells. However, it is also the reason why we can develop diseases in which lymphocytes perceive our own tissues as a threat and start an autoimmune response. My work is primarily dedicated to understanding the autoimmune disease multiple sclerosis (MS) in which the immune system specifically attacks the nerve-isolation in the brain (for review see (3)). As MS is primarily thought to be mediated by self-reactive T cells, I will now discuss the immune regulation and interactions leading to the activation of T cells, albeit at the expense of B lymphocytes which are responsible for their own set of autoimmune disease states.

Three signals to get T cells ready to 'march'

T lymphocytes emerge as precursor cells from the bone marrow (BM) and travel into the thymus where they undergo maturation, somatic recombination of the T cell receptor (TcR) loci and selection for successful gene-rearrangement. T cells develop into either $CD4^+$ T helper cells (T_H) or $CD8^+$ cytotoxic T cells (CTL). Again, for the sake of simplicity I will here focus on T_H cells, but most of the concepts discussed here similarly apply to CTLs. As for T_H cells, prior to their departure from the thymus into the peripheral immune system, thymic education limits the generation of auto-reactive TCRs. TCR specificities for self-tissues should lead to the death of the bearing immature T cell. T cells that leave the thymus should hence tolerate self-tissues and scan the body for anything 'non-self'. However, a small proportion of auto-reactive T lymphocytes inevitably escapes central tolerance, after which they wander into the periphery. Here they can be activated by auto-Ags such as proteins in the nerve insulation, as is the case in MS. This is true also for healthy individuals and the mere presence of auto-reactive T cells is no threat to health. However, in patients with autoimmune disease, these auto-reactive T cells turn into auto-aggressive T cells. So, the study of T cell activation allows us not only to develop a means to prevent unwanted immune responses, but also how to generate better immune responses (in the fight against cancer and infectious diseases).

All protein-Ags (foreign and self) are processed into peptides by so-called Ag-presenting cells (APCs) and presented on the cell surface bound to major histocompatibility complex (MHC)-molecules. APCs are key players during both the initiation and progression of the autoimmune response. During an immune reaction, APCs provide three signals that are required for the activation of Ag-specific T cells (see **Figure 1**). The first signal involves the presentation of Ag on the surface of an MHC class II molecule that allows T cell recognition of their cognate antigen through the T cell receptor. In order for Ag-specific T cells to become activated and expand, a second signal must be

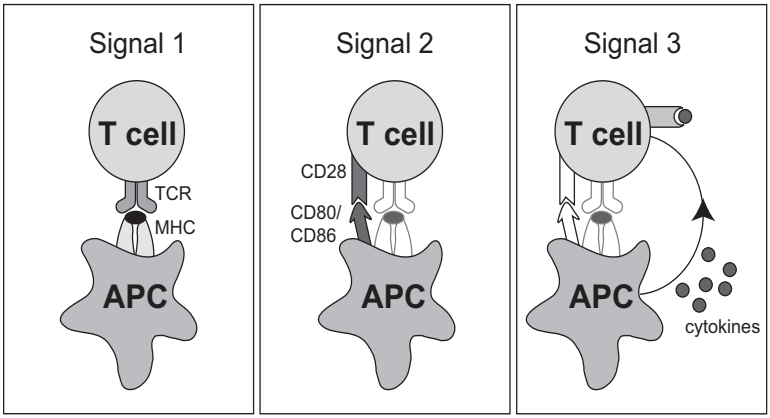

Figure 1: Three signals to start a T cell
T cells only recognize their cognate Ag, when presented in the context of an APC expressing MHC molecules. This signal is an absolute requirement for T cells to become activated. Signal 2 is represented by co-stimulatory molecules which if sufficiently strong will initiate T cell activation. If co-stimulation is absent, the T cell is taught to 'tolerate' the presented Ag. Signal 3 represents the cytokine environment and APC-derived cytokines further direct and polarize the T cells towards a certain effector type. (Modified from Gutcher and Becher, J.Clin. Invest. 2006).

generated through the interaction of adhesion and co-stimulatory B-7 molecules that are present on the APC with the cognate receptor, CD28, on the surface of interacting T cells. The third signal is the secretion of cytokines by APCs that further direct the differentiation of activated

Ag-specific lymphocytes into an effector T cell subtype. The creation of a particular cytokine environment by APCs during immunity is critical for the determination of the appropriate type of immune response. Consistently, during an autoimmune reaction, the development of potentially autoreactive T cells into actively pathogenic effector cells relies critically upon the secretion of soluble cytokines by APCs (For review see (4)). Thus the capacity of cytokines to polarize and instruct T cells is of significant importance as it endows them with the potential to either promote or suppress the development of autoimmune disease.

T_H polarization

20 years ago a model was proposed whereby T_H cells are subdivided into two independent subsets with distinct functions. It was discovered that T_H cells can be segregated into T_H1 and T_H2 subsets on the basis of cytokine expression and bioactivities. T_H1 cells secrete predominantly interferon-g (IFNγ) and control cell-mediated functions such as the activation of macrophages, while the secretion of IL-4 by T_H2 cells leads to the stimulation of B cells to secrete antibodies. Thus, while T_H1 cells induce such pro-inflammatory responses as are involved in autoimmunity, delayed type hypersensitivity, and the elimination of intracellular infections, T_H2 cells dominantly mediate allergic reactions and anti-helminth responses. The differentiation of lymphocytes into T_H1 or T_H2 cells is absolutely dependent upon the production of cytokines by APCs (4). The dominant activity of a precise set of cytokines ultimately results in a specific immune response by instructing T_H cells how to behave. APCs thus bridge the innate and adaptive arms of immunity. Among the most prominent cytokines secreted by APCs is Interleukin (IL)-12, which is certainly one of the most important players in the development of a T_H1 cell response (5, 6).

With regards to autoimmune inflammation, the idea that T_H1 cells cause autoimmunity was proposed on the basis of several observations: i) tissue-invading T_H cells often express IFNγ; ii) T_H1-inducing cytokines are present in the inflammatory lesion and often correlate with disease severity; iii) experimental autoimmune encephalomyelitis (EAE), the

animal model for MS, can be induced by the adoptive transfer of encephalitogenic T_H1 cells (7); and iv) treatment of mice with T_H1-inducing cytokines mostly results in aggravation of autoimmune diseases. However, the generation and immunization of mice deficient in T_H1 cytokines such as IFNγ and TNFα demonstrated that they were not, in contrast to expectations, protected from autoimmune disease (8, 9). This was an utterly surprising result, but at the same time promises to reflect the biology of IFNγ more faithfully. On the other hand, findings supporting a strong role for T_H1 cells mentioned above are almost exclusively correlative, based on extensive manipulation *in vitro* or driven my the 'unnatural' exogenous application of T_H1 cytokines. Despite these contradictory results, autoimmunity has persisted to be defined by the Th1/Th2 paradigm mostly due to the fact that T_H1-inducing cytokines such as IL-12 were held to be critical for the development of autoimmunity.

The IL-12 family of cytokines

Mice deficient in Th1-inducing cytokines, such as IL-12, have been shown to be completely resistant to EAE, again coinciding with reduced T_H1 development. In addition, the treatment of mice with neutralizing antibodies against IL-12p40 effectively prevents disease induction. In MS, patients demonstrate increased levels of IL-12 in serum, cerebrospinal fluid (CSF), CNS lesions and peripheral blood mononuclear cells (PBMC) when compared to controls.

IL-12 is a heterodimeric cytokine of approximately 70 kDa consisting of heavy (p40) and light (p35) subunits, which are covalently linked through disulfide bonds (see also **Figure 2**). The wealth of data pertaining to IL-12's role in autoimmunity originated from using mice deficient in the p40 subunit of IL-12 as well as administering antibodies specific against IL-12p40, both of which abolish expression and/or the function of IL-12. However, a series of reports have led to a revised concept of IL-12 function in autoimmune disease, starting in 1999 with further evidence that the p40 subunit is able to dimerize with subunits other than p35. Oppmann and colleagues identified an IL-6/IL-12-re-

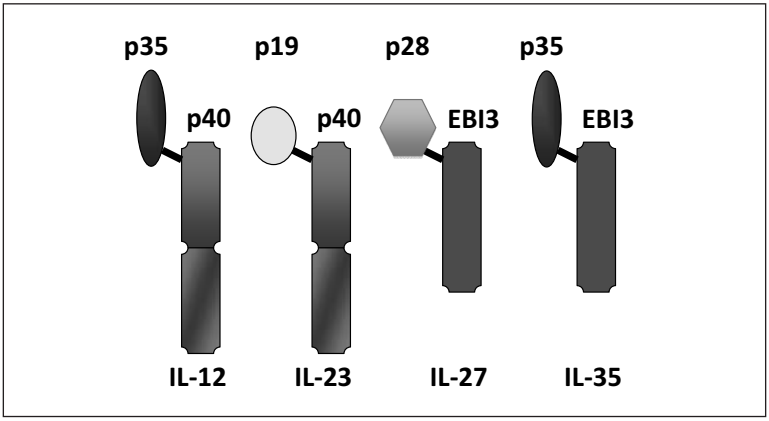

Figure 2: The growing IL-12 cytokine superfamily
Initially, IL12, a heterodimer of p40 and p35 bound by a disulfide bond, was discovered and its biological properties firmly linked to activating NK cells and to polarize T_H1 cells. IL-23 was discovered only 8 years later followed by IL-27 and IL-35.

lated subunit, p19, with which p40 heterodimerizes to form the cytokine IL-23 (10). Ubiquitous transgenic expression of p19 in mice resulted in the induction of multi-organ inflammation in these animals (11). In 2002, we were able to demonstrate that mice deficient in IL-12p35 were surprisingly not resistant to MOG-induced EAE but actually had increased clinical severity, a finding that could also be reproduced by others (12). Interestingly, the conflicting phenotypes were accompanied by a decrease in Th1 immunity in both susceptible p35-deficient mice as well as in resistant p40-deficient mice (12). The discordant effects of IL-12p40 and IL-12p35 clearly suggest an indispensable role for p40 in experimental disease but not for IL-12. The incompatibility of the $p40^{-/-}$ and $p35^{-/-}$ phenotypes seemed likely to result from the promiscuity of the p40 subunit, whereby resistance of p40-deficient mice resulted from the lack of IL-23 and not IL-12 (12, 13). This was later confirmed in mice deficient in the IL-23 subunit p19, and therefore in IL-23 alone, which in resemblance to p40-deficient mice were protected from EAE (14). In contrast to p40-deficient mice, p19 deficiency does not impact on the development of Th1 cells and IFNγ production,

which provides further and overwhelming evidence against a non-redundant pathogenic role for Th1 cells in autoimmune tissue inflammation. At the same time, IL-23 must exert an effect on T cells, which ultimately leads to their ability to induce immunopathology.

Th17 cells

The data thus far support the idea that IL-23 activates and polarizes a population of T_H cells which we would want to call $T_H$path cells, where 'path' stands for pathogenic (**Figure 3**). Understanding exactly which features of $T_H$path cells actually makes them auto-aggressive and encpehalitogenic was the focus of my lab ever since I started working in Zurich in 2003. While we were analyzing the transcriptome (all expressed genes within a cell population) of IL-23-stimulated T cells, Langrish *et al*. already proposed to call this $T_H$path cell population T_H17 cells based on their expression of IL-17. Since then T_H17 cells have then become the focus of attention of cellular immunologists and the number of articles about T_H17 cells and the cytokines produced by them

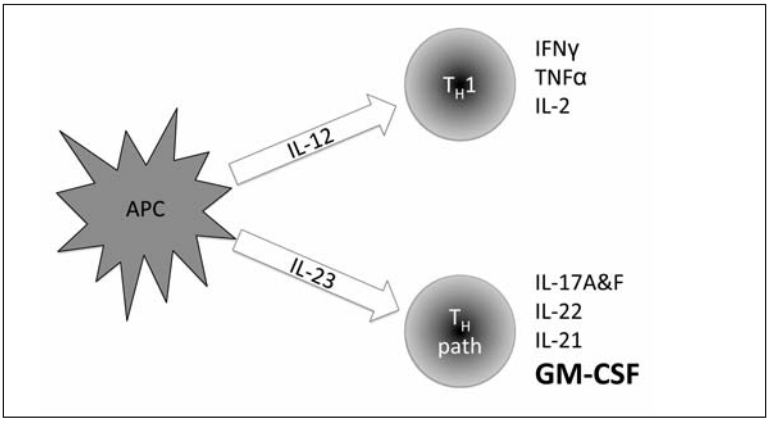

Figure 3: pathogenic T cell populations in autoimmunity
There are a number of polarization patterns described in effector T_H cells (e.g. T_H1, T_H2, T_H17, Treg). IL-12 and IL-23 polarize primarily T_H1 and $T_H$path (or perhaps T_H17 cells) respectively.

increased massively over the past 5 years. T_H17 cells were described to express IL-17A, its sister molecule IL-17F and as well as IL-21. Our group was particularly excited to observe the expression of IL-22 by T_H17 cells. IL-22 is a close relative to the regulatory cytokine IL-10, but in contrast to IL-10, IL-22 has been demonstrated to have powerful pro-inflammatory properties. Also, the receptor of IL-22 is expressed by the endothelium of the blood-brain barrier (15). However, in spite of our expectations that IL-22 would be a critical pathogenic cytokine produced by $T_H$path (i.e. $T_H17$cells), we found it to play absolutely no role in the development of CNS-inflammation (16). This finding taught us to be careful by convicting a particular player just because it is associated with a pathogenic event. Just because IL-23 is pathogenic, it does not mean that IL-22 (which is induced by IL-23) is also pathogenic.

Nonetheless, in light of the inconsistencies in the strict T_H1/T_H2 paradigm in autoimmunity the emergence of an IL-23-driven, proinflammatory T_H effector line was widely greeted as a convenient explanation, which shook up the $T_H1/2$ dogma without discrediting it entirely; the dogma only had to be revised to house this new T_H subset. Many contradictory findings generated while studying T_H1 cells were explained by this new T_H17 cell type. The excitement connected to the discovery was tangible in all of the first articles delineating the causal and exclusive connection between T_H17 activity and autoimmunity. IL-17A was conventionalized to be a master effector molecule particularly active and essential in autoimmunity. A plethora of data was generated henceforth that correlated T_H17 cells and IL-17A expression with the development of autoimmune inflammation in mice and humans (17). Almost forgotten was the fact that in most respects T_H1 cells and T_H1 cytokines correlate just as well with autoimmune disease and that adoptive transfer of T_H1 cells for the passive induction of EAE was the standard procedure for years (18).

The obviously essential role of IL-23 in EAE was explained via the T_H17/IL-17A axis. Langrish *et al*. produced a seminal article in 2005 suggesting that T_H17 cells are far more encephalitogenic in an adoptive transfer model of EAE than their T_H1 counterparts, and that this effect may be due to the secretion of IL-17A (19). IL-17A neutralization in

EAE in C57BL/6 mice by Hofstetter *et al.*, however only revealed a very mild effect of treatment with either monoclonal antibodies against IL-17A or with the soluble receptor of IL-17A and -17F (20). IL-17A$^{-/-}$ mice were generated later and found to be fully susceptible to EAE after active immunization, but demonstrate an alleviated course of clinical EAE in the chronic phase of disease. Upon adoptive transfer of T_H cells the IL-17A deficient T_H performed significantly poorer than the wt cells (21). These findings were then interpreted by others to represent a clear and solid proof that IL-17A was the key player in CNS autoimmune inflammation in mice, and probably also in men.

To our understanding, the slight change in the course of EAE found upon deletion of IL-17A certainly confirms its pro-inflammatory properties at the site of an active autoimmune lesion, but nonetheless, fails to mark it as an essential encephalitogenic factor. Combined with our own data showing that neither IL-22 nor IL-17A nor IL-17F nor the combined loss of IL-17A and -17F critically affect the severity or clinical course of actively induced EAE it becomes apparent that the known T_H17 cytokines do not play vital roles in the induction of CNS autoimmune inflammation (22). This, to some degree also questions the role of T_H17 cells themselves as the exclusive encephalitogenic driving force in autoimmunity.

The field advanced by delineating the factors required to generate T_H17 cells *in vitro*, and an impressive number of articles discussed different means of generating T_H17 cells *in vitro*, proposing different tissue culture methods, cytokine cocktails and means to purify the 'starting population' of T_H17 (23). By now it is, however, relatively clear that T_H17 cells (at least *in vivo*) do not represent a stable lineage, but a highly plastic effector state (24, 25). On transfer, it has been observed that T_H17 cells can turn into IFNγ producers (26). Reported by a great number of labs, the existence of IL-17A/IFNγ double producers under inflammatory conditions has been established which introduces a «grey-zone» at least in the proposed exclusiveness of the T_H1 and T_H17 effector types. The advantage to form a stable committed polarized population of effector T cells is their ability to develop immunological memory, so that upon reencountering their cognate Ag, they would immediately

commence the proper immune response. While T_H1, T_H2 and regulatory T cells (T_{reg}) appear to be able to terminally differentiate, memory T_H17 cells have thus far not been found, raising the possibility that differentiated T_H17 cells simply do not exist. Regardless, the possibility that a T cell population exists, which is dedicated to chronic autoimmune inflammation, needs to be explored in detail.

Hunting for the elusive 'destroy-the-brain'-signal

To this day, not one single cytokine produced by T_H cells could be shown to actually have a mandatory, essential function in the development of autoimmune diseases in mice and men. Even though the occasional report will claim such a notion, as was the case with IL-21 (27), it can often not be reproduced by others (28). It may be that a cocktail of specific cytokines directly translates into 'destroy the brain', or that there is one such cytokine that is actually is critical. I described earlier that we analyzed the levels of all the genes expressed by T_H cells in response to IL-23. One of these genes is the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF). It is a cytokine discovered long ago and thus is not designated an *interleukin*. However, its name is misleading, because we now know that its role in the formation of granulocytes and macrophages is negligible (29). Interestingly, it not only is expressed by $T_H$path cells, but we recently found it to be absolutely critical for T cells to cause brain-inflammation. GM-CSF appears more closely related to T_H17 cells and is linked to the transcriptional regulation of IL-17A, but in contrast to IL-17A, when T cells do not produce GM-CSF, they also do not cause disease (Codarri et al, submitted). Is GM-CSF the elusive 'destroy-the-brain'-signal? Given its absolute requirements for disease induction, it may very well be. However, we feel a more appropriate translation of its function is 'initiate-tissue-invasion-of-APCs'.

As I mentioned in the beginning, T cells only recognize their target, when it is appropriately presented by an APC. The initial target recognition of anti-brain T cells occurs at the level of the blood-brain barrier by specialized APCs (30). GM-CSF is a powerful activator of these

cells. We currently hold it to be the trigger, which licenses these APCs to initiate and sustain inflammation. This notion is currently been tested and we are hopeful that targeting GM-CSF therapeutically will benefit patients suffering from autoimmune diseases, such as MS.

Conclusion and future perspective

Translating the language of the immune syetem remains one of the biggest challenges in modern molecular medicine. The idea that we could one day specifically interfere with immune signals to halt an unwanted immune response is highly attractive. Conversely, pro-inflammatory factors such as GM-CSF or IL-12 can act to initiate immune responses against otherwise immunologically invisible tumors (31, 32). I continue to be optimistic that we shall see more and more so called biologicals (recombinant proteins) in clinical practice and that our management of autoimmune diseases and cancer (where anti-'self'-immunity is desired) will rely on our ability to manipulate the immune system.

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Melanie & Patrick. These were difficult times and the team spirit was what held the group tightly together. I am now surrounded by gifted, hard-working, ambitious, and brilliant students and post-docs, who do give the lab and the team their very best and sustain a highly creative and exciting environment. I am truly grateful to be allowed to have a part in the discoveries made by the individual scientists and the team.

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