# Repositioning Th cell polarization from single cytokines to complex help

**Selma Tuzlak[1*], Anne S. Dejean[2], Matteo Iannacone[3,4], Francisco Quintana[5,6], Ari Waisman[7,8,9], Florent Ginhoux[10,11,12]*, Thomas Korn[13,14,15]* and Burkhard Becher[1*]**

**Affiliations**

1 Institute of Experimental Immunology, University of Zurich, Zurich, Switzerland
2 Institut Toulousain des Maladies Infectieuses et Inflammatoires (INFINITy), INSERM UMR1291 - CNRS UMR5051 - Université Toulouse III, Toulouse, France
3 Division of Immunology, Transplantation and Infectious Diseases, IRCCS San Raffaele Scientific Institute, Milan, Italy.
4 Vita-Salute San Raffaele University, Milan, Italy.
5 Ann Romney Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA;
6 Broad Institute of MIT and Harvard, Cambridge, MA, USA.
7 Institute for Molecular Medicine, University Medical Center of the Johannes Gutenberg-University Mainz, Mainz, Germany.
8 Focus Program Translational Neurosciences, University Medical Center of the Johannes Gutenberg-University Mainz, Mainz, Germany
9 Research Center for Immunotherapy, University Medical Center of the Johannes Gutenberg-University Mainz, Mainz, Germany
10 Singapore Immunology Network (SIgN), Agency for Science, Technology and Research (A*STAR), 8A Biomedical Grove, IMMUNOS Building, Biopolis, Singapore
11 Translational Immunology Institute, Singhealth/Duke-NUS Academic Medical Centre, the Academia, 20 college road, Discovery tower level 8, Singapore
12 Shanghai Institute of Immunology, Shanghai JiaoTong University School of Medicine, 280 South Chongqing Road, Shanghai, China
13 Institute for Experimental Neuroimmunology, Klinikum rechts der Isar, Technical University of Munich
14 Dept. of Neurology, Klinikum rechts der Isar, Technical University of Munich
15 Munich Cluster for Systems Neurology (SyNergy)

***Abstract***

When Th cell polarization was initially described three decades ago, the Th cell universe grew dramatically. New subsets were described based on their expression of few specific cytokines. Beyond Th1 and Th2 cells, this led to the coining of various Th17 and Treg cell subsets as well as Th22, Th25, Tfh, Th3, Th5, and Th9 cells. High-dimensional single cell analysis revealed that a categorization of Th cells into a single cytokine-based nomenclature fails to capture the complexity and diversity of Th cells. We propose that Th cell polarization should be categorized in terms of the help they provide to phagocytes (type 1), to B cells, eosinophils and mast cells (type 2) and to non-immune tissue cells, including stroma and epithelium (type 3). Studying Th cells based on their helper function rather than individual analysed cytokines or transcription factors better captures Th cell plasticity and conversion as well as the breadth of immune responses *in vivo*.

## Introduction

T helper (Th) cell polarization is primarily geared towards the responder cells that synergize, amplify and cooperate towards a distinct type of response, while repressing alternative responses at a certain time point of disease or infection. This is to a large part achieved by a complex and tightly regulated network of activating and inhibiting cytokines. Besides the cytokine pattern captured, the helper properties are further expressed through surface molecules, pattern of migration and the ability to enter specific tissues. Here, we focus on what was traditionally used to define Th cells, namely the individual cytokines proposed to categorize Th cells. The expression of cytokines by Th cells depends on upstream signals from the encounter with antigen presenting cells (APCs). This combination of cytokines lays, together with specific transcription factors (TFs) that control their expression, the foundation for the current classification of Th cell subsets. With the emergence of new technologies enabling us to simultaneously measure literally dozens of cytokines along with other markers such as TFs, integrins or chemokine receptors at the single cell level (Galli et al., 2019a), it is no longer feasible to categorize Th cells based on a dominant cytokine or even a family of cytokines (Tortola et al., 2020). Also, by attempting to categorize every single Th cell based on individual cytokines or transcription factors, we may overlook the actual complex biology of the differential responses and other involved cell types. Here, we focus on how the expanding Th cell universe can be reorganized based on the actual help provided towards the actual cellular targets, rather than on the momentary expression of certain cytokines and TFs.

## Historical perspective

The categorization of T cells by their biological properties has provided us essentially with $CD8^+$ cytotoxic killer and $CD4^+$ Th cells. The latter received a further bifurcation into Th1 and Th2 cells when Mosmann and Coffman described in 1986 that Th cells can be polarized to produce either Interferon (IFN)-γ or Interleukin (IL)-4, depending on their environment and stimulatory context (Mosmann et al., 1986). Later, dominant TFs were found to drive this polarization program, namely Tbet for Th1 cells and GATA-3 for Th2 cells (Szabo et al., 2000; Zhang et al., 1997; Zheng and Flavell, 1997). Importantly, one subset actively suppresses the others' ability to produce its characteristic cytokines and transcription factors (Mosmann et al., 1986).

Another, now well-established Th subset comprises of regulatory T cells (Tregs). Already in the early 1970s, experiments with thymectomized mice showed the development of tissue damage in various organs indicating the presence of a suppressive T cell subset developing in the thymus (Gershon and Kondo, 1970; Nishizuka and Sakakura, 1969). However, due to lack of reliable markers to distinguish these cells from other T cells, Tregs underwent a history from being defined as Tr1, when secreting the suppressive cytokine IL-10 *in vitro*, to being termed Th3, when found to secrete TGF-beta upon induction of oral tolerance (Chen et al., 1994; Groux et al., 1997). Nowadays, thymically hard-wired Tregs are characterized by high expression of the high-affinity IL-2 receptor alpha-chain CD25 (Sakaguchi et al., 1995) and the transcription factor FoxP3 (Fontenot et al., 2003) and known to be of particular importance for maintaining immune homeostasis and preventing autoimmunity (Fontenot et al., 2003).

Whereas the simple Th1/Th2 paradigm provided an easy explanation of immune responses towards intra- and extracellular pathogens, respectively, numerous open questions emerged in the context of chronic inflammation and autoimmunity. The path for extending the Th family was cleared after it was noted that the IFN-γ inducing cytokine IL-12 was not the critical factor for the induction of autoimmune pathology in preclinical models of chronic tissue inflammation, mimicking diseases such as Multiple Sclerosis (MS), Rheumatoid Arthritis (RA) and others. Instead, IL-23, which shares the p40 subunit with IL-12, was actually the main driver of the inflammatory response (Becher et al., 2002; Cua et al., 2003; Murphy et al., 2003). Additionally to being pivotal for the development of pathogenic $CD4^+$ T cells in neuro-inflammation, IL-23 also triggered IL-17 expression (Aggarwal et al., 2003; Langrish et al., 2005). Thus, it was recognized that Th1 cells were not the sole driving force for autoimmune pathology, at least in the context of experimental autoimmune encephalomyelitis (EAE), and the call was out for the identification of the true (pathogenic) T helper cell subset(s) in this disease.

In 2005, IL-17 producing Th cells were described as a new entity (Harrington et al., 2005; Park et al., 2005). This subset was readily accepted as an independent Th subset, probably due to its clear segregation from Th1 and Th2 cells, whose induction seemed to antagonize the production of IL-17

(Harrington et al., 2005). The definition of TGF-β and IL-6 as differentiation factors for these T cells *in vitro* (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006), and the identification of RAR-related orphan receptor gamma (RORγt) as critical transcription factor for IL-17 secretion solidified the standing of an independent Th17 subset (Ivanov et al., 2006). Even though the role of Th17 cells in tissue inflammation in general has been heavily debated, IL-17 producing cells have been clearly implicated in a number of chronic inflammatory diseases like Psoriasis, RA and Crohn's Disease (reviewed in (Zwicky et al., 2020)).

Already in 2000, another new subset was proposed, when two groups showed that B cell help in follicles was provided by specific Th cells that reside close to the B cell zone in secondary lymphoid structures (Breitfeld et al., 2000; Schaerli et al., 2000). These Th cells express the CXC chemokine receptor 5 (CXCR5) that is also expressed on mature B cells and were termed follicular Th cells (Tfh). However, it was not until 2009 that Bcl-6 was identified as the transcription factor necessary for the generation of Tfh cells (Johnston et al., 2009). Even then, the acceptance of Tfh cells as independent entity was strongly debated. Partly, this was due to the observation that the expression of canonical Th1, Th2, or Th17 cytokines like IFN-γ, IL-4 and IL-17, respectively, was necessary to induce a proper class switching reaction in B cells (Reinhardt et al., 2009). Although the regulation of the expression of these cytokines in Tfh cells is not yet clear, it has been proposed that Tfh cells differentiate independently of other Th subsets from naïve $CD4^+$ T cells when interacting with B cells upon initial activation by dendritic cells (DCs) (Crotty, 2011). Interestingly, the generation and retention of Tfh cells appears to depend on the presence of germinal center B cells and *vice versa* (Johnston et al., 2009), which may hint towards a role of specific niches as drivers for T cell diversity and plasticity.

The addition of new cytokines in the analysis workflow of immunology labs led to the description of additional Th subsets, such as Th9 (Dardalhon et al., 2008; Veldhoen et al., 2008), Th22 (Duhen et al., 2009; Eyerich et al., 2009; Trifari et al., 2009) , and Th25 (Wu et al., 2015). To then adjust to this single cytokine-based view on Th cells in immunity, even more subsets were coined. These include pathogenic vs. non-pathogenic Th17, Th17.1 Th17.2 and Th5 cells, among others (Becher et al., 2016; Cosmi et al., 2010; Ghoreschi et al., 2010; van Hamburg and Tas, 2018). During this expanding discovery phase of new Th subsets, several voices warned against the idea that the identification of an individual cytokine expressed by Th cells should not automatically deliver a newly coined subset and that immunologists should keep an eye on the biology of these T cells and their role in immune responses (Locksley, 2009; Zhou et al., 2009). The same holds true for the definition of dominating TF needed to allow the 'discovery' of a new Th subset, especially as most of the subsequent findings were based on *in vitro* studies where specific cytokine cocktails were applied to either naïve or activated purified T cells.

Furthermore, the distinction of subsets requires not only "private" master TFs, but also, and maybe more importantly, stability and the ability to form memory. Stability is largely granted through

epigenetic imprinting, which ensures the maintenance of the cells' identity even after an extended period of time and without persistent antigenic threat. Genetic stability has been best described in Tregs (Huehn et al., 2009), and to some degree in Th1 and Th2 subsets (Avni et al., 2002; Fields et al., 2002), however not so much for Th17 cells (Mukasa et al., 2010) or any of the other described subsets. At the present day, it is needless to say that the diversity of coined Th subsets has become exceedingly complex and also increasingly controversial among immunologists, as the designation of Th subsets beyond Th1, Th2, and Th17 cells remains debated.

**Limitations of the current Th classification**

The current Th subset classification reaches its meaningful limits when trying to categorize Th cells involved in the induction of pathologies. One prominent example is EAE, a preclinical model for the neuroinflammatory disease MS, where the responsible Th subset was not fully elucidated despite decades of research (reviewed in (Kunkl et al., 2020)). Initially, EAE was believed to be a Th1-mediated disease model because of the abundant IFN-γ expressing Th cell infiltration in the central nervous system (Ando et al., 1989; Voskuhl et al., 1993). However, the observation that loss of IL-12 and IFN-γ signalling, respectively, led to EAE aggravation (Becher et al., 2002; Ferber et al., 1996) suggested that Th1 cells were not required for encephalitogenicity, but may even have at least partly a protective role.

Shortly after, it was discovered, that IL-23 signalling was pivotal for EAE induction and simultaneously a potent inducer of numerous cytokines including IL-17 (Langrish et al., 2005). This observation coincided with the claim that Th17 cells represent an independent Th cell subset (Harrington et al., 2005; Park et al., 2005). This association in turn suggested that Th17 cells may represent the pathogenic, disease-initiating population in EAE. However, there are contradicting reports on the effect of the canonical Th 17 cytokines IL-17A and IL-17F on EAE. While one study described a milder course of EAE upon the depletion of IL-17A (Komiyama et al., 2006), others failed to observe a tangible effect on the progression of EAE upon loss of IL-17A or IL-17F (Haak et al., 2009), making conclusions on the involvement of Th17 cells in EAE more difficult. Only recently, it was shown that the effects of IL-17 on the disease course - besides direct effects on the blood brain barrier and perhaps astrocytes (Kang et al., 2010; Kebir et al., 2007) - stem from its ability to shape the microbiome in the gut, thereby indirectly acting on CNS inflammation by shaping the systemic immune compartment (Regen et al., 2021). The same study showed that exclusive IL-17 production by neuro-antigen specific T cells was dispensable for their pathogenic potential. Moreover, although the use of IL-17 fate-mapping mice showed that the use of CFA as an adjuvant does favour the formation of IL-17 expressing Th cells, upon the initiation of immunopathology, these cells showed a high degree of plasticity (Hirota et al.,

2011). After tissue invasion, many of them produced high levels of IFN-γ thereby raising the idea of an intermediate Th17/Th1 phenotype covering the "pathogenic" Th cell subset.

An essential key-player cytokine of this pathogenic Th cell subset is the Granulocyte-Macrophage Colony-Stimulation Factor (GM-CSF). In the context of EAE, GM-CSF is mainly produced by Th cells (Komuczki et al., 2019) and has a dominant function in the development of the inflammatory cascade, as GM-CSF deficient mice are completely resistant to EAE (Komuczki et al., 2019; McQualter et al., 2001; Ponomarev et al., 2007). Furthermore, patients suffering from MS have elevated frequencies of GM-CSF expressing Th cells (Galli et al., 2019b). It appears that GM-CSF, similar to TNF, can be transiently expressed by several Th subsets upon TCR-mediated stimulation (reviewed in (Sheng et al., 2015)) making it difficult to allocate GM-CSF to one of the established Th subsets. Due to the inability to clearly define Th1 or Th17 cells as pathogenic entity in EAE, a new GM-CSF expressing Th subset was discussed (Herndler-Brandstetter and Flavell, 2014; Komuczki et al., 2019; Sheng et al., 2014). This idea was supported by the observation that while GM-CSF was clearly co-expressed with IFN-γ, co-expression with IL-17 was rarely observed (Noster et al., 2014). However, GM-CSF expression has been shown to be regulated by a complex transcriptional network downstream of the TCR including the activity of TFs such as RORγt, NFAT, NFκB, JNK/AP-1, PU.1 and Bhlhe40 (reviewed in (Sheng et al., 2015)), thus no individual dominant TF for GM-CSF expression has been identified so far. The regulation by the different pathways might also indicate the need of tight control of GM-CSF expression to avoid accidental activation of this potent pro-inflammatory cytokine. Regardless as to whether GM-CSF expressing cells represent a new and independent cellular subset, the present categorization of Th cells is not able to unravel the bundle of distinct and overlapping Th subsets, but rather limits the possibilities to define specific (disease-related) processes without colliding with the established nomenclature.

**The power of plasticity**

There is evidence that all Th cells, with the exception perhaps of Tregs, retain a certain degree of plasticity upon differentiation into effector cells. This is a fortuitous feature as it enables immune responses to adapt to changing circumstances based on incoming stimulating or inhibitory cues. Experiments regarding the stability of the single subsets showed that even fully differentiated Th1 and Th2 cells were able to switch their transcriptional signature when challenged under the respective conditions within the first five days of stimulation. Prolonged stimulation, however, induced a more stabilized Th1 or Th2 program (Murphy et al., 1996). This indicates that polarized Th cells retain flexibility in regard to their transcriptional signature for several rounds of expansion, giving them enough time to adjust their response to the stimulation. Especially Th17 cells have a particularly unstable lineage commitment, thus readily converting into Th1-like or Treg-like phenotypes (reviewed

206 in (Lee et al., 2009)). The conversion of Th17 into Th1-like cells has especially been associated with
207 the occurrence of organ-specific autoimmune diseases. Importantly, a high degree of Th flexibility
208 cannot only be observed in laboratory animals under strictly defined experimental conditions, but also
209 in the human immune system. One example is the development of different vaccine-specific Th subsets,
210 that were not only diverse directly upon immunisation but even able to change their "fate" with
211 following rounds of expansion (Becattini et al., 2015).

212 Taken together, the flexibility of Th cells makes their classification based on cytokine patterns alone
213 opaque and bulky. In a review article by O'Shea and Paul (O'Shea and Paul, 2010), the authors
214 acknowledged this challenge and proposed a continuum model where Th cells are positioned across an
215 orbital shape of states with the three transcription factors, ROR**γ**t, Tbet and GATA-3 as the three
216 extreme positions.

217 This 'continuum model' was certainly a step in the right direction, but with increasing numbers of
218 transcription factors and cytokines analysed simultaneously, the anchor points of this orbital model
219 extend into the multidimensional space and can no longer help the visualization and conceptualization
220 of T cell states. Therefore, we believe that the continual bifurcation of Th subsets no longer contributes
221 to the understanding of the plasticity and functionality these cells adduce, but rather unnecessarily
222 complicates our appreciation of dynamic immune responses. Current state-of the art methods such as
223 single cell RNA-sequencing, ATAC-sequencing, and high-dimensional cytometry also failed to capture
224 canonical polarized Th cells, particularly in *vivo*. Instead, the data support the notion that Th-cell driven
225 immune responses in mammals are highly diverse and complex. This apparent breadth of Th cell states
226 could be explained by a) Th cells are primed towards a certain lineage, but then retain a high level of
227 plasticity, or b) Th cells are primed towards a diverse continuum and that they are no dedicated
228 canonical lineages. Either way, dividing Th cells into increasing numbers of subsets, based on the
229 cytokine production measured, may only apply to specific experimental conditions at a certain time
230 point, but does not contribute significantly to a better understanding of Th cell biology. Hence, we
231 propose to take one step back and focus again on the actual helper function of Th cells and consider
232 their polarization based on the target cells they 'help', akin to the designation of Treg and Tfh cells,
233 designations based on function rather than phenotype.

234

235 **Reframing Th cell subsets**

236 In 2018, Eberl and Pradeu proposed a unifying theory that is taking the bigger physiological picture
237 into account (Eberl and Pradeu, 2018). They started by picking up on the idea that the immune system
238 is not activated by recognizing non-self *per se*, but by the change in "normality" – the so called
239 "discontinuity theory" (Pradeu et al., 2013) (that builds upon the danger model that was proposed by
240 Polly Matzinger in the 1990s (Matzinger, 1994)). The new theory considers three levels of immune

responses: activation of the immune system by different ways (e.g. intracellular, tissular, extracellular), regulation of the immune response by cross-inhibition of the different types of immune response (Figure 1), and integration of the immune response into other vital processes necessary for maintaining homeostasis at the level of the whole organism (Eberl and Pradeu, 2018). The three types of responses they described are loosely associated with the known concept of type 1, 2, and 3 immunity (Annunziato et al., 2015). Accordingly, type 1 responses are induced by intracellular discontinuities, type 2 responses are involved in tissue repair mechanisms to prevent entrance of pathogens, and type 3 responses are activated by discontinuities affecting the extracellular space, such as fungi and bacteria in barrier tissues (Eberl, 2016).

We propose to extend this concept towards the initial definition of Th cells; namely their primary function – to provide help. Th cells are not predominantly killers or cleaners, but as their name says, they support and enable other cells in the execution of their tasks. Depending on the context of activation, Th cells interact with different other cell types and produce a variety of cytokines, probably in varying concentrations and for a certain duration. This in turn acts on a palette of cell types including macrophages, DCs, monocytes, B cells or non-immune cell subsets that cross-regulate each other to achieve the desirable/adequate type of response. Therefore, we propose to define Th cells by the type of the responding cells they target (Figure 2). This classification based on function rather than phenotype is then further refined by the continuum model of O'Shea and Paul (O'Shea and Paul, 2010), to acknowledge the plastic nature of Th cell states. However, while plasticity can be extensive, it is also limited by two major principles: First cross-inhibitory interaction between type 1, type 2, and type 3 responses (as also suggested by Eberl (Eberl and Pradeu, 2018)), and second auto-amplification of established T helper cell responses. Auto-amplification loops have been described for type 1, type 2, and type 3 responses - mostly based on T cell derived cytokines that directly add back on their sources, re-enforcing their functional phenotype. IFN-γ (Bradley et al., 1996), IL-4 (Kurtjones et al., 1987), and IL-21 (Korn et al., 2007) are examples of such autocrine feed-forward loop drivers for type 1, type 2, and type 3 responses, respectively.

### *Type 1 response*

Type 1 responses are executed primarily by mononuclear myeloid cells, such as monocytes, macrophages and DCs. The most canonical type 1 cytokines produced by Th cells are IFN-γ and GM-CSF. The IFN-γ effects in responder cells depend on the nature of the responding cell type (de Veer et al., 2001). The IFN-γ receptor (IFNGR) is a tetramer of two ligand binding IFNGR1 chains and two signal-transducing IFNGR2 chains. While IFNGR1 is constitutively expressed on the surface of most cell types, IFNGR2 expression is more tightly regulated and predominantly found in phagocytes. More than 2000 IFN-γ responsive genes have been identified, including MHCI, MHCII, NOS2, various

CAMs like VCAM1, and CD44, IRF1-9 and different TRIM genes (Hertzog et al., 2011). IFN-γ is particularly important for APCs, as it not only induces the upregulation of MHC-I and -II molecules but also slows lysosomal function in macrophages in order to enhance antigen processing (Trost et al., 2009; Yates et al., 2007). Interestingly, other pro-inflammatory stimuli like type I IFN, LPS, and TNF can initiate a similar signalling cascade to IFN-γ (Ahn et al., 1997; Kovarik et al., 1999), thereby modulating the IFN-γ response, but also possibly accounting for the mild phenotype of *IFNG-/-* and *IFNGR-/-* mice (Snapper et al., 1987). However, loss of IFN-γ signalling in mice led to impaired clearance of several intracellular pathogens and a shift in the Th1/Th2 response (reviewed in (Tau and Rothman, 1999)).

GM-CSF similarly acts as a potent communication conduit between T cells and myeloid cells (Croxford et al., 2015; Darrieutort-Laffite et al., 2014; Kobayashi, 2005; Zhan et al., 2012). The GM-CSF receptor is a heterodimer composed of the cytokine-specific α-chain and a β-chain that is shared with receptors for IL-3 and IL-5 (reviewed in (Barreda et al., 2004)). Its cellular expression is even more restricted than the expression of the IFNGR since the GM-CSF receptor is almost exclusively expressed by myeloid cells. *In vitro* stimulation with GM-CSF initiates the differentiation of DCs, granulocytes, and macrophages, depending on the concentration of the cytokine (Sun et al., 2018). The situation *in vivo* is more complex, although there is evidence that also *in vivo* GM-CSF has dose- and time-dependent effects (Guthridge et al., 2006). In general, GM-CSF is promoting survival, differentiation and activation of monocytes, macrophages, and other phagocytes by engaging the JAK2/STAT5 and ERK pathways (Guthridge et al., 1998). Under certain inflammatory conditions, GM-CSF can be regarded as a pro-inflammatory mediator between Th cells and phagocytes (reviewed in (Becher et al., 2016)) and it can be speculated that blocking GM-CSF will alleviate type 1 driven inflammatory diseases. Hence, it is not surprising that GM-CSF blocking antibodies are prominently used in clinical trials, e.g. recently in the context of COVID-19 (Mehta et al., 2020).

Of note, among others, GM-CSF expression is induced by IL-23, which has also been shown to be important for the modulation of "Th17" responses (Aggarwal et al., 2003; Komuczki et al., 2019), and other type 3 immune responses (see below), making IL-23 both, a type 1 and type 3 response-inducing cytokine depending on the circumstances (perhaps linked to its ability to signal through both STAT4 and STAT3). In this regard, it will be interesting to decipher the additional factors causing a mainly destructive GM-CSF-driven type 1 response versus a protective IL-17-mediated type 3 response upon IL-23 exposure. Although it was argued that GM-CSF might serve as a marker for "destructive, or pathogenic" Th17 (or Th1/17, or Th17.1) cells, GM-CSF producing cells preferably co-express IFN-γ over IL-17 (Galli et al., 2019b; Herndler-Brandstetter and Flavell, 2014; Komuczki et al., 2019; Noster et al., 2014). Nevertheless, the relationship with IFN- γ appears to be a complex one, since both, IFN-γ and its driver, IL-12, effectively suppress GM-CSF production in T cells (Komuczki et al., 2019). Of note, whereas T cells can sense IFN-γ, which has long been considered to aid in the maintenance of the

Th1 phenotype, GM-CSF is not sensed by lymphocytes themselves. In spite of the apparent contradictions which emerge, when Th cells are categorized by individual cytokines expression, the categorization of Th cells by the target cells they help, alleviates that problem and permits a better understanding of the actual properties of Th cells in type 1 immunity.

Taken together, in type 1 responses Th cells mainly target and activate phagocytic cells. While this communication aids in the elimination of intracellular pathogens, aberrant (dysregulated) type 1 responses - through persistent recruitment of phagocytes - can be drivers of immunopathology.

***Type 2 response***

Type 2 immune responses have been initially described to primarily foster humoral immunity, and that Th derived type 2 cytokines help predominantly the B cell compartment and the involved intricacies to generate potent high-affinity antibodies. However, here again, the pure categorization of Th cell by their cytokine profile makes it much harder to capture the function of IL-4 secreting Th2- and Tfh cells alike. As such, type 2 Th cells include not only Th2 and Tfh, but also Th1 cells, since all of them have been shown to be necessary for humoral (type 2) immunity (Crotty, 2015; Smith et al., 2000). Typical type 2 cytokines are IL4, IL-5 and IL-13. IL-4 was the first factor that was recognized to be crucial for B cell maturation and class switching, therefore recognizing Th2 cells as main providers of B cell help (Howard et al., 1982). However, the deletion of Th2 associated genes did not cause loss of germinal centers and later, it became apparent that IL-4 was solely needed for IgE class-switch recombination (Kopf et al., 1995), and that additional factors like CD40L and IL-21 were needed for fully functional B cell responses, which were attributed to Tfh cells (reviewed in (Crotty, 2015)).

Another important function of type 2 immunity beyond the engagement of B lymphocytes is the attraction and activation of eosinophils, mast cells and basophils during inflammatory responses. This is mainly achieved by the cytokines IL-5 and IL-13, which induce Eosinophilia and Goblet cell hyperplasia during helminth-infections (Koyasu and Moro, 2011). However, eosinophils, mast cells and basophils are not only type 2 effector cells, but they are also involved in the amplification of type 2 immunity by producing IL-4 and other type 2 mediators themselves. Eosinophil-recruitment, for instance can occur prior to the infiltration of Th cells, which in turn stimulates APCs to initiate a type 2 promoting Th phenotype (Shinkai et al., 2002; Yang et al., 2008). Although it is not fully understood which cell types induce the initial attraction of eosinophils, tissue-resident group 2 innate lymphoid cells (ILC2s) might be involved as they can react before the adaptive response is initiated (Gasteiger et al., 2015) making them important early phase type 2 players. Furthermore, it has been shown that the presence of ILC2s was required for a complete Th response, at least in the context of allergic inflammation (Halim et al., 2014, 2016).

Deregulated expression of type 2 cytokines, especially IL-4 and IL-13 may contribute to inflammatory diseases, one of the most prominent being atopic dermatitis (Brunner et al., 2017). Thereby, IL-4 suppresses the expression of genes involved in barrier function in keratinocytes (Sehra et al., 2010), and alarmins (i.e. IL-25 and IL-33) produced by keratinocytes, which trigger an ILC2 mediated expression of IL-13 which attracts Th cells into the irritated skin, thereby amplifying the inflammation (reviewed in (Bieber, 2020)). Hence, it is not surprising that blocking IL-4 and IL-13 significantly improves clinical symptoms in atopic dermatitis patients (Beck et al., 2014).

The alarmin IL-25, also known as IL-17E, was first reported to be secreted by Th2 cells and subsequently led to the coining of Th25 cells as IL-25 producing entity that is boosting type 2 responses by enhancing IL-4, IL-5 and IL-9 production via STAT-5 activation (Fort et al., 2001). Now we know that it can be produced by many different hematopoietic and non-hematopoietic cell types, such as mast cells, alveolar epithelial cells, brain capillary endothelial cells and others (reviewed in (Liu et al., 2018)). The exact mechanisms by which these cells induce and enhance type 2 responses are not fully understood yet, however, there is strong evidence that ILC2s act as type 2 response amplifiers (Moro et al., 2010; Neill et al., 2010; Price et al., 2010).

Another type 2 cytokine that has defined an independent Th subset is IL-9 (Dardalhon et al., 2008; Veldhoen et al., 2008). Initially believed to be a T cell growth factor (Renauld et al., 1993), IL-9 was soon recognized to be crucial for mast cell expansion and recruitment (Townsend et al., 2000). In this context, it is involved in the clearance of parasitic infections but may also play a role in promoting allergic inflammation (reviewed in (Noelle and Nowak, 2010)).

Taken together, type 2 T cells including Tfh cells target primarily B cells to aid in GC formation and class switch, whereas dysregulated type 2 immunity leads to allergic inflammation involving eosinophils, mast cells and basophils.

***Type 3 response***

Type 3 responses have been very well defined as barrier-tissue specific reactions to extracellular disturbances. Receptors for the critical cytokines IL-17 and IL-22 are expressed throughout the stromal and immune compartment, but dysregulated expression of these cytokines (IL-17A, IL-17F, IL-22 etc.) leads to dramatic immunopathology across barrier tissues (skin, lung, gut) with little to no signs of internal organ specific effects (Conti et al., 2009; Haak et al., 2009; O'Connor et al., 2009; Sonnenberg et al., 2010). Ectopic IL-17 expression has the most dramatic effect upon the engagement of the IL-17R complex in epithelial cells of the skin. Apart from the production of anti-microbial peptides, IL-17 activated keratinocytes produce a set of chemokines and cytokines that in turn attract neutrophils into the skin (reviewed in (Perera et al., 2012)). Dysregulation of IL-17 in mammals also triggers psoriasiform inflammation, characterized by the cellular expansion of keratinocytes, and the influx of

neutrophils. Targeting the type 3 immune response in patients suffering from psoriasis through neutralization of IL-17 or IL-23 dramatically alleviates the clinical symptoms (reviewed in (Zwicky et al., 2020)). Strikingly, IL-23 is critical for both, GM-CSF and IL-17 production in inflammatory conditions (as discussed above). This poses interesting questions about the regulation of IL-23R signalling within different inflammatory conditions and cell types. In line with this, IL-23 has also been shown to be released in response to nociceptor activation (Kashem et al., 2015; Riol-Blanco et al., 2014), linking the immune system with the neuronal network. The notion that there is more to the immune system than simple host defence is applying not only for type 3 immunity and pain sensation. A growing scientific field tries to decipher the interplay of the immune system and other physiological processes like the neuronal network and the enteric system (reviewed in (Rankin and Artis, 2018)).

In line with the notion that type 3 immune responses predominantly involve barrier tissues, physiological amounts of type 3 cytokines (such as IL-17A, IL-17F and IL-22) are involved in the control of mucosal pathogens, in particular fungi (reviewed in (Sparber and Leibundgut-Landmann, 2019)). Hence, the most dramatic side effect of IL-17 neutralization is the development of uncontrolled fungal infections.

IL-22 producing cells can be easily "reprogrammed" into IFN-γ, or IL-4 expressing Th cells illustrating one more time the dynamics of Th cell plasticity and indicates the importance of a flexible and collaborative environment for a functional immune system (Plank et al., 2017). Furthermore, it was shown recently that Th cells isolated from the lamina propria could not be attributed to the "classical" Th1 or 17 subsets but rather expressed a continuum of different (signature) cytokines (Kiner et al., 2021). In support of the idea that type 3 immunity is an evolutionary hard-wired mechanism of barrier-protection, is the source of type 3 cytokines outside of Th cells. The production of IL-17 and IL-22 for instance is readily observed in γδ-T cells and ILC3 cells, which are prominent and early responders in barrier tissue immunity.

In summary, in contrast to type 1 and type 2 responses, type 3 responses are less targeted to distinct immune effector cells but activate and regulate non-immune cells. The code, which is used by type III responses (including e.g. IL-17 and the IL-20 family of cytokines) is a code which likely is phylogenetically old that is used by tissue resident immune cells (like ILC3s and γδ-T cells) to communicate with their non-hematopoietic environment and has been co-opted by the adaptive immune system for host defence at lining tissues.

## Summary and conclusion

The establishment of advanced single cell analysis tools such as sc-RNAseq and high-dimensional cytometry revealed that the hitherto known classification of the Th cell universe based on previously established cytokine patterns (Galli et al., 2019a; Tortola et al., 2020), does not adequately capture the

diversity and complexity of the mammalian immune system. Here, we aimed to take a step back in order to acknowledge the bigger picture instead of focusing on small Th subsets that might simply represent an intermediate stage within their differentiation. By expanding the concept initially proposed by Eberl and Pradeu (Eberl and Pradeu, 2018) and integration of the until now described subsets into a more comprehensive capture of immunity based on the target cells of the Th response (Figure 2), we propose the following nomenclature:

Type 1 Th cells that primarily activate and attract mononuclear phagocytes such as monocytes, macrophages and DCs

Type 2 Th cells targeting B cells and polymorphonucleated granulocytes such as mast cells, basophils, and eosinophils

Type 3 Th cells acting on non-hematopoietic cells at barrier tissue sites, including epithelial cells and stromal cells.

This categorization is in our opinion superior to the coining of ever new subsets and sub-subsets. We acknowledge that this concept is also imperfect in that it does not capture all the possible cellular states and their individual role in immune responses. Furthermore, we would hope to have solid molecular markers of Th cell states to better describe their biology. In lieu of such a 'super-marker' or molecular pattern of Th cell states, this simplified contextual 'help' framework proposed here is also not overly rigid. While polarized Th cells will in general fall into one of the three categories, this does not mean that their role in immunity is by any means inflexible. There is solid evidence of plasticity in memory T cells and the ability to respond to different challenges with speed and agility. Hence, all attempts to categorize single Th cells observed during a snapshot within a complex immune response cannot truly give an account of the actual function and the role of individual Th cell in the development of a dynamic immune response. The physiological importance of Th differentiation must be the outcome of the response – the activation/attraction/modulation of responder cells. We hope that this perspective may help to establish a more intuitive classification of Th cell function, which will help to understand the growing complexity in this field.

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**Competing interests**

The authors declare no competing interests.

**Figure legends**

**Figure 1: Cross-inhibition model.** From the perspective of a pathogenic insult type 1 immune responses are typically triggered by intracellular pathogens. Multicellular organisms that cannot easily be phagocytosed induce type 2 responses which support the development of humoral immunity. Type 3 responses are initiated upon extracellular activation at barrier sites like the skin, gut and other mucosal tissue. In this model, the three types of immune response inhibit each other and are strengthened by auto-amplification.

**Figure 2: Orbital model based on Th cell targets.** Th cells can be classified by the primary target cells engaged. Type 1 responses target mononuclear phagocytes including macrophages and monocytes. The responding cells of type 2 immunity are predominantly mast cells, eosinophils and basophils, as well as B cells (in particular in germinal centers). Type 3 cytokines engage predominantly non-immune cells, such as epithelial cells across barrier tissues. In this model, the three types of immunity are interconnected, plastic and allow cross-talk when necessary.

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# Repositioning Th cell polarization from single cytokines to complex help

**Selma Tuzlak[1*], Anne S. Dejean[2], Matteo Iannacone[3,4], Francisco J. Quintana[5,6], Ari Waisman[7,8,9], Florent Ginhoux[10,11,12]*, Thomas Korn[13,14,15]* and Burkhard Becher[1*]**

**Affiliations**

1 Institute of Experimental Immunology, University of Zurich, Zurich, Switzerland

2 Institut Toulousain des Maladies Infectieuses et Inflammatoires (INFINITy), INSERM UMR1291 - CNRS UMR5051 - Université Toulouse III, Toulouse, France

3 Division of Immunology, Transplantation and Infectious Diseases, IRCCS San Raffaele Scientific Institute, Milan, Italy.

4 Vita-Salute San Raffaele University, Milan, Italy.

5 Ann Romney Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA;

6 Broad Institute of MIT and Harvard, Cambridge, MA, USA.

7 Institute for Molecular Medicine, University Medical Center of the Johannes Gutenberg-University Mainz, Mainz, Germany.

8 Focus Program Translational Neurosciences, University Medical Center of the Johannes Gutenberg-University Mainz, Mainz, Germany

9 Research Center for Immunotherapy, University Medical Center of the Johannes Gutenberg-University Mainz, Mainz, Germany

10 Singapore Immunology Network (SIgN), Agency for Science, Technology and Research (A*STAR), 8A Biomedical Grove, IMMUNOS Building, Biopolis, Singapore

11 Translational Immunology Institute, Singhealth/Duke-NUS Academic Medical Centre, the Academia, 20 college road, Discovery tower level 8, Singapore

12 Shanghai Institute of Immunology, Shanghai JiaoTong University School of Medicine, 280 South Chongqing Road, Shanghai, China

13 Institute for Experimental Neuroimmunology, Klinikum rechts der Isar, Technical University of Munich

14 Dept. of Neurology, Klinikum rechts der Isar, Technical University of Munich

15 Munich Cluster for Systems Neurology (SyNergy)

***Abstract***

When Th cell polarization was initially described three decades ago, the Th cell universe grew dramatically. New subsets were described based on their expression of few specific cytokines. Beyond Th1 and Th2 cells, this led to the coining of various Th17 and Treg cell subsets as well as Th22, Th25, Tfh, Th3, Th5, and Th9 cells. High-dimensional single cell analysis revealed that a categorization of Th cells into a single cytokine-based nomenclature fails to capture the complexity and diversity of Th cells. Similar to the simple nomenclature used to describe innate lymphoid cells (ILCs), we propose that Th cell polarization should be categorized in terms of the help they provide to phagocytes (type 1), to B cells, eosinophils and mast cells (type 2) and to non-immune tissue cells, including stroma and epithelium (type 3). Studying Th cells based on their helper function and the cells they help rather than phenotypic features such as individual analysed cytokines or transcription factors, better captures Th cell plasticity and conversion as well as the breadth of immune responses *in vivo*.

## Introduction

T helper (Th) cell polarization is primarily geared towards the responder cells that synergize, amplify and cooperate towards a distinct type of response, while repressing alternative responses at a certain time point of disease or infection. This is to a large part achieved by a complex and tightly regulated network of activating and inhibiting cytokines. Besides the cytokine pattern captured, the helper properties are further expressed through surface molecules, pattern of migration and the ability to enter specific tissues. Here, we focus on what was traditionally used to define Th cells, namely the individual cytokines proposed to categorize Th cells. The expression of cytokines by Th cells depends on upstream signals from the encounter with antigen presenting cells (APCs). This combination of cytokines lays, together with specific transcription factors (TFs) that control their expression, the foundation for the current classification of Th cell subsets. With the emergence of new technologies enabling us to simultaneously measure literally dozens of cytokines along with other markers such as TFs, integrins or chemokine receptors at the single cell level (Galli et al., 2019a), it is no longer feasible to categorize Th cells based on a dominant cytokine or even a family of cytokines (Tortola et al., 2020). Also, by attempting to categorize every single Th cell based on individual cytokines or transcription factors, we may overlook the actual complex biology of the differential responses and other involved cell types. Here, we focus on how the expanding Th cell universe can be reorganized based on the actual help provided towards the actual cellular targets, rather than on the momentary expression of certain cytokines and TFs.

## Historical perspective

The categorization of T cells by their biological properties has provided us essentially with $CD8^+$ cytotoxic killer and $CD4^+$ Th cells. In 1971, an inverse relationship between humoral and cell-mediated immunity was observed by Chris Parish and Eddy Liew and others (Parish and Liew, 1972), laying the foundation for Th cell bifurcation (Bottomly et al., 1978; Kappler and Marrack, 1977; Tada et al., 1978). Eventually, Mosmann and Coffman described in 1986 that Th cells can be polarized to produce either Interferon (IFN)-γ or Interleukin (IL)-4, depending on their environment and stimulatory context (Mosmann et al., 1986). Later, dominant TFs were found to drive this polarization program, namely Tbet for Th1 cells and GATA-3 for Th2 cells (Szabo et al., 2000; Zhang et al., 1997; Zheng and Flavell, 1997). Importantly, one subset actively suppresses the others' ability to produce its characteristic cytokines and transcription factors (Mosmann et al., 1986).

Another, now well-established Th subset comprises of regulatory T cells (Tregs). Already in the early 1970s, experiments with thymectomized mice showed the development of tissue damage in various organs indicating the presence of a suppressive T cell subset developing in the thymus (Gershon and Kondo, 1970; Nishizuka and Sakakura, 1969). However, due to lack of reliable markers to distinguish these cells from other T cells, Tregs underwent a history from being defined as Tr1, when secreting the suppressive cytokine IL-10 *in vitro*, to being termed Th3, when found to secrete TGF-beta upon induction of oral tolerance (Chen et al., 1994; Groux et al., 1997). Nowadays, thymically hard-wired Tregs are characterized by high expression of the high-affinity IL-2 receptor alpha-chain CD25 (Sakaguchi et al., 1995) and the transcription factor FoxP3 (Fontenot et al., 2003) and known to be of particular importance for maintaining immune homeostasis and preventing autoimmunity (Fontenot et al., 2003).

Whereas the simple Th1/Th2 paradigm provided an easy explanation of immune responses towards intra- and extracellular pathogens, respectively, numerous open questions emerged in the context of chronic inflammation and autoimmunity. The path for extending the Th family was cleared after it was noted that the IFN-γ inducing cytokine IL-12 was not the critical factor for the induction of autoimmune pathology in preclinical models of chronic tissue inflammation, mimicking diseases such as Multiple Sclerosis (MS), Rheumatoid Arthritis (RA) and others. Instead, IL-23, which shares the p40 subunit with IL-12, was actually the main driver of the inflammatory response (Becher et al., 2002; Cua et al., 2003; Murphy et al., 2003). Additionally to being pivotal for the development of pathogenic $CD4^+$ T cells in neuro-inflammation, IL-23 also triggered IL-17 expression (Aggarwal et al., 2003; Langrish et al., 2005). Thus, it was recognized that Th1 cells were not the sole driving force for autoimmune pathology, at least in the context of experimental autoimmune encephalomyelitis (EAE), and the call was out for the identification of the true (pathogenic) T helper cell subset(s) in this disease.

In 2005, IL-17 producing Th cells were described as a new entity (Harrington et al., 2005; Park et al., 2005). This subset was readily accepted as an independent Th subset, probably due to its clear segregation from Th1 and Th2 cells, whose induction seemed to antagonize the production of IL-17 (Harrington et al., 2005). The definition of TGF-β and IL-6 as differentiation factors for these T cells *in vitro* (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006), and the identification of RAR-related orphan receptor gamma (RORγt) as critical transcription factor for IL-17 secretion solidified the standing of an independent Th17 subset (Ivanov et al., 2006). Even though the role of Th17 cells in tissue inflammation in general has been heavily debated, IL-17 producing cells have been clearly implicated in a number of chronic inflammatory diseases like Psoriasis, RA and Crohn's Disease (reviewed in (Zwicky et al., 2020)) (Bacher et al., 2019; Borghi et al., 2014). Of note, neutralization of IL-17 in patients triggers fungal infection as a major frequent side effect, demonstrating the importance of IL-17 and IL-17 producing cells (such as Th17 cells) in anti-fungal control in mucosal tissues.

Already in 2000, another new subset was proposed, when two groups showed that B cell help in follicles was provided by specific Th cells that reside close to the B cell zone in secondary lymphoid structures (Breitfeld et al., 2000; Schaerli et al., 2000). These Th cells express the CXC chemokine receptor 5 (CXCR5) that is also expressed on mature B cells and were termed follicular Th cells (Tfh). However, it was not until 2009 that Bcl-6 was identified as the transcription factor necessary for the generation of Tfh cells (Johnston et al., 2009). Even then, the acceptance of Tfh cells as independent entity was strongly debated. Partly, this was due to the observation that the expression of canonical Th1, Th2, or Th17 cytokines like IFN-γ, IL-4 and IL-17, respectively, was necessary to induce a proper class switching reaction in B cells (Mitsdoerffer et al., 2010; Olatunde et al., 2021; Reinhardt et al., 2009). Although the regulation of the expression of these cytokines in Tfh cells is not yet clear, it has been proposed that Tfh cells differentiate independently of other Th subsets from naïve $CD4^+$ T cells when interacting with B cells upon initial activation by dendritic cells (DCs) (Crotty, 2011). Interestingly, the generation and retention of Tfh cells depends on the same antagonistic TFs needed for germinal center B cell differentiation, namely Bcl6 and Blimp-1 (Johnston et al., 2009), which may hint towards a role of specific niches as drivers for T cell diversity and plasticity.

The addition of new cytokines in the analysis workflow of immunology labs led to the description of additional Th subsets, such as Th9 (Dardalhon et al., 2008; Veldhoen et al., 2008), Th22 (Duhen et al., 2009; Eyerich et al., 2009; Trifari et al., 2009) , and Th25 (Wu et al., 2015). To then adjust to this single cytokine-based view on Th cells in immunity, even more subsets were coined. These include pathogenic vs. non-pathogenic Th17, Th17.1 Th17.2 and Th5 cells, among others (Cosmi et al., 2010; Ghoreschi et al., 2010; van Hamburg and Tas, 2018). During this expanding discovery phase of new Th subsets, several voices warned against the idea that the identification of an individual cytokine

expressed by Th cells should not automatically deliver a newly coined subset and that immunologists should keep an eye on the biology of these T cells and their role in immune responses (Locksley, 2009; Zhou et al., 2009). The same holds true for the definition of dominating TF needed to allow the 'discovery' of a new Th subset, especially as most of the subsequent findings were based on *in vitro* studies where specific cytokine cocktails were applied to either naïve or activated purified T cells.

Furthermore, the distinction of subsets requires not only "private" master TFs, but also, and maybe more importantly, stability and the ability to form memory. Stability is largely granted through epigenetic imprinting, which ensures the maintenance of the cells' identity even after an extended period of time and without persistent antigenic threat. Even though there is some evidence that Tregs can develop into Tfh cells (Tsuji et al., 2009) or intestinal intraepithelial cells (Sujino et al., 2016), genetic stability has been best described in Tregs (Rubtsov et al., 2010). Some level of stability has been observed in Th1 and Th2 subsets (Avni et al., 2002; Fields et al., 2002), however not so much for Th17 cells (Mukasa et al., 2010) or any of the other described subsets. At the present day, it is needless to say that the diversity of coined Th subsets has become exceedingly complex and also increasingly controversial among immunologists, as the designation of Th subsets beyond Th1, Th2, and Th17 cells remains debated.

**Limitations of the current Th classification**

The current Th subset classification reaches its meaningful limits when trying to categorize Th cells involved in the induction of pathologies. One prominent example is EAE, a preclinical model for the neuroinflammatory disease MS, where the responsible Th subset was not fully elucidated despite decades of research (reviewed in (Kunkl et al., 2020)). For simplicity, we will here focus on tissue inflammation rather than immunity elicited by pathogens. As a frequently studied preclinical model for tissue inflammation, EAE was believed to be a Th1-mediated disease model because of the abundant IFN-γ expressing Th cell infiltration in the central nervous system (Ando et al., 1989; Voskuhl et al., 1993). However, the observation that loss of IL-12 and IFN-γ signalling, respectively, led to EAE aggravation (Becher et al., 2002; Ferber et al., 1996; Krakowski and Owens, 1996) suggested that Th1 cells were not required for encephalitogenicity, but may even have at least partly a protective role.

Shortly after, it was discovered, that IL-23 signalling was pivotal for EAE induction and simultaneously a potent inducer of numerous cytokines including IL-17 (Langrish et al., 2005). This observation coincided with the claim that Th17 cells represent an independent Th cell subset (Harrington et al., 2005; Park et al., 2005). This association in turn suggested that Th17 cells may represent the pathogenic, disease-initiating population in EAE. However, there are contradicting reports on the effect of the canonical Th 17 cytokines IL-17A and IL-17F on EAE. While one study

described a milder course of EAE upon the depletion of IL-17A (Komiyama et al., 2006), others failed to observe a tangible effect on the progression of EAE upon loss of IL-17A or IL-17F (Haak et al., 2009), making conclusions on the involvement of Th17 cells in EAE more difficult. Only recently, it was shown that the effects of IL-17 on the disease course - besides direct effects on the blood brain barrier and perhaps astrocytes (Kang et al., 2010; Kebir et al., 2007) - stem from its ability to shape the microbiome in the gut, thereby indirectly acting on CNS inflammation by shaping the systemic immune compartment (Regen et al., 2021). The same study showed that exclusive IL-17 production by neuro-antigen specific T cells was dispensable for their pathogenic potential. Moreover, although the use of IL-17 fate-mapping mice showed that the use of CFA as an adjuvant does favour the formation of IL-17 expressing Th cells, upon the initiation of immunopathology, these cells showed a high degree of plasticity (Hirota et al., 2011). After tissue invasion, many of them produced high levels of IFN-γ thereby raising the idea of an intermediate Th17/Th1 phenotype covering the "pathogenic" Th cell subset.

An essential key-player cytokine of this pathogenic Th cell subset is the Granulocyte-Macrophage Colony-Stimulation Factor (GM-CSF). In the context of EAE, GM-CSF is mainly produced by Th cells (Komuczki et al., 2019) and has a dominant function in the development of the inflammatory cascade, as GM-CSF deficient mice are completely resistant to EAE (Komuczki et al., 2019; McQualter et al., 2001; Ponomarev et al., 2007). Furthermore, patients suffering from MS have elevated frequencies of GM-CSF expressing Th cells (Galli et al., 2019b). It appears that GM-CSF, similar to TNF, can be transiently expressed by several Th subsets upon TCR-mediated stimulation (reviewed in (Sheng et al., 2015)) making it difficult to allocate GM-CSF to one of the established Th subsets. Due to the inability to clearly define Th1 or Th17 cells as pathogenic entity in EAE, a new GM-CSF expressing Th subset was discussed (Herndler-Brandstetter and Flavell, 2014; Komuczki et al., 2019; Sheng et al., 2014). This idea was supported by the observation that while GM-CSF was clearly co-expressed with IFN-γ, co-expression with IL-17 was rarely observed (Noster et al., 2014). However, GM-CSF expression has been shown to be regulated by a complex transcriptional network downstream of the TCR including the activity of TFs such as RORγt, NFAT, NFκB, JNK/AP-1, PU.1 and Bhlhe40 (reviewed in (Sheng et al., 2015)), thus no individual dominant TF for GM-CSF expression has been identified so far. The regulation by the different pathways might also indicate the need of tight control of GM-CSF expression to avoid accidental activation of this potent pro-inflammatory cytokine. Regardless as to whether GM-CSF expressing cells represent a new and independent cellular subset, the present categorization of Th cells is not able to unravel the bundle of distinct and overlapping Th subsets, but rather limits the possibilities to define specific (disease-related) processes without colliding with the established nomenclature.

**The power of plasticity**

There is evidence that all Th cells, with the exception perhaps of Tregs, retain a certain degree of plasticity upon differentiation into effector cells. This is a fortuitous feature as it enables immune responses to adapt to changing circumstances based on incoming stimulating or inhibitory cues. Experiments regarding the stability of the single subsets showed that even fully differentiated Th1 and Th2 cells were able to switch their transcriptional signature when challenged under the respective conditions within the first five days of stimulation (Hegazy et al., 2010; Murphy et al., 1996). Prolonged stimulation, however, induced a more stabilized Th1 or Th2 program (Murphy et al., 1996). This indicates that polarized Th cells retain flexibility in regard to their transcriptional signature for several rounds of expansion, giving them enough time to adjust their response to the stimulation. Especially Th17 cells have a particularly unstable lineage commitment, thus readily converting into Th1-like or Treg-like phenotypes (reviewed in (Lee et al., 2009)). The conversion of Th17 into Th1-like cells has especially been associated with the occurrence of organ-specific autoimmune diseases. Importantly, a high degree of Th flexibility cannot only be observed in laboratory animals under strictly defined experimental conditions, but also in the human immune system. One example is the development of different vaccine-specific Th subsets, that were not only diverse directly upon immunisation but even able to change their "fate" with following rounds of expansion (Becattini et al., 2015).

Taken together, the flexibility of Th cells makes their classification based on cytokine patterns alone opaque and bulky. In a review article by O'Shea and Paul (O'Shea and Paul, 2010), the authors acknowledged this challenge and proposed a continuum model where Th cells are positioned across an orbital shape of states with the three transcription factors, ROR t, Tbet and GATA-3 as the three extreme positions.

This 'continuum model' was certainly a step in the right direction, but with increasing numbers of transcription factors and cytokines analysed simultaneously, the anchor points of this orbital model extend into the multidimensional space and can no longer help the visualization and conceptualization of T cell states. Therefore, we believe that the continual bifurcation of Th subsets no longer contributes to the understanding of the plasticity and functionality these cells adduce, but rather unnecessarily complicates our appreciation of dynamic immune responses. Current state-of the art methods such as single cell RNA-sequencing, ATAC-sequencing, and high-dimensional cytometry also failed to capture canonical polarized Th cells, particularly in *vivo* (Kiner et al., 2021; Tortola et al., 2020). Instead, the data support the notion that Th-cell driven immune responses in mammals are highly diverse and complex. Kiner et al recently also challenged the utility of Th archetypes in that unbiased analysis of intestinal Th cells shows that their phenotype is moulded by the microbes they encounter (Kiner et al., 2021). This apparent breadth of Th cell states could be explained by a) Th cells are primed towards a certain lineage, but then retain a high level of plasticity, or b) Th cells are primed towards a diverse continuum and that they are no dedicated canonical lineages. Either way,

dividing Th cells into increasing numbers of subsets, based on the cytokine production measured, may only apply to specific experimental conditions at a certain time point, but does not contribute significantly to a better understanding of Th cell biology. Hence, we propose to take one step back and focus again on the actual helper function of Th cells and consider their polarization based on the target cells they 'help', akin to the designation of Treg and Tfh cells, designations based on function rather than phenotype.

**Reframing Th cell subsets**

In 2018, Eberl and Pradeu proposed a unifying theory that is taking the bigger physiological picture into account (Eberl and Pradeu, 2018). They started by picking up on the idea that the immune system is not activated by recognizing non-self *per se*, but by the change in "normality" – the so called "discontinuity theory" (Pradeu et al., 2013) (that builds upon the danger model that was proposed by Polly Matzinger in the 1990s (Matzinger, 1994)). The new theory considers three levels of immune responses: activation of the immune system by different ways (e.g. intracellular, tissular, extracellular), regulation of the immune response by cross-inhibition of the different types of immune response (Figure 1), and integration of the immune response into other vital processes necessary for maintaining homeostasis at the level of the whole organism (Eberl and Pradeu, 2018). The three types of responses they described are loosely associated with the known concept of type 1, 2, and 3 immunity (Annunziato et al., 2015). Accordingly, type 1 responses are induced by intracellular discontinuities, type 2 responses are involved in tissue repair mechanisms to prevent entrance of pathogens, and type 3 responses are activated by discontinuities affecting the extracellular space, such as fungi and bacteria in barrier tissues (Eberl, 2016). Such a simple classification would mirror that of other lymphocytes with helper function, namely ILCs (for review see (Spits et al., 2013))

We propose to extend this concept towards the initial definition of Th cells; namely their primary function – to provide help. Th cells are not predominantly killers or cleaners, but as their name says, they support and enable other cells in the execution of their tasks. Depending on the context of activation, Th cells interact with different other cell types and produce a variety of cytokines, probably in varying concentrations and for a certain duration. This in turn acts on a palette of cell types including macrophages, DCs, monocytes, B cells or non-immune cell subsets that cross-regulate each other to achieve the desirable/adequate type of response. Therefore, we propose to consider Th cells by the type of the responding cells they target (Figure 2). This classification based on function rather than phenotype is then further refined by the continuum model of O'Shea and Paul (O'Shea and Paul, 2010), to acknowledge the plastic nature of Th cell states. However, while plasticity can be extensive, it is also limited by two major principles: First cross-inhibitory interaction between type 1, type 2, and type 3 responses (as also suggested by Eberl (Eberl and Pradeu, 2018)), and second auto-

amplification of established T helper cell responses. Auto-amplification loops have been described for type 1, type 2, and type 3 responses - mostly based on T cell derived cytokines that directly add back on their sources, re-enforcing their functional phenotype. IFN-γ (Bradley et al., 1996), IL-4 (Kurtjones et al., 1987), and IL-21 (Korn et al., 2007) are examples of such autocrine feed-forward loop drivers for type 1, type 2, and type 3 responses, respectively.

***Type 1 response***

Type 1 responses are executed primarily by mononuclear myeloid cells, such as monocytes, macrophages and DCs. The most canonical type 1 cytokines produced by Th cells are IFN-γ and GM-CSF. The IFN-γ effects in responder cells depend on the nature of the responding cell type (de Veer et al., 2001). The IFN-γ receptor (IFNGR) is a tetramer of two ligand binding IFNGR1 chains and two signal-transducing IFNGR2 chains. While IFNGR1 is constitutively expressed on the surface of most cell types, IFNGR2 expression is more tightly regulated and predominantly found in phagocytes. More than 2000 IFN-γ responsive genes have been identified, including MHCI, MHCII, NOS2, various CAMs like VCAM1, and CD44, IRF1-9 and different TRIM genes (Hertzog et al., 2011). IFN-γ is particularly important for APCs, as it not only induces the upregulation of MHC-I and -II molecules but also slows lysosomal function in macrophages in order to enhance antigen processing (Trost et al., 2009; Yates et al., 2007). Interestingly, other pro-inflammatory stimuli like type I IFN, LPS, and TNF can initiate a similar signalling cascade to IFN-γ (Ahn et al., 1997; Kovarik et al., 1999), thereby modulating the IFN-γ response, but also possibly accounting for the mild phenotype of *IFNG-/-* and *IFNGR-/-* mice (Snapper et al., 1987). However, loss of IFN-γ signalling in mice led to impaired clearance of several intracellular pathogens and a shift in the Th1/Th2 response (reviewed in (Tau and Rothman, 1999)).

GM-CSF similarly acts as a potent communication conduit between T cells and myeloid cells (Croxford et al., 2015; Darrieutort-Laffite et al., 2014; Kobayashi, 2005; Zhan et al., 2012). The GM-CSF receptor is a heterodimer composed of the cytokine-specific α-chain and a β-chain that is shared with receptors for IL-3 and IL-5 (reviewed in (Barreda et al., 2004)). Its cellular expression is even more restricted than the expression of the IFNGR since the GM-CSF receptor is almost exclusively expressed by myeloid cells. *In vitro* stimulation with GM-CSF initiates the differentiation of DCs, granulocytes, and macrophages, depending on the concentration of the cytokine (Sun et al., 2018). The situation *in vivo* is more complex, although there is evidence that also *in vivo* GM-CSF has dose- and time-dependent effects (Guthridge et al., 2006). In general, GM-CSF is promoting survival, differentiation and activation of monocytes, macrophages, and other phagocytes by engaging the JAK2/STAT5 and ERK pathways (Guthridge et al., 1998). Under certain inflammatory conditions, GM-CSF can be regarded as a pro-inflammatory mediator between Th cells and phagocytes (reviewed

315 in (Becher et al., 2016)) and may also act on astrocytes to promote CNS pathology (Sanmarco et al.,
316 2021; Wheeler et al., 2020). Hence, it is not surprising that GM-CSF blocking antibodies are
317 prominently used in clinical trials, e.g. recently in the context of COVID-19 (Mehta et al., 2020).

318 Of note, among others, GM-CSF expression is induced by IL-23, which has also been shown to be
319 important for the modulation of "Th17" responses (Aggarwal et al., 2003; Komuczki et al., 2019), and
320 other type 3 immune responses (see below), making IL-23 both, a type 1 and type 3 response-
321 inducing cytokine depending on the circumstances (perhaps linked to its ability to signal through both
322 STAT4 and STAT3). In this regard, it will be interesting to decipher the additional factors causing a
323 mainly destructive GM-CSF-driven type 1 response versus a protective IL-17-mediated type 3
324 response upon IL-23 exposure. Although it was argued that GM-CSF might serve as a marker for
325 "destructive, or pathogenic" Th17 (or Th1/17, or Th17.1) cells, GM-CSF producing cells preferably
326 co-express IFN-γ over IL-17 (Galli et al., 2019b; Herndler-Brandstetter and Flavell, 2014; Komuczki
327 et al., 2019; Noster et al., 2014). Nevertheless, the relationship with IFN- γ appears to be a complex
328 one, since both, IFN- γ and its driver, IL-12, effectively suppress GM-CSF production in T cells
329 (Komuczki et al., 2019). Of note, whereas T cells can sense IFN-γ, which has long been considered to
330 aid in the maintenance of the Th1 phenotype, GM-CSF is not sensed by lymphocytes themselves. In
331 spite of the apparent contradictions which emerge, when Th cells are categorized by individual
332 cytokines expression, the categorization of Th cells by the target cells they help, alleviates that
333 problem and permits a better understanding of the actual biology of Th cells in type 1 immunity.

334 Taken together, in type 1 responses Th cells mainly target and activate phagocytic cells. While this
335 communication aids in the elimination of intracellular pathogens, aberrant (dysregulated) type 1
336 responses - through persistent recruitment of phagocytes - can be drivers of immunopathology.

337

338 ***Type 2 response***

339 Type 2 immune responses have been initially described to primarily foster humoral immunity, and
340 that Th derived type 2 cytokines help predominantly the B cell compartment and the involved
341 intricacies to generate potent high-affinity antibodies. However, here again, the pure categorization of
342 Th cell by their cytokine profile makes it much harder to capture the function of IL-4 secreting Th2-
343 and Tfh cells alike. As such, type 2 Th cells include not only Th2 and Tfh, but also Th1 cells, since all
344 of them have been shown to be necessary for humoral (type 2) immunity (Crotty, 2015; Smith et al.,
345 2000). Typical type 2 cytokines are IL4, IL-5 and IL-13. IL-4 was the first factor that was recognized
346 to be crucial for B cell maturation and class switching, therefore recognizing Th2 cells as main
347 providers of B cell help (Howard et al., 1982). However, the deletion of Th2 associated genes did not
348 cause loss of germinal centers and later, it became apparent that IL-4 was solely needed for IgE class-
349 switch recombination (Kopf et al., 1995), and that additional factors like CD40L and IL-21 were

needed for fully functional B cell responses, which were attributed to Tfh cells (reviewed in (Crotty, 2015)). Of course, there are various flavours of Tfh cells, which may warrant a Tfh cell-specific nomenclature as suggested by Eisenbarth et al. (Eisenbarth et al., 2021). Nevertheless, in this perspective, we consider their target, namely B cells the reason why Tfh cells are primarily type 2 Th cells.

Another important function of type 2 immunity beyond the engagement of B lymphocytes is the attraction and activation of eosinophils, mast cells and basophils during inflammatory responses. This is mainly achieved by the cytokines IL-5 and IL-13, which induce Eosinophilia and Goblet cell hyperplasia during helminth-infections (Koyasu and Moro, 2011). However, eosinophils, mast cells and basophils are not only type 2 effector cells, but they are also involved in the amplification of type 2 immunity by producing IL-4 and other type 2 mediators themselves. Eosinophil-recruitment, for instance can occur prior to the infiltration of Th cells, which in turn stimulates APCs to initiate a type 2 promoting Th phenotype (Shinkai et al., 2002; Yang et al., 2008). Although it is not fully understood which cell types induce the initial attraction of eosinophils, tissue-resident group 2 ILCs (ILC2s) might be involved as they can react before the adaptive response is initiated (Gasteiger et al., 2015) making them important early phase type 2 players. Furthermore, it has been shown that the presence of ILC2s was required for a complete Th response, at least in the context of allergic inflammation (Halim et al., 2014, 2016).

The alarmin IL-25, also known as IL-17E, was first reported to be secreted by Th2 cells and subsequently led to the coining of Th25 cells as IL-25 producing entity that is boosting type 2 responses by enhancing IL-4, IL-5 and IL-9 production via STAT-5 activation (Fort et al., 2001). Now we know that it can be produced by many different hematopoietic and non-hematopoietic cell types, such as mast cells, alveolar epithelial cells, brain capillary endothelial cells and others (reviewed in (Liu et al., 2018)). The exact mechanisms by which these cells induce and enhance type 2 responses are not fully understood yet, however, there is strong evidence that ILC2s act as type 2 response amplifiers (Moro et al., 2010; Neill et al., 2010; Price et al., 2010).

Another type 2 cytokine that has defined an independent Th subset is IL-9 (Dardalhon et al., 2008; Veldhoen et al., 2008). Initially believed to be a T cell growth factor (Renauld et al., 1993), IL-9 was soon recognized to be crucial for mast cell expansion and recruitment (Townsend et al., 2000). In this context, it is involved in the clearance of parasitic infections but may also play a role in promoting allergic inflammation (reviewed in (Noelle and Nowak, 2010)).

Taken together, type 2 T cells including Tfh cells target primarily B cells to aid in GC formation and class switch, whereas dysregulated type 2 immunity leads to allergic inflammation involving eosinophils, mast cells and basophils.

***Type 3 response***

Type 3 responses have been very well defined as barrier-tissue specific reactions to extracellular disturbances. Receptors for the critical cytokines IL-17 and IL-22 are expressed throughout the stromal and immune compartment, but dysregulated expression of these cytokines (IL-17A, IL-17F, IL-22 etc.) leads to dramatic immunopathology across barrier tissues (skin, lung, gut) with little to no signs of internal organ specific effects (Conti et al., 2009; Haak et al., 2009; O'Connor et al., 2009; Sonnenberg et al., 2010). Ectopic IL-17 expression has the most dramatic effect upon the engagement of the IL-17R complex in epithelial cells of the skin (Croxford et al., 2014). Apart from the production of anti-microbial peptides, IL-17 activated keratinocytes produce a set of chemokines and cytokines that in turn attract neutrophils into the skin (reviewed in (Perera et al., 2012)). Dysregulation of IL-17 in mammals also triggers psoriasiform inflammation, characterized by the cellular expansion of keratinocytes, and the influx of neutrophils. Targeting the type 3 immune response in patients suffering from psoriasis through neutralization of IL-17 or IL-23 dramatically alleviates the clinical symptoms (reviewed in (Zwicky et al., 2020)). Strikingly, IL-23 is critical for both, GM-CSF and IL-17 production in inflammatory conditions (as discussed above). This poses interesting questions about the regulation of IL-23R signalling within different inflammatory conditions and cell types. In line with this, IL-23 has also been shown to be released in response to nociceptor activation (Kashem et al., 2015; Riol-Blanco et al., 2014), linking the immune system with the neuronal network. The notion that there is more to the immune system than simple host defence is applying not only for type 3 immunity and pain sensation. A growing scientific field tries to decipher the interplay of the immune system and other physiological processes like the neuronal network and the enteric system (reviewed in (Rankin and Artis, 2018)).

In line with the notion that type 3 immune responses predominantly involve barrier tissues, physiological amounts of type 3 cytokines (such as IL-17A, IL-17F and IL-22) are involved in the control of mucosal pathogens, in particular fungi (reviewed in (Sparber and Leibundgut-Landmann, 2019)). However, IL-22 producing cells can be easily "reprogrammed" into IFN-γ, or IL-4 expressing Th cells illustrating one more time the dynamics of Th cell plasticity and indicating the importance of a flexible and collaborative environment for a functional immune system (Plank et al., 2017).

Importantly, IL-17 and IL-22 production is readily observed in ILC3 cells and thymic educated γδ-T cells, which are prominent and early responders in barrier tissue immunity, supporting the idea that a major portion of type 3 immunity is an evolutionary hard-wired mechanism of barrier-protection (Kubick et al., 2021).

In summary, in contrast to type 1 and type 2 responses, type 3 responses are less targeted to distinct immune effector cells but activate and regulate non-immune cells. The code, which is used to induce type 3 responses (through e.g. IL-17 and the IL-20 family of cytokines) is likely phylogenetically old

and is used by tissue resident immune cells (like ILC3s and γδ-T cells) to communicate with their non-hematopoietic environment. Eventually, it has been co-opted by the adaptive immune system for host defence at lining tissues.

**Summary and conclusion**

The establishment of advanced single cell analysis tools such as sc-RNAseq and high-dimensional cytometry revealed that the hitherto known classification of the Th cell universe based on previously established cytokine patterns (Galli et al., 2019a; Kiner et al., 2021; Tortola et al., 2020), does not adequately capture the diversity and complexity of the mammalian immune system. For example, it was shown recently that Th cells isolated from the lamina propria could not be attributed to the "classical" Th1 or 17 subsets but rather expressed a continuum of different (signature) cytokines (Kiner et al., 2021). Hence, we propose to take a step back in order to acknowledge the bigger picture instead of focusing on small Th subsets that might simply represent an intermediate stage within their differentiation. By expanding the concept initially proposed by Eberl and Pradeu (Eberl and Pradeu, 2018) and integration of the until now described subsets into a more comprehensive capture of immunity based on the target cells of the Th response (Figure 2), we propose the following nomenclature:

Type 1 Th cells that primarily activate and attract mononuclear phagocytes such as monocytes, macrophages and DCs

Type 2 Th cells targeting B cells and polymorphonucleated granulocytes such as mast cells, basophils, and eosinophils

Type 3 Th cells acting on non-hematopoietic cells at barrier tissue sites, including epithelial cells and stromal cells.

This categorization is in our opinion superior to the coining of ever new Th subsets and sub-subsets. We acknowledge that this concept is however also imperfect in that it does not capture all the possible cellular states and their individual role in immune responses. Furthermore, we would hope to have solid molecular markers of Th cell states to better describe their biology. In lieu of such a 'super-marker' or molecular pattern of Th cell states, this simplified contextual 'help' framework proposed here is also not overly rigid. While polarized Th cells will in general fall into one of the three categories, this does not mean that their role in immunity is by any means inflexible. There is solid evidence of plasticity in memory Th cells and the ability to respond to different challenges with speed and agility. Hence, all attempts to categorize single Th cells observed during a snapshot within a complex immune response cannot truly give an account of the actual function and the role of individual Th cell in the development of a dynamic immune response. The physiological importance

of Th differentiation must be the outcome of the response – the activation/attraction/modulation of responder cells. We hope that this perspective may help to establish a more intuitive classification of Th cell function, which will help to understand the growing complexity in this field. Lastly, this perspective here is not meant to cast a new nomenclature for Th cells, but instead is to initiate the discussion to consider help function over phenotype as a potential stratifier for a more function-based categorization of Th cells.

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**Competing interests**

The authors declare no competing interests.

**Figure legends**

**Figure 1: Cross-inhibition model.** From the perspective of a pathogenic insult type 1 immune responses are typically triggered by intracellular pathogens. Multicellular organisms that cannot easily be phagocytosed induce type 2 responses which support the development of humoral immunity. Type 3 responses are initiated upon extracellular activation at barrier sites like the skin, gut and other mucosal tissue. In this model, the three types of immune response inhibit each other and are strengthened by auto-amplification.

**Figure 2: Orbital model based on Th cell targets.** Th cells can be classified by the primary target cells engaged. Type 1 responses target mononuclear phagocytes including macrophages and monocytes. The responding cells of type 2 immunity are predominantly mast cells, eosinophils and basophils, as well as B cells (in particular in germinal centers). Type 3 cytokines engage predominantly non-immune cells, such as epithelial cells across barrier tissues. In this model, the three types of immunity are interconnected, plastic and allow cross-talk when necessary.

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Figure 1

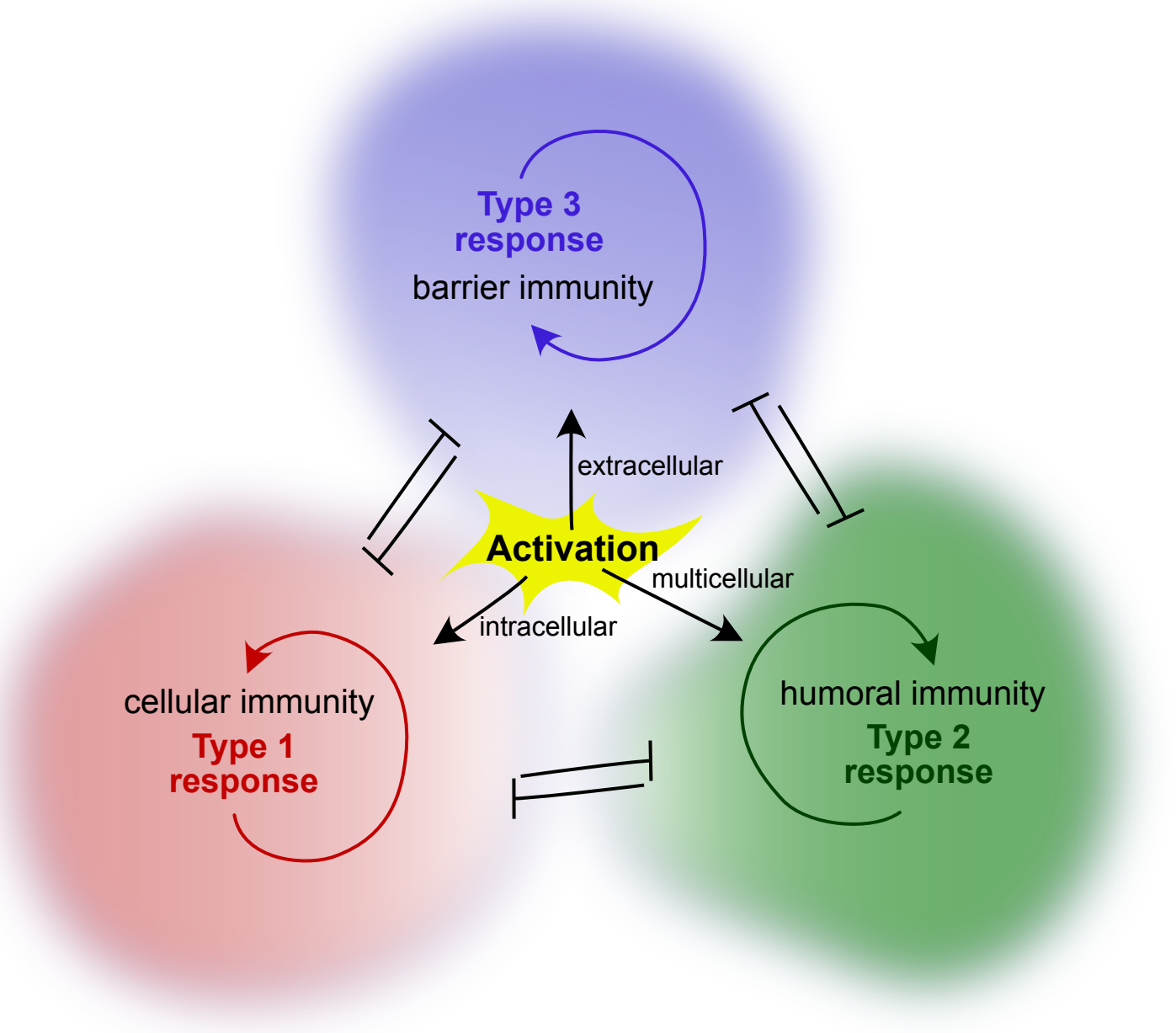
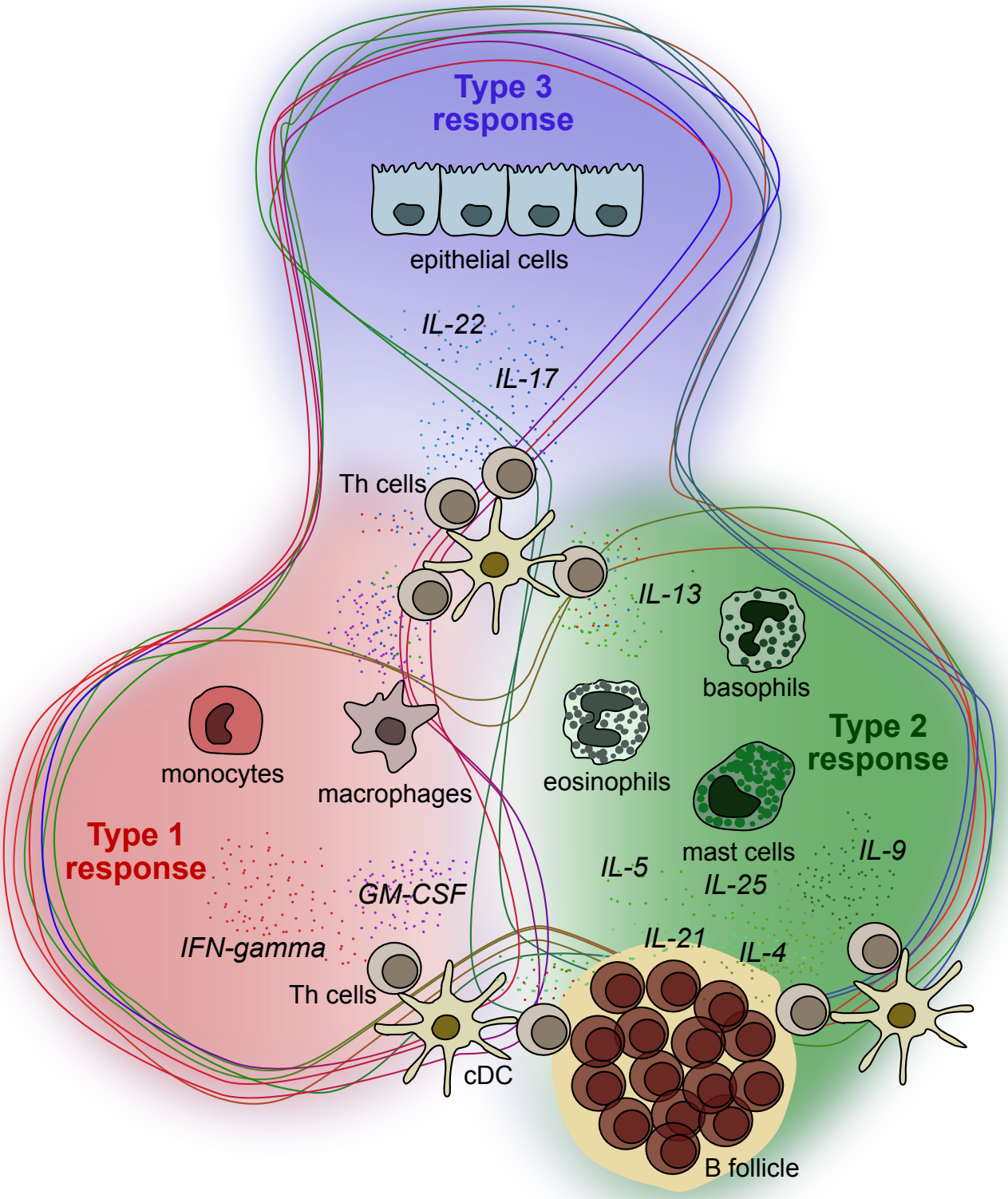

Type 3
response
barrier immunity
extracellular
Activation
multicellular
intracellular
cellular immunity
Type 1
response
humoral immunity
Type 2
response

Figure 2


Type 3
response
epithelial cells
IL-22
IL-17
Th cells
IL-13
basophils
Type 2
response
eosinophils
mast cells
IL-9
IL-5
IL-25
monocytes
macrophages
Type 1
response
GM-CSF
IFN-gamma
IL-21
IL-4
Th cells
cDC
B follicle