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Commentary

Disentangling Tfr cells from Treg cells and Tfh cells: How to untie the Gordian knot

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T follicular regulatory (Tfr) cells are a subpopulation of Treg cells that have adopted the T follicular helper cell program to localize to the B-cell follicle. Because of the difficulties in generating mouse models in which Tfr cells are selectively affected, determining where and how Tfr cells regulate the germinal center response remains to be resolved. In this issue of the *European Journal of Immunology*, Dent and colleagues [Eur. J. Immunol. 2016. 46: 1152–1161] describe a simple, elegant mouse model to conditionally delete Tfr cells without impacting on the Treg- and Tfh-cell populations. Their initial studies suggest that Tfr cells have a more complex role than previously thought, particularly with respect to the regulation of immunoglobulin isotype switching to IgA.

Keywords: Bcl6 · Follicular T cells · Germinal Center Response · IgA · Regulatory T cells

See accompanying article by Wu et al.

Legend has it that when Gordias, a peasant farmer, rode his oxcart into the capital he was proclaimed the new king of Phrygia by an oracle. In gratitude, he tied his oxcart to a post and dedicated it as an offering to Zeus. The knot was so intricate that it resisted all attempts to untie it, and the oracle prophesied that whoever could untie it would rule over all of Asia. In 333 BC, Alexander the Great came upon the knot and, realizing it had no free ends, took his sword and cut it free. Since their first description in 2004 [1], $CD4^+$ T follicular regulatory (Tfr) cells have also resisted all attempts to disentangle them from T follicular helper (Tfh) cells and regulatory T (Treg) cells (Fig. 1). In subsequent landmark studies, Tfr cells were defined as a subset of thymic-derived, $FOXP3^+$ natural Treg cells that are dependent on BCL6 and expression of the chemokine receptor CXCR5 to localize in the germinal center (GC) of the B-cell follicle [2–4]. However, while there was initial consensus on the origin and phenotype of these cells, there was some disagreement on their actual role in the antibody response. This discrepancy may have arisen because these studies necessitated complicated strategies involving mixed fetal liver chimeras and adoptive transfers into lymphopenic mice to either deplete [2] or reconstitute [3, 4] Tfr cells without also directly impacting the Treg or Tfh cells in the respective systems. In this issue of the *European Journal of Immunology*, Dent and colleagues [5] describe a simple new method to selectively deplete Tfr cells, which may go some way toward reconciling some of the inconsistencies in the literature.

Tfr cells are present in secondary lymphoid organs in the steady state and proliferate following immunization with T-dependent antigens such as sheep red blood cells [2] and the hapten (4-hydroxy-3-nitrophenyl)acetyl [2, 3]. Tfr cells simultaneously express both Treg-associated genes, such as *Foxp3, Ctla4, Gitr, Klrg1,* and *Prdm1*, and Tfh-associated genes, such as *Cxcr5, Pdcd1, Bcl6, Cxcl13,* and *Icos* [2]. Thus, Tfr cells can be described as suppressive $Ki67^+$ effector Treg cells, which have co-opted the Tfh-cell programme to localize to the GC [6]. Importantly, while they express high levels of IL-10, Tfr cells do not express IL-21, the T helper cytokine critical for GC B-cell responses [7]. Nevertheless, it is still unknown how Tfr cells are able to express *Bcl6, Prdm1,* and *Foxp3* at the same time, since some of these transcriptional regulators are considered mutually antagonistic [8]. Lineage

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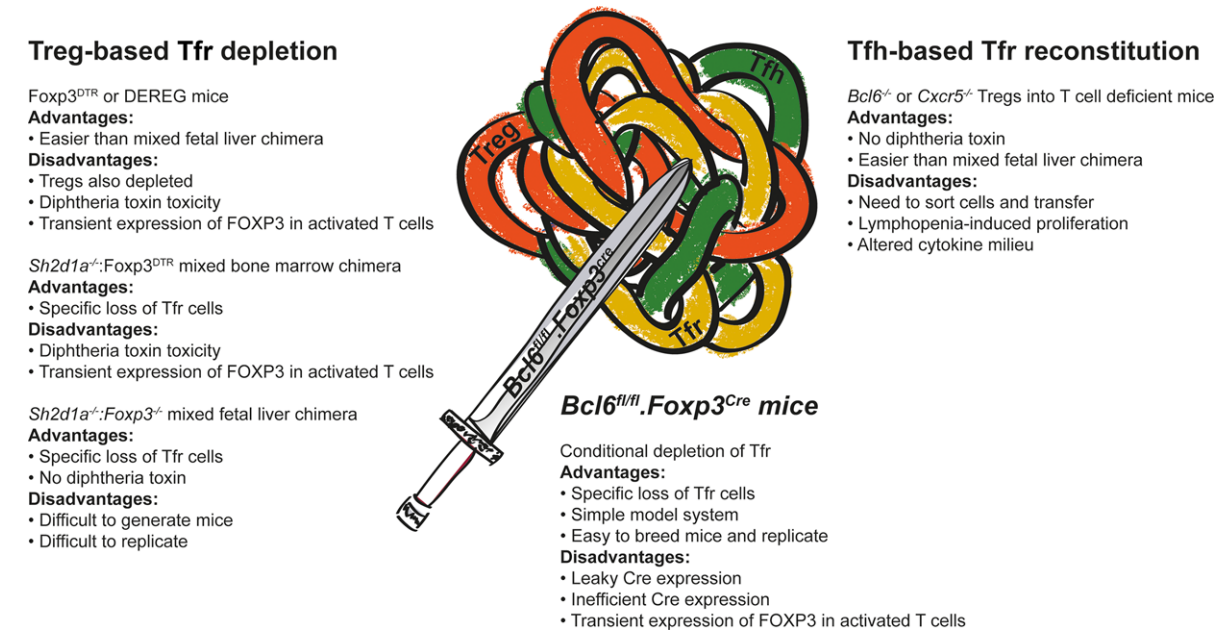

Figure 1. Disentangling Tfr cells from Treg cells and Tfh cells. A Gordian knot and Alexander the Great's sword are depicted to represent Treg cells, Tfh cells, and Trf cells and the *Bcl6*$^{fl/fl}$.*Foxp3*^{Cre} mouse, respectively. Tfr cells (yellow) are FOXP3$^+$ Treg cells (red) that have turned on Tfh (green) genes such as *Bcl6* and *Cxcr5* to localize to the B-cell follicle, providing a means to investigate their function in vivo. Shown are current strategies to manipulate Tfr cells and their advantages and disadvantages in comparison to the *Bcl6*$^{fl/fl}$.*Foxp3*^{Cre} mouse.

tracing experiments using donor cells from Foxp3^{GFP} mice have shown that Tfr cells are derived from FOXP3$^+$ precursors and not Tfh cells [2–4]. Tfr cells also express the transcription factor *Helios*, suggesting that they are derived from natural Treg cells [2, 3]. Similar to Tfh cells, Tfr cells require the transcription factor BCL6 for their development. Tfr cells are also dependent on interactions with B cells, which are mediated by the signaling lymphocytic activation molecule-associated protein (SLAM-associated protein) encoded by the gene *Sh2d1a*. It is notable that while expression of CXCR5 is required for the follicular localization of Tfr cells [3, 4], it is unclear whether it is needed for Tfr-cell expansion (via interactions with B cells at the T-B border), or for Tfr-mediated suppression (via interactions with Tfh or B cells in GCs) [6, 9]. Another key consideration is the transient induction of FOXP3 in activated nonregulatory T cells [10–12] and the stability of Foxp3 expression in Treg cells [13]. For example, TGF-β signaling can induce [11] and also maintain FOXP3 [14] expression in nonregulatory T cells. Interestingly, TGF-β also promotes Tfh-cell differentiation by downregulating IL-2Rα (CD25), thereby "insulating" T cells from the effects of IL-2 in mucosal sites [15], but not in secondary lymphoid organs [16].

The regulation of GC responses by Tfr cells has been demonstrated by a number of investigators taking advantage of Tfr-cell expression of Treg and Tfh molecules to perturb them. One approach has been to use mice in which FOXP3$^+$ cells can be deleted by injection of diphtheria toxin, such as Foxp3^{DTR} and DEREG mice (Fig. 1). In early studies using Foxp3^{DTR} mice, it was reported that injection of diphtheria toxin resulted in increased numbers of Tfh cells but not GC B cells compared to injection of saline [2]. Surprisingly, this was associated with decreased titres of low- and high-affinity antibodies following prime-boost immunization with NP-keyhole limpet haemocyanin (NP-KLH) in alum, indicating failure of GC selection despite the increase in the number of Tfh cells [2]. Similar results were obtained in a recent study using influenza virus infection, indicating that Tfr cells may augment the GC response [17]. However, this system will not only deplete Tfr cells but also all FOXP3$^+$ Treg cells [2]. This was addressed by making mixed bone marrow chimeras containing a 1:1 mix of *Sh2d1a*$^{-/-}$ and Foxp3^{DTR} donor cells to specifically delete Tfr cells [2] (Fig. 1). A caveat with the use of diphtheria toxin is that it induces nonspecific inflammation even in wild-type mice, and this limitation has recently been reported for DEREG mice [18]. Therefore, investigators went on to make *Sh2d1a*$^{-/-}$:*Foxp3*$^{-/-}$ mixed fetal liver chimeras and this also showed abundant GCs with decreased antigen-specific GC B cells, BM plasma cells, and memory B cells 21 days after immunization with NP-CGG [2] (Fig. 1). In contrast, in studies in which the Tfr compartment was reconstituted with WT, *Cxcr5*$^{-/-}$ or *Bcl6*$^{-/-}$ Treg cells in *Tcrb*- [3] or *Tcra*-deficient mice [4], Treg cells failed to localize in the GC and the opposite result was obtained (Fig. 1). This CXCR5-dependent localization of Tfr cells appears to be driven by NFAT2 [19]. Thus, immunization of mice with KLH in CFA following adoptive transfer of a 1:9 mix of Treg cells and naïve CD4$^+$ T cells showed increased numbers of GC B cells but not Tfh cells in mice that had received *Cxcr5*$^{-/-}$ or *Bcl6*$^{-/-}$ Treg cells, compared with those that had received WT Treg cells [3]. Significantly, affinity maturation and antibody production was much greater in mice that had received *Bcl6*-deficient Treg cells following NP-KLH immunization. Similarly, cotransfer of a 1:1 mix of OT2 *Rag2*$^{-/-}$ T cells and *Cxcr5*$^{-/-}$ Treg cells and immunization with OVA in

alum resulted in larger GCs and higher titres of anti-OVA antibodies compared to cotransfer with WT Treg cells [4]. Consistent with these results, Tfr cells are expanded in PD-1 deficient compared to WT mice, and transfer of circulating Tfr cells from blood into $Tcra^{-/-}$ recipients also resulted in a marked decrease in the serum antibody response to NP-OVA immunization [20]. Taken together, these and other reconstitution experiments [21] suggest that Treg and Tfr cells suppress the GC response. However, the results from these experiments may have been influenced by the cytokine environment in T-cell deficient hosts and the lymphopenia-induced proliferation of transferred cells, particularly Treg cells, which are highly sensitive to IL-2 levels [22].

To overcome these limitations, Dent and colleagues [5] have crossed $Bcl6^{fl/fl}$ with $Foxp3^{Cre}$ mice to generate mice with conditional depletion of Tfr cells (Fig. 1). Remarkably, depletion of Tfr cells in $Bcl6^{fl/fl}.Foxp3^{Cre}$ mice had no impact on the Tfh-cell or GC B-cell compartment, suggesting that Tfr cells do not control the size of the GC response [5]. Furthermore, while IgG antibody titres were decreased in $Bcl6^{fl/fl}Foxp3^{Cre}$ mice following immunization with SRBC and NP-KLH, there was a corresponding but smaller increase in IgA antibody titres. This striking pattern of immunoglobulin isotype switching was also seen in the pristane-induced lupus model in which there was more IgA and possibly less IgM and IgG anti-dsDNA autoantibodies [5]. In addition, the authors showed decreased affinity maturation following prime-boost vaccination with the gp120 vaccine. These data point to a more nuanced regulation of the GC response by Tfr cells than previously thought.

So why are the results so discordant with previous studies? One possibility is that depletion of Treg cells with diphtheria toxin in $Foxp3^{DTR}$ mice resulted in more efficient extrafollicular priming of Tfh cells, and lymphopenia-induced proliferation of Treg cells in T-cell deficient mice resulted in more pronounced suppression of Tfh cells in the reconstitution systems. Alternatively, it is possible that Tfr cells in $Bcl6^{fl/fl}.Foxp3^{Cre}$ mice are unable to access the GC and accumulate at extrafollicular sites where they can still exert their regulatory function. In addition, the transient expression of FOXP3 in some activated Tfh-cell precursors may lead to their depletion in this system, particularly in mucosal sites where TGF-β is abundant. Thus, future examination of Peyer's patches in this system will be informative, especially with regard to the elevated IgA antibody response. As with all conditional Cre systems, $Bcl6^{fl/fl}.Foxp3^{Cre}$ mice may also have issues with leaky off-target deletion and inefficient deletion. Nevertheless, the simplicity and accessibility of $Bcl6^{fl/fl}.Foxp3^{Cre}$ mice is a clear advantage and will make it easy for others to replicate and extend these findings.

So where and how do Tfr cells regulate the GC response? Imaging of $Foxp3^{GFP}$ cells in the steady state by two-photon microscopy shows two distinct subpopulations of $Foxp3^{+}$ Treg cells, which are confined to the B-cell follicle and T-cell zone [23]. However, it remains to be seen if these "follicular Treg cells" are the same as Tfr cells and if they relocalize to the T-B border or GC to interact with B cells or Tfh cells following immunization. Recently, inducible global [24] and conditional Treg-specific deletion of CTLA-4 [25] was shown to result in increased Tfr cells with poor suppressive function and increased numbers of Tfh and GC B cells. Interestingly, CTLA-4 regulates expression of the costimulatory molecules CD80 (B7-1) [25] and CD86 (B7-2) [24, 25] in pre-GC but not GC B cells, suggesting the Treg cells or Tfr cells exert their suppressive action outside of GCs. This would be consistent with data suggesting that interactions between T cells and B cells at the T-B border were mediated by CD28 and CD80/CD86 [26]. Whether similar mechanisms that impair the capacity of GC B cells to costimulate and acquire T-cell help also operate in the GC remains to be seen. Dent and colleagues, however, did not examine expression of costimulatory molecules by GC B cells. Another possibility that was not explored was the role of limiting IL-2 availability by Tfr cells in Tfh-cell development and maintenance [17]. Dent and colleagues show that Tfr cells express lower levels of IL-2Rα than Treg cells, suggesting that they may not be as efficient at competing for IL-2 [5]. It is unknown if this is due to exposure to increased levels of TGF-β. Regardless, Tfr cells expressed higher levels of T cell immunoreceptor with Ig and ITIM domains (TIGIT) than Treg cells and this may be another mechanism of suppression. Dent and colleagues also measured cytokine secretion by Tfh cells and found increased levels of interferon-γ, IL-10, and IL-21 but not IL-4 [5]. IL-10 may promote IgA class switching but it would be useful to know if TGF-β, a major IgA switch factor, was also elevated [27]. Tfr cells can egress from the lymph node and circulate in the blood [20] and circulating Tfr cells have memory-like properties [28]. The trafficking pattern of primary and secondary Tfh cells is quite distinct [29] and it will also be interesting to see if similar differences also apply to Tfr cells.

While $Bcl6^{fl/fl}.Foxp3^{Cre}$ mice are a significant advance for the field, a recent study has reported that it is also possible, under very specific experimental conditions, to generate antigen-specific Tfr cells in the periphery from naïve Foxp3-negative precursors [30]. These "induced Tfr cells" express low levels of neuropilin-1 suggesting that they are not natural Treg cells. These data are in stark contrast to the studies described above. How these and $CD8^{+}$ Treg cells [31] contribute to regulation of the GC response by Tfr cells remains to be seen. Like Alexander the Great, it is hoped that $Bcl6^{fl/fl}.Foxp3^{Cre}$ and other similarly elegant mouse models will be utilized to address these outstanding questions and go on to conquer the field.

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