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Published in:
Journal of Autoimmunity

DOI:
10.1016/j.jaut.2024.103174

Publication date:
2024

Document version
Publisher's PDF, also known as Version of record

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Citation for published version (APA):
Repeated immunization with ATRA-containing liposomal adjuvant transdifferentiates Th17 cells to a Tr1-like phenotype

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https://doi.org/10.1016/j.jaut.2024.103174
Received 31 August 2023; Received in revised form 17 January 2024; Accepted 31 January 2024
Available online 19 February 2024

ABSTRACT

In many autoimmune diseases, autoantigen-specific Th17 cells play a pivotal role in disease pathogenesis. Th17 cells can transdifferentiate into other T cell subsets in inflammatory conditions, however, there have been no attempts to target Th17 cell plasticity using vaccines. We investigated if autoantigen-specific Th17 cells could be specifically targeted using a therapeutic vaccine approach, where antigen was formulated in all-trans retinoic acid (ATRA)-containing liposomes, permitting co-delivery of antigen and ATRA to the same target cell. Whilst ATRA was previously found to broadly reduce Th17 responses, we found that antigen formulated in ATRA-containing cationic liposomes only inhibited Th17 cells in an antigen-specific manner and not when combined with an irrelevant antigen. Furthermore, this approach shifted existing Th17 cells away from IL-17A expression and transcriptomic analysis of sorted Th17 lineage cells from IL-17 fate reporter mice revealed a shift of antigen-specific Th17 cells to exTh17 cells, expressing functional markers associated with T cell regulation and tolerance. In the experimental autoimmune encephalomyelitis (EAE) mouse model of MS, vaccination with myelin-specific (MOG) antigen in ATRA-containing liposomes reduced Th17 responses and alleviated disease. This highlights the potential of therapeutic vaccination for changing the phenotype of existing Th17 cells in the context of immune mediated diseases.

1. Introduction

T helper (Th)17 cells are a vital component of the immune system protecting the host against foreign pathogens, but their implication in many inflammatory and autoimmune diseases including multiple sclerosis (MS), rheumatoid arthritis (RA) and type 1 diabetes (T1D) (reviewed in Ref. [1]), makes them an important target for therapeutic intervention. In MS, Th17 cells disrupt the junction proteins in the endothelial cells of the central nervous system (CNS) and migrate through the brain-blood barrier (BBB) [2] to accumulate in the cerebrospinal fluid [3]. Originally, it was believed that Th cells were determined to their specific lineage after differentiation, but an important feature of especially Th17 cells is their plasticity and capability to transdifferentiate into other CD4 T cell subsets, e.g. Th1-like cells, Th2-like cells or T regulatory cells (Tregs) [4,5].

Th1-like exTh17 cells derive from Th17 cells under inflammatory conditions, such as in the experimental autoimmune encephalomyelitis (EAE) model where nearly all IFN-γ expressing cells originate from Th17 cells [6]. Under the influence of e.g. interleukin (IL)-1β, IL-12, IL-23, prostaglandin E2 (PGE2) and TNF-α, Th17 cells can convert into cells expressing the Th1-associated transcription factor T-bet (reviewed in Ref. [7]). Circulating CD4+ cells producing IL-4 and IL-17 and expressing both RORγt and GATA3 have been observed in patients with allergic asthma, but it is not completely understood if this
Th2/Th17-like phenotype derives from Th2 or Th17 cells [8]. Furthermore, Th17 cells can differentiate into FoxP3+ Tregs and FoxP3<sup>−</sup> T regulatory type 1 (Tr1) cells and in IL-17A fate reporter mice, εTh17 cells from the intestine expressed markers related to both FoxP3+ Tregs and IL-10-producing FoxP3<sup>−</sup> Tr1 cells at steady state [9]. During a non-resolved immune response, εTh17 cells acquired a Th1-like phenotype but became Tr1-like during self-limiting inflammation [9]. Therefore, utilizing Th17 cell plasticity could be a potential strategy for therapeutic vaccines against autoimmune disease. Importantly, since Th17 cells play a vital role in the protection against infectious diseases, it would be advantageous to develop a therapeutic vaccine that specifically targets disease-driving autoantigen-specific Th17 cells without compromising the protective function of Th17 cells.

Studies have demonstrated that the vitamin A metabolite All-trans Retinoic Acid (ATRA) can inhibit Th17 polarization while enhancing FoxP3+ T regulatory cell differentiation in vitro [10-14]. ATRA was reported to operate directly on T cells by reducing the expression of RORγt [11] and ATRA stimulation of CD4 T cells isolated from patients with systemic sclerosis increased the frequencies of FoxP3+ Tregs [15]. We hypothesized that ATRA could be used to target disease-causing Th17 cells in autoimmune disease. However, due to its poor solubility, the administration of ATRA as a parenteral formulation is challenging [16]. In this study, we investigated if CAF®1 liposomes composed of the cationic surfactant DDA (dimethylidioctadecylammonium bromide) stabilized with the synthetic immunostimulator TDB (trehalose 6,6′-dibehenate) could function as a delivery system for ATRA, and whether this adjuvant (named CAF®16) could be used to change the phenotype of existing Th17 cells. We found that repeated immunization with antigen formulated in CAF16 liposomes led to a shift of pre-existing antigen-specific Th17 cells towards a Tr1-like phenotype with gene signatures associated with T cell regulation and tolerance. In the experimental autoimmune encephalomyelitis (EAE) model, mice receiving CAF16 formulated with recombinant MOG (rMOG) protein or MOG(35–55) peptide were partially protected against disease progression.

2. Results

2.1. ATRA incorporated in cationic liposomes inhibits Th17 responses

Previous studies have demonstrated that ATRA inhibits Th17 responses [10,12]. Using murine in vitro cultures, we confirmed that ATRA prevented differentiation of isolated murine CD4+ T cells into Th17 cells. Thus, whereas on average 22.7 % IL-17A-producing cells were observed in splenocyte cultures under Th17 cell-promoting conditions, adding ATRA significantly reduced the frequencies by 4.5 fold (p < 0.0001) (Fig. 1A and B). ATRA is a poorly soluble compound [16], making parental formulation challenging. Liposomes can serve as carriers for lipophilic compounds, and in a previous study, we were able to incorporate ATRA into the bilayer of a cationic DDA/TDB adjuvant [17]. This ATRA-containing adjuvant, here named CAF16, also reduced the differentiation of CD4+ T cells into Th17 cells (p < 0.0001) in vitro, whereas Th17 differentiation was intact when adding control DDA/TDB liposomes (CAF01) without ATRA (Fig. 1C). The CAF01 adjuvant elicits high-magnitude Th17 responses when administered with various antigens in vivo [18]. We tested if these responses were retained when ATRA was incorporated in CAF01. We used the recombinant Mycobacterium tuberculosis (MtB) hybrid protein 56 (H56) as a model antigen and injected H56 s.c. with either CAF01 (CAF01:H56) or CAF01+ATRA (CAF16:H56) in mice (Fig. 1D). Incorporation of ATRA in CAF01 completely abrogated antigen-specific Th17 and Th1 responses (Fig. 1E and F). Furthermore, the frequency of cells expressing the Th17 transcription factor RORγt was significantly reduced in CAF16:H56 immunized mice compared to those receiving CAF01:H56 (p < 0.05) (Fig. 1G), whereas the secretion of the anti-inflammatory cytokine IL-10 from restimulated splenocytes was significantly increased (p < 0.05) (Fig. 1H). Using well-characterized tetramers for ESAT-6 and Ag85b (antigen subunits in H56 [19]), we observed that antigen-specific cells were still induced when ATRA was incorporated in CAF01 (Fig. 1I and J) and expression of the classical regulatory T cell marker FoxP3, was significantly increased on antigen-specific CD4+ T cells (Fig. 1K). These results indicate that CAF16:H56, in comparison to CAF01:H56, induces a non-inflammatory and possibly regulatory T cell subset.

In summary, CAF01 served as a promising carrier for ATRA, and ATRA-containing CAF16 reduced IL-17A responses while inducing antigen-specific T cell responses with a regulatory phenotype. Regulatory T cells may be induced by tolerogenic antigen presenting cells at the site of immunization [20]. To investigate how CAF16 affected cells taking up the vaccine at the site of injection (SOI), we injected fluororescently labeled antigen (OVA-AF647) formulated in CAF16 or CAF01 and investigated the phenotype of antigen-positive cells one day later. The frequency of OVA+ cells was slightly higher (statistically significant, p < 0.001) in mice that had received CAF01:OVA compared to CAF16:OVA. Interestingly, markedly fewer of the OVA+ cells expressed MHC-II in the CAF16:OVA group compared to the CAF01:OVA group (statistically significant, p < 0.0001) (Suppl. Figs. 1A–B). The OVA+ cells in the CAF16:OVA group also had significantly reduced expression levels (MFI) of CD86 (p < 0.05), whilst CD80 expression was higher than in the CAF01:OVA group (p < 0.01) (Suppl. Figs. 1C–D). We also investigated the local cytokine milieu at the site of injection, and while there was no measurable IL-10 in either of the groups (not shown), the anti-inflammatory cytokine TGF-β was increased in the CAF16:OVA group. Furthermore, this group had significantly reduced levels of the pro-inflammatory cytokines IL-12 (IL12p70), IL-6, IL-1β, IFNγ and a tendency towards reduced TNFα compared to the CAF01:OVA group (Suppl. Figs. 1E–J). Overall, this suggests that incorporating ATRA in CAF01 (CAF16), inhibits pro-inflammatory cytokine production at the site of injection and changes the phenotype of locally recruited APCs taking up the vaccine.

2.2. CAF16 regulates preexisting Th17 responses

Th17 cells are known to be plastic. Persistent inflammation can drive Th17 cells into εTh17 cells with a Th1-like phenotype [5,21], whilst a subset of Th17 cells acquires a FoxP3<sup>−</sup> Tr1 phenotype during resolution of inflammation [9]. Therefore, we investigated if established antigen-specific Th17 cells could also be targeted using CAF16 in a therapeutic vaccine approach. Mice were immunized with CAF01:H56, to induce a strong Th17 response, followed by repeated vaccination with CAF16:H56 (Fig. 2A). The H56-specific Th17 response, as measured by IL-17A secretion in H56-destimulated splenocytes between the two groups, is significantly reduced in mice that received CAF01:H56 (twice) followed by CAF16:H56 vaccinations (four doses) compared to the group that only received two doses of CAF01:H56 (p < 0.001) (Fig. 2B). The frequency and absolute number of splenic antigen-specific IL-17A+ T cells in the mice that received CAF01:H56 followed by CAF16:H56 was also significantly reduced (p < 0.0001) compared to those that only received CAF01:H56 (Fig. 2C and D). The effect of CAF16:H56 on existing Th17 cell responses appeared to be unique, since the administration of H56 alone or H56 formulated in the oil-in-water emulsion AddaVax did not reduce IL-17A secretion from stimulated PBMCs when administered following CAF01:H56 immunization (Fig. 2A and E). In contrast, CAF01-induced Th1 responses, measured by restimulating splenocytes and assessing secreted IFN-γ (Fig. 2F), or intracellular IFN-γ (Fig. 2G and H), were not affected by subsequent CAF16:H56 vaccination, suggesting that Th17 cells were the primarily targeted subset. CAF16:H56 administration after CAF01:H56 also reduces secretion of the Th17-related inflammatory cytokines IL-22 (p < 0.001) and GM-CSF (p < 0.01), whilst there was a trend towards reduced IL-17F responses (p = 0.20) (Suppl. Fig. 2). Staining for antigen-specific T cells with the Ag85b-tetramer, we found similar frequencies of Ag85b-specific CD4 T cells in mice immunized with CAF01:H56 followed by CAF16:H56.
Fig. 1. Incorporating ATRA in CAF01 abrogates Th17 responses. A–C) Murine CD4+ T cells were in vitro stimulated under Th17 polarizing conditions (TGF-β and IL-6) with or without ATRA in DMSO added at the indicated concentrations. The frequencies of IL-17A and IFN-γ producing cells were measured by intracellular cytokine staining. A) Representative plots of IL-17A+ vs. IFN-γ+ cells. B) Frequency of IL-17A+ cells in Th17-stimulated cultures, with or without addition of ATRA in DMSO. Data represent triplicates. C) Frequency of IL-17A+ cells in Th17-stimulated cultures with or without addition of DDA/TDB (CAF01) or DDA/TDB/ATRA (CAF16), using a final ATRA concentration of 10 nM. Data points indicate triplicates and are representative of three individual experiments. Statistically significant differences are indicated by *** (p < 0.001) or **** (p < 0.0001) (Ordinary one-way ANOVA with Holm-Šidák’s multiple comparisons test, using the Th17-stim. group as reference). D–K) Splenocytes were harvested 10 days after the last of three immunizations with antigen (H56 = ESAT-6, Ag85b, Rv2660c) formulated in CAF01 or CAF16. D) Experimental setup. E) IL-17A measured by intracellular cytokine staining (ICS) after antigen-restimulation, F) IFN-γ measured by ICS after antigen-restimulation. G) RORγt measured by flow cytometry. H) IL-10 cytokine secretion measured in antigen-stimulated supernatants. I-J) Representative plots and barplot of MHCII tetramers surface staining on antigen (Ag)-specific cells; ESAT-6+ and Ag85b+ cells, K) FoxP3+ Ag-specific cells. E-F, I-K) Data represent two independent experiments, G-H) Data are from one experiment. Statistically significant differences are indicated by *, **, *** or ****p < 0.05, 0.01, 0.001 or 0.0001, respectively (Unpaired t-test between immunized groups). There was no statistically significant difference amongst groups if not otherwise indicated. Data are presented as mean ± SEM with data points displaying responses from individual animals.
Fig. 2. CAF16 inhibits existing antigen-specific Th17 responses. A) Experimental setup. Mice were immunized s.c. with CAF01:H56 two times followed by four immunizations with either CAF01:H56, CAF16:H56, H56 alone or AddaVax:H56 (all using H56 at 5 μg/dose). PBMCs were isolated from blood harvested 10 days after the second (bleed 1), fourth (bleed 2) and sixth (bleed 3) immunization. B) Secreted IL-17A. C) Representative plots of IL-17A+ vs. IFN-γ+ T cells after restimulation of splenocytes with H56. T cells were gated from CD44hi of CD3+CD4+Live singlet lymphocytes. D) Frequency of IL-17A-producing T cells. E) IL-17A cytokine secretion as measured in the supernatants of H56-stimulated PBMCs from bleeds 1, 2 and 3. F) Secreted IFN-γ. G) Frequency of IFN-γ-producing T cells. H) MHCI tetramers surface staining on antigen (Ag)-specific splenocytes (Ag85b+ cells). I) Frequency of Ag-specific RORγt+ cells and J) Ag-specific FoxP3+ cells. B-D and F-G) Data represent at least five individual experiments. Statistically significant differences are indicated by *, **, ***, or ****, p < 0.05, 0.01, 0.001 or 0.0001, respectively (Unpaired t-test between immunized groups). E) Secreted IL-17A from PBMCs. I) Ag-specific cells. J) Ag-specific FoxP3+ cells.
compared to mice immunized only with CAF01:H56 (Fig. 2H), indicating that CAF16 regulates Th17 responses without significantly altering the number of antigen-specific T cells. Amongst tetramer−/+ ROsy expression was significantly reduced in mice immunized with CAF01:H56 followed by CAF16:H56 compared to mice immunized with CAF01:H56 alone (Fig. 2I), whereas FoxP3 was unaffected (Fig. 2I). This indicates that vaccination with antigen in CAF16 following CAF01:H56, leads to reduced frequencies of ROsy expressing Th17 cells, but not induction of FoxP3− Tregs. Th17 cells are important for protection against infectious diseases, and it is thus not desirable to broadly dampen non-pathogenic Th17 responses. To test if CAF16 reduced overall Th17 responses, we established Th17 responses against H56 (by CAF01:H56 immunization) followed by vaccination with CAF16 with either the same antigen (H56) or an unrelated antigen (CTH522, a Chlamydia trachomatis antigen [22]) (Fig. 2K). Mice that received CAF01:H56 followed by CAF16:H56 had significantly reduced Th17 responses compared to those that had only received CAF01:H56. In contrast, immunizations with CAF01:H56 followed by CAF16:CTH522 had no apparent impact on H56-specific Th17 responses (Fig. 2L and M). This indicates that CAF16 must be administered with the homologous antigen in order to dampen antigen-specific Th17 responses. In summary, CAF16 regulates not only de novo generation of Th17 cells, but also preexisting Th17 responses in an antigen-specific manner, while Th1- and FoxP3+ Treg-responses are unaffected.

2.3. CAF16-promoted exTh17 cells display distinct markers associated with immune regulation

Our results demonstrate that CAF16 can regulate preexisting Th17 responses. We next asked whether CAF16 eliminates the Th17 cells or induces transdifferentiation into a non-IL-17A-expressing “exTh17” cell subset, as has previously been described in the context of infectious or autoimmune diseases [4–6,9]. We therefore examined the phenotype of the exTh17 cells arising from CAF16 immunization, given after Th17 priming (using CAF01:H56), in the IL-17A fate reporter mouse strain (IL17AγCX3CR1YFP/eYFP) [6]. In these mice, transcription of IL17A at any point during a cell’s lifespan results in continuous yellow fluorescent protein (YFP) expression irrespective of current IL17A production allowing us to track cells that have previously produced IL17A (Fig. 3A). Immunization of IL17A+ fate reporter mice with CAF01:H56 only or CAF01:H56 followed by four immunizations with CAF16:H56 induced similar numbers of splenic YFP+ cells (Th17-primed cells) (Fig. 3B). However, the latter group had significantly lower frequencies of YFP+ cells producing IL-17A (p < 0.0001) (Fig. 3C). These cells also had significantly reduced CCR6 and CXCR3 surface expression compared to YFP+ cells from mice that had received CAF01:H56 only (Suppl. Figs. 3A and 3B), thus indicating that immunization with CAF16: H56 diverts existing Th17 cells into exTh17 cells.

To further characterize the CAF16-induced exTh17 cells, we used the IL-17A fate reporter mice to perform bulk RNA-sequencing, comparing unstimulated cells generated from immunization with CAF01:H56 followed by CAF16:H56 (Fig. 3A). We found a total of 94 genes significantly upregulated in YFP+ cells compared to mice that only received CAF01:H56. These included several genes related to regulators of T cell signaling e.g. Hvac2, Rorc, Pdcd1 (PD-1), Cila4, Mmp9, Mt1, Rgs16, Dusp2, Il10ra and Sostdc1 (Fig. 3B–Suppl. Table 1) [23–28]. These results correspond to the gene ontology (GO) analysis, showing significant enrichment of pathways related to regulation of T cell activation and cytokine signaling (Suppl. Fig. 4B).

To understand the fate of the exTh17 cells in more detail, we performed gene set enrichment analysis (GSEA) comparing CAF16:H56 boosting with CAF01:H56 alone, using a described core set of signature genes expressed in Th1, Th2, Th17, or Treg cells [29] (Suppl. Table 2). Of the four tested gene lists, only Tregs had a positive normalized enrichment score (NES) with a false discovery rate (FDR) < 0.05, indicating CAF16:H56 booster immunizations significantly upregulated Treg signature genes (Fig. 3G). Similarly, using the “MyGeneSet” feature (Immgen database), significantly upregulated genes from the CAF16: H56 boosted YFP+ cells compared to YFP+ cells from mice that had received CAF01:H56 only, revealed a phenotype resembling Tregs (Treg-4_FP3+_Nrp1_0) (Suppl. Fig. 4I).

We next sought to validate aspects of the gene-expression analysis at the protein level. Focusing on the Treg signature, we found that CAF16: H56 booster immunization did not increase Foxp3 on the YFP+ cells (Fig. 3H), suggesting that CAF16:H56 booster immunization did not shift Th17 cells towards classical Foxp3+ Tregs. However, several of the upregulated transcripts, e.g. Havcr2, Pdcd1, Il10ra and Cila4 are associated with Tr1 cells [24,28] (Suppl. Fig. 4C). Boosting with CAF16:H56 significantly increased IL-10 responses after antigen restimulation of splenocytes (Suppl. Fig. 4D), but did not appear to upregulate expression of the other Tr1-associated markers Lag-3 and Tgf-β among YFP+ cells (Suppl. Figs. 4E–G) [28,30–32]. We also found increased expression and production of IFN-γ, which can be produced by Th1 and Tr1 cells (Fig. 3I, Suppl. Fig. 4C) [33], but it is unlikely this represents skewing towards Th1 cells as T-bet expression was significantly decreased in YFP+ cells from CAF16:H56 boosted mice (p < 0.05) (Suppl. Fig. 4H) and IFN-γ levels in restimulated splenocytes were unaltered (Fig. 2F and G). Altogether, this indicates that CAF16:H56 boost transdifferentiates Th17 cells into Tr1-like exTh17 cells with markers associated with tolerance. Consistent with this, additional genes associated with T cell tolerance were found to be induced in YFP+ T cells after boost with CAF16:H56, including Isumo1r (encoding Folate Receptor 4 (FR4), Rnf128 (encoding “gene related to anergy in lymphocytes” (GRAIL)), Tox, Tox2, Cx3cr1, Il12r1, Cds8, Il21 and Slamf7 (Fig. 3B–Suppl. Table 1) [34–38]. Among these, we confirmed that PD-1, CD38 and KLRG1 were all significantly upregulated amongst YFP+ cells in mice boosted with CAF16:H56 (p < 0.05) (Fig. S3–L).}

To further investigate the phenotype of CAF16-induced exTh17 cells and explore potential heterogeneity in the arising cell populations, we performed single-cell RNA sequencing of YFP+ splenocytes from IL-17A reporter mice receiving CAF01:H56 versus CAF01:H56 followed by CAF16:H56. In this study, the cells were re-stimulated with antigen to also probe cytokine responses. Subsequently, CD4+ YFP+ cells were sorted and subjected to single-cell RNA sequencing using the BD Rhapsody system (Fig. 4A). The integrated UMAP of the two groups were organized into 8 clusters (resolution 0.5) (Fig. 4B) with cluster 6 representing the highest frequency of classical Th17 cells (Il17a/I17f) (Fig. 4C and D). Separating the two immunization groups, YFP+ cells from CAF16:H56 boosted mice had a higher frequency of cluster 0, 1, 2 and 4, and a lower frequency of cluster 3, 6 and 7, compared to cells from mice that had only received CAF16:H56 (Fig. 4E). There was a low frequency of cells from CAF01:H56 boosted group occupying cluster 6, in line with CAF16:H56 booster immunization suppressing Il17a and Il17f expression. Other top markers in clusters 6 included Il6, Il13, Tf, Il22, Rorc and Il17f (Fig. 4D). Cluster 3, enriched in Il6, Il22, Rorc and Gam1 transcripts associated with pathogenic Th17 cells [39], was also reduced following CAF16:H56 booster immunizations. Another top transcript for cluster 3 was Trdc (Fig. 4D), indicating a population of y8 T cells, but only a subpopulation of cells in cluster 3 was positive for Trdc, and there was no difference in Trdc between the two groups upon
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A) Week

Immunization s.c.

IL-17a

CAF01:H56

CAF01:H56

CAF01:H56

CAF16:H56

CAF01:H56

CAF16:H56

YFP+ cells

sorted

Ag-stimulation.

Flow cytometry

B) CAF01:H56

Th17 primed cells

% of CD3+CD4+CD45R0+YFP+

p<0.054

# of Th17 primed cells

p<0.048

C) CAF01:H56

IL-17A producing YFP+ cells

22.8

7.19

**

D) Up and down-regulated genes

22

94

E) CAF01:H56 + CAF16:H56

Heatmap

Groups

CAFO1:H56

CAF16:H56

F) Heatmap

Groups

G) Enrichment score

CAFO1:H56 + CAF16:H56

CAFO1:H56

CAFO1:H56 + CAF16:H56

CAFO1:H56

H) FoxP3 of YFP+ cells

p<0.057

I) IFN-γ of YFP+ cells

60

J) PD-1 of YFP+ cells

p<0.054

K) CD38 of YFP+ cells

L) KLRG-1 of YFP+ cells

(p<0.05) (caption on next page)
sub-clustering (Suppl. Figs. 5A and 5B). The highest Tr1 score (previously defined as cells expressing a combination of IL10, Lag3, Havcr2, Pdcd1, Clda4, and Tigit [40]) was seen in clusters 0, 1, and 2, which were all upregulated after CAF16:H56 booster immunization (Fig. 4F).

Furthermore, comparing all YFP+ cells from CAF01:H56 alone and CAF16:H56-boostered mice, the Tr1 score was significantly increased in the YFP+ cells derived from CAF16:H56-boostered mice (Fig. 4G). Supervised pseudotime trajectory analysis on scRNAseq data was performed with the IL-17A-expressing cluster (cluster 6) as start cluster. The analysis revealed three resulting lineages (Suppl. Fig. 5C) in which the clusters with the highest Tr1 score were relatively close to cluster 6.

In summary, boosting with CAF16:H56 transdifferentiated Th17 cells into non-IL17a+ (exTh17) cells, some of which expressed markers associated with Tr1 cells and tolerance/anergy.

2.4. Vaccination with CAF16:rMOG reduces IL-17A responses and disease activity in EAE

Our studies demonstrated that CAF16:H56 can divert existing antigen-specific Th17 cells into cells expressing gene programs and cell surface markers associated with T cell tolerance. To investigate whether Th17 responses could be inhibited using an autoimmune-relevant antigen, we immunized mice twice with CAF01 together with the recombinant myelin oligodendrocyte glycoprotein (rMOG) or with CAF01:rMOG followed by CAF16:rMOG (Fig. 5A).

Consistent with our H56 data (Figs. 2 and 3), the frequency of IL-17A+ cells was significantly reduced in rMOG-stimulated splenocytes and lymph nodes (LNs) from mice that received CAF01:rMOG followed by CAF16:rMOG compared to mice that only received CAF01:rMOG (Fig. 5B) (p < 0.05). These results show that CAF16 together with rMOG attenuate an existing rMOG-specific Th17 response.

Next, we investigated the potential of CAF16:rMOG to block pathogenic Th17 cells in two similar models of EAE conducted in separate independent labs to ensure robustness of results [41]. In Exp. 1, EAE was initiated with 200 μg MOG(35–55) in CFA (300 μg Mtbe per dose) administered sc and 100 μg/dose Pertussis toxin (Ptx) administered ip on day 0 followed by another Ptx dose on day 2 (Fig. 5C, left panel). In Exp. 2, EAE was initiated similarly to exp. 1, but with 100 μg MOG (35–55) in CFA (800 μg Mtbe per dose) (Fig. 5C, right panel). In both experiments, CAF16:rMOG immunizations were administered on days 3 and 10. As control, mice received the EAE initiation regimen, but no immunizations, since we suspected that CAF01:rMOG might worsen EAE symptoms, by further increasing Th17 responses (Fig. 2E).

In Exp. 2 CAF16 with the myelin oligodendrocyte glycol-peptide (CAF16:MOG (35–55)) was also tested, using an identical vaccination schedule. Mice were scored from 0 to 8 or 0–5 based on degree of paralysis in Exp. 1 and Exp. 2, respectively. Consistent with the ability of rMOG to regulate Th17 responses, we observed an improvement in EAE score across both experiments after CAF16:rMOG or CAF16:MOG(35–55) administration compared to the unvaccinated group (reduction in AUC, p = 0.055 in Exp. 1 and p < 0.01 in Exp. 2) (Fig. 5D). In addition, the vaccinated groups had reduced IL-17A secretion from MOG(35–55) stimulated splenocytes in EAE mice compared to the control group (trend for significance, p = 0.09, in Exp. 1, and p < 0.001 in Exp. 2) (Fig. 5E). These results showed that rMOG or MOG (35–55) in CAF16 given as a two-dose regimen reduced IL-17A responses and partially ameliorated disease in the EAE model.

3. Discussion

Th17 cells are plastic and can transdifferentiate into other cell subsets [6,8,42] including Treg cells [9]. We hypothesized that we could utilize this plasticity to transdifferentiate pathogenic Th17 cells into a non-inflammatory cell type by a therapeutic vaccine approach. Our study describes delivery of antigens in ATRA-containing liposomes, CAF16, as an approach for antigen-specific therapeutic vaccination. ATRA has been shown to reduce Th17 cell development and increase induction of FoxP3+ cells, making it an interesting molecule to investigate in terms of shifting Th17 and Treg balances [10,12,13]. As ATRA is poorly soluble and difficult to formulate, we investigated the potential of ATRA-containing liposomes (CAF16) [17] for antigen-specific targeting of Th17 cells. Interestingly, repeated vaccination with antigen formulated in CAF16 shifted pre-existing antigen-specific IL-17A-producing Th17 cells to a non-IL-17A-producing exTh17 subset, which also had reduced secretion of the Th17-related cytokines GM-CSF and IL-22. In contrast, repeated immunizations with antigen alone or antigen formulated in an oil-in-water emulsion adjuvant (AddaVax) did not reduce IL-17A responses, and additional CAF01:H56 immunizations rather potentiated IL-17A responses (Fig. 3E), suggesting that the ability to inhibit existing Th17 responses may be unique to CAF16 and possibly other ATRA-containing adjuvants. The resulting non-IL-17A-producing exTh17 cells did not express the classical Treg transcription factor FoxP3+ but rather had higher expression of co-inhibitory receptors present on Th17 cells (Havcr2, Pdcd1, Clda4, Tigit [24,28,40]) and markers associated with tolerance (Izumo1r, Rnf128, Tox, Cd38, Klr1g, Tox [34,37]).

Th17 cells have been demonstrated to be important for the pathogenicity of several autoimmune diseases (reviewed in Ref. [1]). As opposed to vaccines for most infectious diseases, which are administered to immune-naive individuals, therapeutic vaccines against autoimmune diseases must be given to patients with a self-reactive immune response and should aim to resolve or alleviate this. Favorable vaccine targets include therapeutic vaccines against Multiple Sclerosis (MS). IL-17A-producing Th17 cells are abundant in brain tissue of MS patients and their levels correlate with disease activity [3,43]. In the EAE mouse model of MS, transfer of antigen-specific IL-17A-producing Th17 cells to non-IL-17A-producing exTh17 subset, which also had reduced secretion of the Th17-related cytokines GM-CSF and IL-22. In contrast, repeated immunizations with antigen alone or antigen formulated in an oil-in-water emulsion adjuvant (AddaVax) did not reduce IL-17A responses, and additional CAF01:H56 immunizations rather potentiated IL-17A responses (Fig. 3E), suggesting that the ability to inhibit existing Th17 responses may be unique to CAF16 and possibly other ATRA-containing adjuvants. The resulting non-IL-17A-producing exTh17 cells did not express the classical Treg transcription factor FoxP3+ but rather had higher expression of co-inhibitory receptors present on Th17 cells (Havcr2, Pdcd1, Clda4, Tigit [24,28,40]) and markers associated with tolerance (Izumo1r, Rnf128, Tox, Cd38, Klr1g, Tox [34,37]).
therapeutic vaccine regimen. This led to reduced Th17 responses both systemically and in the brains of mice. Previous studies have also demonstrated that continuous i.p. injections of ATRA in DMSO decreased disease score in the EAE model, which correlated with reduced Th17 responses [10,13]. In line with this, we tested rMOG and MOG(35–55) delivered in ATRA-containing liposomes in the EAE model and found improved disease scores, which correlated with reduced Th17 responses. In studies where we initially immunized with antigen formulated in CAF01 to induce Th17 responses, followed by vaccination with homologous antigen formulated in CAF16, we found that four doses of the latter was needed to obtain maximum reduction of Th17 responses (Fig. 2E). Since EAE is a relatively rapidly evolving autoimmune disease we decided to evaluate the effect of CAF16 delivered antigen on EAE using only a two-dose regimen. It is possible that CAF16-adjuvanted vaccines could give a further improved effect if tested in autoimmune diseases with a slower progression, which would allow for repeated dosing. The EAE model demonstrate proof-of-concept that CAF16:Ag immunization can inhibit disease symptoms in an established model of autoimmunity, although other clinically relevant models of autoimmune disease are important for further studies.

Fig. 4. Single-cell RNA sequencing. A) Experimental setup in IL-17A fate reporter mice. Mice were immunized s.c. with CAF01:H56 two times or CAF01:H56 two times followed by four immunizations with CAF16:H56. Spleens were harvested 10 days after the last immunization and restimulated with H56 (1 μg/ml) for 6 h. CD4+ YFP+ cells were subjected to scRNAseq. B) Integrated UMAP of the two groups (resolution 0.5) showing clusters. C) Feature plots of Il17a and Il17f in UMAP. D) Dotplot of the top cluster markers and their average expression and the percentage of cells expressing the gene. E) UMAP of YFP+ cells separated into mice immunized with CAF01:H56 followed by CAF16:H56 (green) compared to mice immunized with only CAF01:H56 (red) (left panel) and the distribution of clusters within the two groups (right panel). F) UMAP displaying cells with a Tr1-signature score (calculated using Seurat’s method AddModulScore with Il10, Lag3, Haver2, Pdcd1, Ctla4, and Tigit) (left panel) and Violin plot of Tr1-signature score in clusters (right panel). G) Violin plot comparing Tr1 score in all YFP+ cells from CAF01:H56 alone and CAF16:H56-boosted mice. Statistically significant differences are indicated by **** p < 0.0001 (Wilcoxon test).
(Foxp3, Ikr2, and Clda4) as well as markers for exhausted antigen-specific cells [46], but it was not investigated whether the Th17 cells were directly targeted and changed phenotype into a less inflammatory subset. Using IL-17A fate reporter mice initially primed to induce antigen-specific IL-17A-producing Th17 cells and subsequently vaccinated with homologous antigen formulated in CAF16, we demonstrated that existing Th17 cells could be directly targeted and converted by a therapeutic vaccine approach (Fig. 3) to decrease their IL-17A expression and upregulate markers associated with Tr1 cells (e.g. Havcr2, Pdcd1, Clda4, Tigit, Rgs16 and Il10ra) [24,28,40]. A GSEA analysis against a described core set of signature genes expressed in Th1, Th2, Th17, or Treg cells indicated that exTh17 cells from mice boosted with CAF16:H56 were most similar to Tregs (Fig. 3G and Suppl. Fig. 4I). Similarly, the Tr1 score (combination of Il10, Lag3, Havcr2, Pdc1, Clda4, and Tigit [40]) was significantly increased in YFP+ cells derived from CAF16:H56-boosted mice compared to CAF01:H56 only immunized mice (Fig. 4G). Together with the lack of increased FoxP3 on YFP+ cells from CAF16:H56-boosted mice, this indicated that CAF16:H56 converted Th17 cells to FoxP3neg Tr1-like cells. Notably, CAF16 only reduced IL-17A responses against the homologous antigen, indicating that overall Th17 responses were not compromised (Fig. 2K–M). The mechanism for this is an important subject for future studies.

Fig. 5. Effect of CAF16:MOG in experimental autoimmune encephalomyelitis (EAE). Two EAE experiments were conducted in two different laboratories. A) Non-EAE experiment where mice were immunized s.c. with recombinant MOG protein (1–125) (rMOG) in CAF01 two times or CAF01:rMOG two times followed by four immunizations with CAF16:rMOG (n = 8). Spleens and lymph nodes (LNs) were harvested 10 days after the last immunization and restimulated with 0, 0.5, 5 or 50 μg/ml rMOG. Cytokine secretion was measured in the supernatants with IL-17A ELISA. B) Secreted IL-17A in the spleen (left panel) and in LNs (right panel).

C–E) EAE mouse model experimental setup performed at two separate locations. C) Exp. 1 and Exp. 2 experimental setups. Left panel, Exp. 1: EAE was induced in female C57BL/6 mice with 200 μl Complete Freund's Adjuvant (CFA) (300 μg Mtb) together with myelin oligodendrocyte glycoprotein (MOG) peptide (MOG35–55), 200 μg/dose) at day 0 as well as Pertussis Toxin (Ptx) on days 0 and 2. The untreated control group (n = 12) was immunized s.c. with buffer and the vaccinated mice (n = 12) were immunized s.c. with CAF16:recombinant-MOG (rMOG) protein two times on days 3 and day 10 after EAE induction. Right panel, Exp. 2: EAE was induced in female C57BL/6 mice with 100 μl Complete Freund's Adjuvant (CFA) (800 μg Mtb) together with MOG(35–55) (100 μg/dose) at day 0 as well as Pertussis Toxin (Ptx) on days 0 and 2. The untreated control group (n = 6) was immunized s.c. with buffer and the vaccinated mice (n = 8) were immunized s.c. with CAF16:MOG(35–55) two times on days 3 and day 10 after EAE induction. D) Exp. 1 EAE score (0–8) based on degree of paralysis evaluated from day 5 and resulting area under curve (AUC) of the EAE score (left panel) and Exp. 2 EAE score (0–5) based on degree of paralysis evaluated from day 0 and resulting AUC of the EAE score (right panel). E) On day 26 and day 22, the spleens from Exp. 1 and Exp. 2 were harvested, respectively, and stimulated with 50 μg/ml MOG(35–55), and IL-17A cytokine secretion was measured on supernatants from Exp. 1 (left panel) and Exp. 2 (right panel). Data are presented as mean ± SEM. Statistically significant differences are indicated by *, **, *** or ****, p < 0.05, 0.01, 0.001 or 0.0001, respectively (unpaired t-test when comparing two groups and ordinary one-way ANOVA with Holm-Sidak's multiple comparisons test using the control as reference when comparing more groups).
cationic structure of CAF16, antigens can be electrosstatically adsorbed to the surface, allowing for simultaneous delivery of antigen and ATRA to the same target cell. It is possible that this leads to generation of tolerogenic dendritic cells in turn influencing the fate of Th17 cells [47, 48]. In line with this, we observed that CA16:Ag reduced the frequency of MHC-II and the expression of CD86 on cells taking up the delivered antigen at the site of injection and decreased the levels of pro-inflammatory cytokines (Suppl. Fig. 1).

CAF16 shifted Th17 cells to non-IL-17A producing exTh17 cells with upregulation of markers related to Tr1-like cells and anergy/tolerance. In the context of chronic infections or cancer, T cells are continuously exposed to antigen, which may drive them into anergy or exhaustion [34,49]. Notably, our studies suggest that vaccination with ATRA-containing protein in adjuvant vaccines may drive CD4 T helper cells to upregulate inhibitory markers, associated with anergy/tolerance [34,38,50], which may hold promise in the context of autoimmune diseases. In summary, this study demonstrates that it is possible to target antigen-specific targeting using ATRA-containing liposomes holds great promise for alleviating autoimmune diseases that are initiated and/or driven by dysregulated Th17 responses.

4. Materials and methods

4.1. Mice

C57BL/6J-OlaHsd female mice were obtained from ENVIGO (Huntingdon, UK). IL-17A fate reporter mice were made by crossing the Il17a tm1.1(icre)Stck/J (IL-17cre) strain with the B6.129X1-Gr(ROSA)26So+tm1(EYFP)Cos/J (R26R-EYFP) strain, both purchased from The Jackson Laboratory (Bar Harbor, USA). Mice were bred and handled at the experimental animal facility at Statens Serum Institut by authorized personnel. Experimental work was conducted in accordance with the regulations of the Danish Ministry of Justice and the Danish National Experiment Inspectorate under permit 2017-15-0201-01363 and in compliance with the European Community Directive 2010/63/EU for the care and use of laboratory animals. Experimental autoimmune encephalomyelitis (EAE) studies were performed by Redoxis AB (Lund, Sweden) and at University of California, San Francisco (UCSF, US), using female C57Bl/6 mice obtained from Janvier, France, or Jackson Laboratory, US, respectively. The experiments were approved by the local animal ethics committee (Malmö/Lund, Sweden under the license: 12369/2018 and UCSF, US under license AN192722).

4.2. Immunization

Mice were injected s.c. three times with two-weeks intervals using the specified antigen at 5 μg/dose in CAFO1 or CAF16. CAFO1 (DDA/TDB) was given as 250/50 μg per dose and CAF16 (DDA/TDB/ATRA/cholesterol) as 200/50/300/200 μg per dose. Alternatively, mice were injected two times with two-week intervals with 5 μg antigen/dose in CAFO1 for Th17 induction followed by four immunizations (with two-weeks intervals) using 5 μg antigen/dose formulated in CAF16. To investigate responses at the site of injection, CAFO1 or CA16 were administered I.m. with Ovalbuminin, Alexa Fluor 647 Conjugate (OVA-AF647, Invitrogen).

4.3. EAE induction

EAE was induced using 200 μg/dose of MOG(35–55) peptide (MBDiosciences, Redoxis exp.) or 100 μg MOG(35–55) (Genemed Synthesis, Inc., UCSF exp.) emulsified with complete Freund’s Adjuvant (CFA) containing IFA (Difco) with M. Tuberculosis H37RA (300 μg or 800 μg H37RA/dose (BD Difco) in the Redoxis experiment (Exp. 1) or UCSF experiment (Exp. 2), respectively). Mice were anaesthetized with isoflurane and the emulsion was injected subcutaneously (volume 100 μl or 200 μl in the Redoxis experiment or UCSF experiment, respectively) in the flank. Pertussis toxin (Ptx, 200 ng/dose, MerckMillipore or List Biological) was administered intraperitoneally (i.p.) on days 0 and 2. CA16:rMOG (25 μg/dose) and CA16:MOG(35–55) (25 μg/dose) vaccine was prepared with 5 nM Sodium acetate buffer and 10 mM Tris-buffer with 2 % glycerol, respectively and administered s.c. in 200 μl on days 3 and 10 following EAE induction. The control group received 5 nM Sodium acetate buffer with 2 % glycerol or PBS in Exp. 1 or Exp. 2, respectively. The mice were scored from 0 to 8 (Exp. 1) or 0–5 (Exp. 2) based on the degree of paralysis.

4.4. Statistical analysis

Statistical significance was calculated using GraphPad Prism software (v9.3.1, GraphPad, CA, USA). Unpaired t-test was used to compare two groups and ordinary one-way ANOVA with treatment groups against a control group. A p-value <0.05 was considered statistically significant and indicated by *, **, *** or **** for p < 0.05, 0.01, 0.001 or 0.0001, respectively.

Data availability


Acknowledgements

We wish to acknowledge the excellent assistance received from Rune Fledelius Jensen and Julia Sid Hansen at the Department of Infectious Disease Immunology at Statens Serum Institut. Furthermore, we are grateful to the experimental animal facilities at Statens Serum Institut for animal handling and in particular to Tia Maria Villumsen, Nete Junkuhn Rosendal and Sabina Christina Kopp Altenburg for their help with breeding of the IL-17A fate reporter mice strain. In addition, we wish to acknowledge Redoxis (Lund, Sweden) and CD Genomics (NY, USA) for running the EAE experiment and bulk RNA sequencing, respectively. We wish to thank Noah Perlmutter from the University of California for assistance with the EAE model. All figures were created with Biorender.com. The study was supported by Independent Research Fund Denmark (FTP1, grant no. 9041-00160B) to G.K.P., a Novo Nordisk Foundation Pioneer Innovator grant (grant no. 0068804) to D.C. and G. K.P., a National Institute of Health grant (no. K08AR072144) to J.F.A., a UCSF Center for the Rheumatic Diseases grant to J.F.A and Nora Eccles Treadwell Foundation to J.F.A.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2024.103174.

References


