Journal of Autoimmunity 97 (2019) 70–76

Contents lists available at ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

# Targeting interferon activity to dendritic cells enables *in vivo* tolerization and protection against EAE in mice

Anje Cauwels[a], Sandra Van Lint[a], Dominiek Catteeuw[a], Shengru Pang[b], Franciane Paul[c], Elke Rogge[a], Annick Verhee[a], Marco Prinz[b,d], Niko Kley[e], Gilles Uzé[c], Jan Tavernier[a,e,*]

[a] Cytokine Receptor Laboratory, VIB Medical Biotechnology Center, Ghent University, 9000, Ghent, Belgium
[b] Institute of Neuropathology, Faculty of Medicine, University of Freiburg, 79106, Freiburg, Germany
[c] CNRS UMR 5235, University Montpellier, 34095, Montpellier, France
[d] BIOSS Centre for Biological Signalling Studies, University of Freiburg, 79106, Freiburg, Germany
[e] Orionis Biosciences, 9052, Gent, Belgium

ARTICLE INFO

*Keywords:*
EAE
Interferon
AcTaferon
Dendritic cells
Tolerization

ABSTRACT

Type I Interferon (IFN) is widely used for multiple sclerosis (MS) treatment, but its side effects are limiting and its mechanism of action still unknown. Furthermore, 30–50% of MS patients are unresponsive, and IFN can even induce relapses. Fundamental understanding of the cellular target(s) of IFN will help to optimize treatments by reducing side effects and separating beneficial from detrimental effects. To improve clinical systemic IFN usage, we are developing AcTaferons (Activity-on-Target IFNs = AFNs), optimized IFN-based immunocytokines that allow cell-specific targeting.

In experimental autoimmune encephalitis (EAE) in mice, high dose WT mIFNα could delay disease, but caused mortality and severe hematological deficits. In contrast, AFN targeted to dendritic cells (DC, via Clec9A) protected without mortality or hematological consequences. Conversely, CD8-targeted AFN did not protect and exacerbated weight loss, indicating the presence of both protective and unfavorable IFN effects in EAE. Comparing Clec9A-, XCR1-and SiglecH-targeting, we found that targeting AFN to plasmacytoid (p) and conventional (c) DC is superior and non-toxic compared to WT mIFN. DC-targeted AFN increased pDC numbers and their tolerogenic potential, evidenced by increased TGFβ and IDO synthesis and regulatory T cell induction. In addition, both regulatory T and B cells produced significantly more immunosuppressive TGFβ and IL-10.

In conclusion, specific DC-targeting of IFN activity induces a robust *in vivo* tolerization, efficiently protecting against EAE, without noticeable side effects. Thus, dissecting positive and negative IFN effects via cell-specific targeting may result in better and safer MS therapy and response rates.

## 1. Introduction

Multiple sclerosis (MS) is an enigmatic inflammatory demyelinating disease of the central nervous system (CNS) leading to multifocal demyelination and axonal loss mostly in the white matter, but also in the grey matter, of both brain and spinal cord, causing a significant decrease in life quality as well as permanent disabilities. Clinical manifestations, which typically start in the third and fourth decade of life, are heterogeneous and depend on the anatomical location of the inflammatory lesions. Several subtypes are defined, with relapsing-remitting MS (RR-MS) representing around 85% of MS cases [1]. If untreated, about half of the people with RR-MS transition to secondary progressive SP-MS within a decade of diagnosis. About 2.5 million people worldwide, primarily women and Caucasians, are affected with MS. MS is more common farther from the equator, possibly linked to sunlight exposure and vitamin D deficiency. What exactly causes MS is still an enigma, probably genetic predisposition combined with environmental and/or viral factors.

First line RR-MS treatment consisted for a long time only of type I IFN and Glatiramer Acetate, a synthetic myelin basic protein amino acid copolymer. Recently, newly approved oral drugs (Teriflunomide, Dimethyl Fumarate) have gained importance as disease modifying therapies (DMTs). However, their exact modes of action are not fully understood, and side effects including itching, alopecia, digestive problems and liver toxicity are frequent. Natalizumab (anti-VLA4), Alemtuzumab (anti-CD52), Ocrelizumab (anti-CD20), Mitoxantrone

* Corresponding author. A. Baertsoenkaai 3, 9000, Ghent, Belgium.
*E-mail address:* jan.tavernier@vib-ugent.be (J. Tavernier).

https://doi.org/10.1016/j.jaut.2018.10.010
Received 11 September 2018; Received in revised form 15 October 2018; Accepted 16 October 2018
Available online 19 November 2018
0896-8411/ 

(topoisomerase inhibiting chemotherapeutic) and Fingolimod (sphingosine 1-phosphate receptor modulator) are second-line DMTs. They typically display better effectiveness, but at the cost of higher risks [2].

The first indication for the clinical use of IFN to treat EAE/MS dates back to 1982. Currently, IFN is still frequently used [3], but the exact mechanism of its action remains unknown. Most patients suffer from mild to moderate side effects including flu-like symptoms, fever and fatigue, but leukopenia, elevated liver enzymes and depression are not uncommon [4,5]. In addition, up to 50% of MS patients are unresponsive to IFN, and in a subset of patients IFN treatment even induces relapses [6,7]. It is conceivable that the current IFN therapy, using WT IFN (signaling in all type I IFN Receptor (IFNAR)-positive cells in the body), causes a mixed response, with protective effects in one cell type but negative and undesirable effects in another. Understanding the specific cellular target of beneficial IFN effects will help us to design better and safer IFN-based treatments for MS. To specifically deliver IFN activity on selected cell types only, we are developing AcTaferons (AFNs). They consist of a mutant type I IFN, with strongly reduced IFNAR affinity, coupled to camelid single domain antibodies (sdAbs) or ligands selectively recognizing cell-specific surface markers. We have recently demonstrated their efficacy as safe, non-toxic antitumor treatments [8,9].

## 2. Materials and methods

### 2.1. EAE model and treatments

All animal experiments followed the Federation of European Laboratory Animal Science Association (FELASA) guidelines and were approved by the Ethical Committee of Ghent University. Male 8 weeks old C57Bl/6J mice were immunized s.c. with 200 μg MOG35-55 in Complete Freund's Adjuvant (CFA) containing 1 mg heat-killed *Mycobacterium tuberculosis*. Two hours and 2 days later 50 ng Pertussis Toxin was injected i.p. First signs of disease typically started on day 10–12. IFN or AFN i.p. treatments were initiated on day 7 or 12 and lasted till day 25. Mice were daily weighed and scored. A score ranging from 0 to 2 indicates progressive tail paralysis, with 1.5 for a partially limp tail, 2 for a completely limp tail. Score 2.5 was given if the animal no longer spreads its hind toes, score 3 for a waddled walk. Scores above 3 indicate increasing paralysis, with 4 for complete hind limb paralysis. If fore limb paralysis was evident, score 5 was given and the animal was euthanized. Batf3$^{-/-}$ and CD11c-IFNAR1$^{-/-}$ mice were used to determine cDC1 involvement and IFN signaling, anti-PDCA1 treatment (BioXCell, 250 μg/mouse given on days 7, 8, 10, 12 after immunization [10,11]) for pDC contribution. To evaluate immune checkpoint involvement, mice were treated with anti-PDL1 sdAb (20 mg/kg daily) or anti-CTLA4 Ab (BioXCell, 10 mg/kg, every 2–3 days). Treatment groups number 5–7, experiments were repeated at least once. Differences were assessed using one-way or two-way ANOVA followed by Dunnett's or Tukey's multiple-comparison test. Survival curves were compared using the log-rank test. GraphPad Prism software was used for statistical analysis. All values depicted are mean ± s.e.m.; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ and $****P < 0.0001$ compared with PBS treated animals, unless otherwise indicated.

### 2.2. AcTaferons

We generated sdAbs binding mouse Clec9A and CD8α. Importantly, they did not interfere with cross-presentation of DC or T cell activation, respectively. In addition, cDC1 were targeted using XCL1, the ligand for XCR1, engineered for better XCR1 binding (removing 25 C-terminal amino acids and mutating V21C and A59C [12]), while pDC were specifically targeted with SiglecH sdAbs [13]. Our lead IFNα2 mutant in mice is hIFNαQ124R, a human IFNα mutant breaching the cross-species barrier and thus weakly active on murine cells (1/100 vs WT mIFNα). The generation and purification of AFNs was described before [8,9].

### 2.3. Hematological and FACS analysis

One day after the last treatment, blood was collected from the tail vein in EDTA-coated microvette tubes (Sarstedt), and analyzed in a Hemavet 950FS whole blood counter (Drew Scientific, Waterbury, USA). Spinal cord sections were dissected and stained with H&E, Luxol fast blue (LFB, for myelination assessment), and antibodies against amyloid precursor protein APP (evaluating axonal damage) or CD3, B220 or MAC-3 for visualizing infiltrating T and B cells and macrophages, respectively. Flow cytometry was done for spleen and lymph node (LN) cells. Doublets were excluded and living cells were selected based on live-dead stain (Invitrogen). pDC (CD3$^-$ CD19$^-$ B220$^+$ SiglecH$^+$) and cDC1 (CD3$^-$ CD19$^-$ CD11b$^-$ CD11c$^+$ MHCII$^+$ XCR1$^+$ Clec9A$^+$) percentages were determined, and the intracellular expression of designated cytokines determined. For regulatory T cells (Tregs), the CD3$^+$ CD4$^+$ CD25$^+$ FoxP3$^+$ population was analyzed. For regulatory B cells (Bregs), the CD3$^-$ CD19$^+$ CD5$^+$ CD1d$^+$ population. Fc receptors were blocked using anti-CD16/CD32 Ab. Fluorescence minus one (FMO) and isotype controls were included to allow adequate analysis. Samples were acquired on an Attune Nxt Acoustic Focusing Cytometer (Life Technologies) and analyzed using FlowJo software.

## 3. Results

### 3.1. Wild type mIFNα11 dose-dependently protects against EAE, associated with severe toxicity

The active EAE model using C57BL/6 mice immunized with MOG35-55 peptide is very robust and uniform and hence widely employed for understanding disease pathology and validating potential novel treatments [14]. In this model, we first defined our positive standard by daily treatment with WT mIFNα11. 5000 IU WT mIFNα had a minor disease-delaying effect (Fig. 1A and B), comparable to mIFNβ effects published before [15–17]. A very high dose of WT mIFNα (1.000.000 IU) was capable of significantly delaying the onset and progression of disease (Fig. 1A and B), but the treatment itself caused 60% mortality (Fig. 1C) and severe hematological deficits in the 40% surviving mice, including dramatic lymphopenia (Fig. 1D), neutropenia (Fig. 1E), monocytopenia (Fig. 1F), anemia (Fig. 1G), as well as severe thrombocytopenia (Fig. 1H), and increased mean platelet volume (MPV, Fig. 1I), indicative of platelet destruction.

### 3.2. Targeting IFN activity to DC protects even better than WT mIFNα, without toxicity

In the classic MS/EAE scenario [18], processing and presentation of self-antigen(s) by antigen presenting cells (APC) causes activation and expansion of peripheral T cells. These auto-reactive T cells migrate across the blood brain barrier (BBB), enter the CNS, and induce disease by activating glial cells and attracting and activating other immune cells, ultimately resulting in severe damage of the central neurons and their myelin sheaths and axons. It was long believed that CD4$^+$ T cells are primary inducers and mediators in MS. However, not CD4$^+$ but CD8$^+$ T cells are the predominant population in human MS lesions, and a role for myelin-specific CD8$^+$ T cells was suggested [19]. Intriguingly, these may develop into either cytotoxic or immunosuppressive CD8$^+$ lymphocytes [20]. Several cell types have been suggested to be responsible for the beneficial effects of IFN in MS [21]. These include T lymphocytes and APC such as dendritic cells (DC). To evaluate their involvement, we targeted AFN to CD8$^+$ cells or to Clec9A$^+$ DC. In contrast to WT mIFNα, 5000 IU Clec9A-AFN significantly protected (Fig. 2A–D) without mortality (Fig. 2E) or hematological problems (Fig. 2F–H). Interestingly, CD8-AFN had no influence on clinical score,

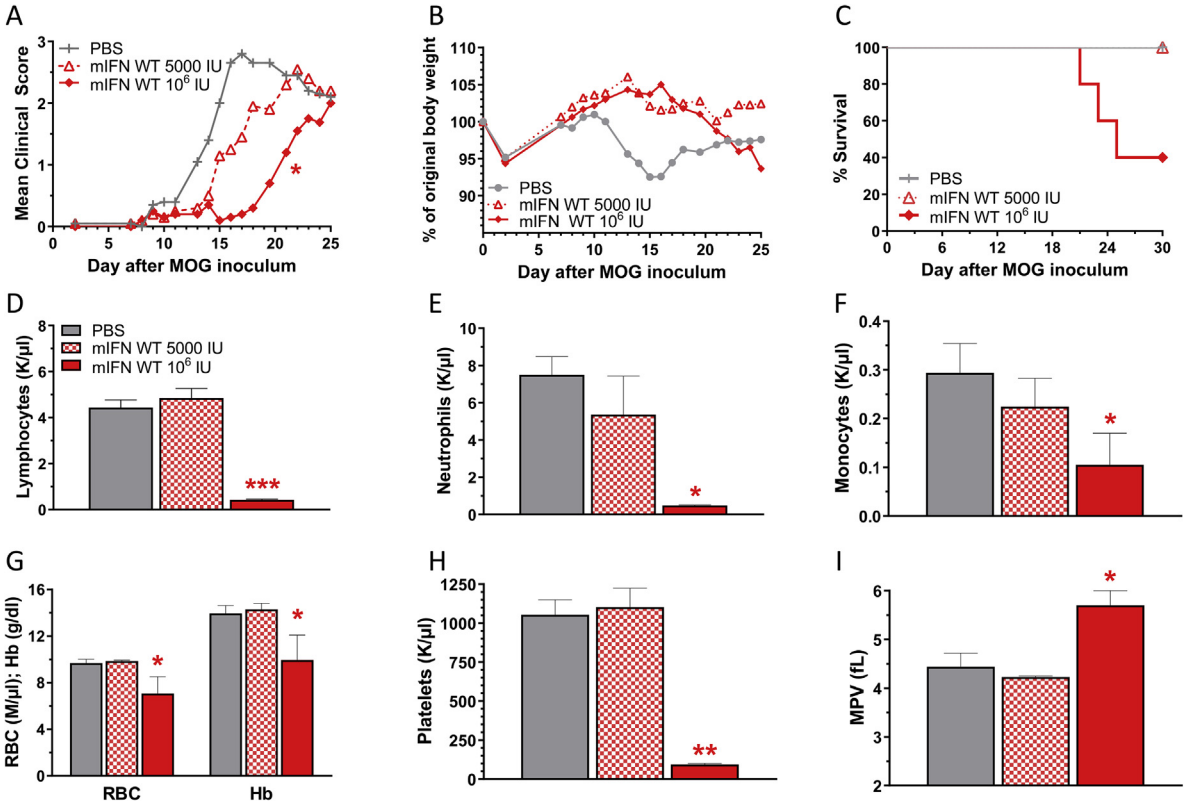


**Fig. 1. WT mIFNα protects dose-dependently, but is associated with severe toxicity.** Mice were immunized on d0, and treated d7-25 with 5000 or $10^6$ IU mIFNα daily. Clinical score (A) and body weights (B) were determined daily. High dose mIFNα caused mortality (C) and severe hematological deficits (D–I). Shown is a representative experiment (n = 5). Differences were assessed using two-way ANOVA followed by Dunnett's multiple-comparison test; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with PBS treated animals.

onset of disease, or paralysis (Fig. 2A–C), but had a detrimental effect on body weight loss (Fig. 2D). Notably, Clec9A-AFN treatment provided long-term protection against EAE progression and the development of paralysis (Fig. 2I), also when given after disease onset (Fig. 2J).

### 3.3. DC-targeted protection is dependent on PDL-1 and CTLA-4

For APC to activate T lymphocytes, a well-defined set of interactions and signals is necessary. Positive signals include TCR-MHC/antigen interaction, binding of costimulatory molecules ($CD28^-$CD80/CD86), and the production of stimulatory cytokines. However, the absence of negative interactions (immune checkpoints) is at least equally important. In MS patients, IFNβ therapy increases PDL-1 levels [22]. In addition, blocking CTLA-4 can exacerbate EAE sensitivity [23,24] or induce EAE sensitivity in non-susceptible mice [25]. Hence, we treated Clec9A-AFN treated EAE mice simultaneously with neutralizing anti-PDL1 sdAbs, or anti-CTLA-4 Abs. Both immune checkpoint inhibitors prevented the protective effect of Clec9A-AFN (Fig. 2K).

### 3.4. DC-targeted protection is mainly mediated by pDC

DCs are typically divided in three major subsets: $Clec9A^+$ $XCR1^+$ type 1 conventional/myeloid DC (cDC1), $CD11b^+$ $SIRP\alpha^+$ type 2 conventional/myeloid DC (cDC2), and plasmacytoid pDC. In humans, only cDC1 are $Clec9A^+$ [26,27]. In mice, however, not only $XCR1^+$ cDC1, but also pDC are $Clec9A^+$. To verify the involvement of the $XCR1^+$ cDC1, we specifically targeted them using XCL1-AFN. Surprisingly, comparison of Clec9A-AFN and XCL1-AFN indicated that not only cDC1 but also pDC may be important (Fig. 2L). As cDC1 specifically require the Batf3 transcription factor for their differentiation, deletion of Batf3 ablates their development [28]. Experiments in $Batf3^{-/-}$ mice indicated not only their susceptibility for EAE, but also the ability of Clec9A-AFN to prevent disease progression, spinal cord damage and inflammation in $Batf3^{-/-}$ animals even better than in WT mice (Fig. 3A–C), suggesting the critical involvement of pDC rather than cDC1. Consequently, we depleted pDC using anti-PDCA1 antibodies [10,11]. Unexpectedly, pDC depletion did not affect Clec9A-AFN protection, not in WT mice (Fig. 3D), but also not in $Batf3^{-/-}$ mice (Fig. 3E). To clarify these puzzling contradictory results, we analyzed the presence of cDC1 and pDC in our various mice and experimental conditions. Surprisingly, whereas naive $Batf3^{-/-}$ mice were indeed cDC1-deficient, cDC1 were present in the EAE-developing $Batf3^{-/-}$ animals (Fig. 3F), probably due to compensatory Batf-induced cDC1 development, which has already been reported in response to intracellular pathogens, mediated by IL-12 and IFN-γ [29]. Hence, the results obtained in $Batf3^{-/-}$ mice are not conclusive with regard to cDC1 involvement. In addition, when analyzing pDC after anti-PDCA1 treatment, we found evidence for pDC depletion in lymph nodes (LNs) during EAE progression in mice receiving PBS therapy (Fig. 3G), but not in spleens (Fig. 3H), or in LNs of mice treated with Clec9A-AFN (Fig. 3G). Together, these results indicate that the pDC depletion experiments cannot provide conclusive interpretations either. Nevertheless, in CD11c-$IFNAR1^{-/-}$ mice, where IFNAR1 and IFN signaling are absent in cDC but present in pDC [30], Clec9A-AFN can still protect, clearly providing evidence for critical pDC involvement in Clec9A-AFN mediated protection.

### 3.5. Targeting pDC is superior to targeting cDC1

To further corroborate the involvement of cDC1 versus pDC, we generated sdAbs specific for SiglecH, a validated pDC marker in mice [13]. SiglecH-AFN treatment was equally proficient as Clec9A-AFN to

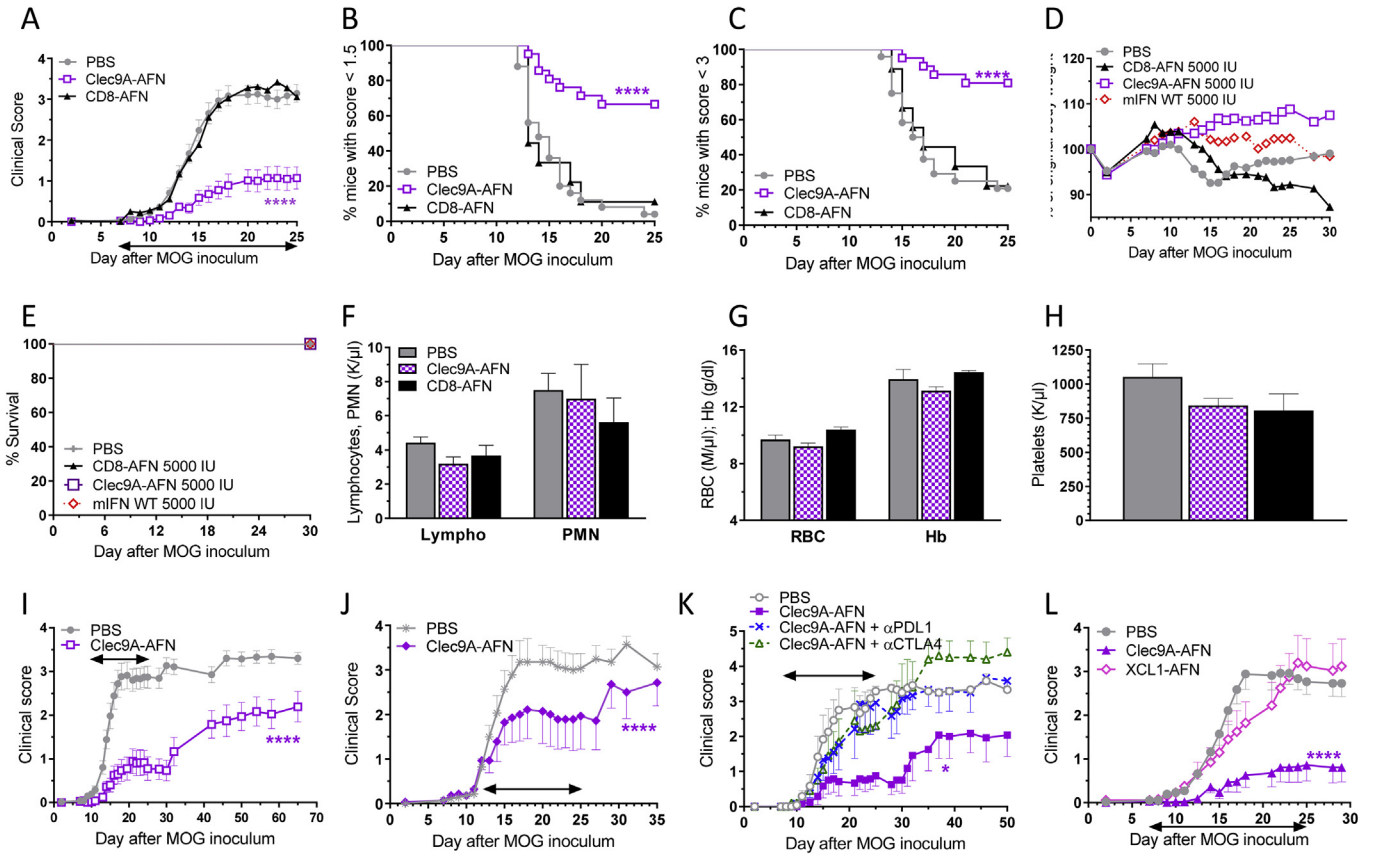


**Fig. 2. Clec9A-AFN, but not CD8-AFN, efficiently protects without toxicity.** Shown are clinical scores (A), % of diseased (B) or paralyzed (C) mice, body weight (D), mortality (E), hematological parameters (F–H). (A–C) Shown are pooled data from 3 experiments (n = 15–18), (D–H) shown is a representative experiment (n = 5). Clec9A-AFN protection is long lasting (I), effective if started after disease onset (J), reversed by anti-PDL1 or anti-CTLA4 (K), and better than XCL1-AFN treatment (L). (I–L) Shown is a representative experiment (n = 6). The black horizontal arrow indicates the treatment period. Differences were assessed using two-way ANOVA followed by Dunnett's multiple-comparison test; $*P < 0.05$, $****P < 0.0001$ compared with PBS treated animals.

protect against EAE development, at least till day 12 (Fig. 3J). Combining SiglecH-AFN with Clec9A-AFN did not add to Clec9A-AFN efficacy (Fig. 3J). These results suggest the critical early involvement of pDC, as well as the additional need for cDC1 later during disease progression. To investigate this possibility, we added XCL1-AFN (targeting only cDC1) to the SiglecH-AFN therapy (targeting only pDC), starting on day 11. Additional XCL1-AFN treatments enabled SiglecH-AFN therapy to become as effective as Clec9A-AFN (Fig. 3K). Corroborating the specificity of XCL1-AFN, its additive effect on SiglecH-AFN therapy was absent in CD11c-IFNAR1$^{-/-}$ mice that lack IFNAR1 on cDC only [30] (Fig. 3L). Thus, targeting IFN activity to pDC and cDC1, using DC-specific AFNs, can very efficiently protect against EAE progression, without the development of toxic side effects.

### 3.6. Targeting DC induces a systemic tolerogenic response

To evaluate how DC-targeted AFN could provide protection in EAE, we analyzed pDC and cDC1 numbers as well as tolerization markers, and evidence of additional tolerogenic cells. While splenic pDC numbers were increased after Clec9A-AFN (Figs. 3H and 4A), there was no increase in LN (Fig. 3G). Determination of SiglecH$^+$ pDC numbers after SiglecH-AFN treatment showed very little pDC, probably due to the endocytic nature of SiglecH, which is very efficiently internalized after engagement [31]. To analyze the tolerogenic nature of the pDCs, we determined their production of the potent immunosuppressive determinants TGFβ, IL-10, and indoleamine 2,3-dioxygenase (IDO) [32–34]. While there was no increase in IL-10, Clec9A-AFN and SiglecH-AFN robustly enhanced both TGFβ and IDO in splenic pDC (Fig. 4B). In contrast to pDC, cDC1 numbers were not different in spleen or LNs (not shown). To dampen immune responses, also other regulatory cell types such as Treg and/or Breg may be involved. Treg numbers were increased in spleens of Clec9A-AFN treated EAE mice, and the percentage of TGFβ and IL-10 producing Tregs was enhanced by both Clec9A-AFN and SiglecH-AFN (Fig. 4C). While Breg numbers were not statistically increased, significantly more of them produced immunosuppressive IL-10 and TGFβ (Fig. 4D).

## 4. Discussion

The results described herein clearly demonstrate the superior protective potential of specifically targeting type I IFN activity to DCs when compared to WT IFN. Indeed, targeting low dose AFN to Clec9A$^+$ or SiglecH$^+$ DCs resulted in a significantly better but non-toxic response compared to the same, or even a much higher (and hence extremely toxic), dose of WT mIFNα. Thus, via specific targeting to DCs, IFN therapy may not only be improved, it is also devoid of systemic side effects. In addition, it is important to realize that, since the same low dose of CD8-targeted AFN actually exacerbated long-term body weight loss, this implies that IFN treatment as currently used in the clinic to treat MS patients may result in disease-retarding effects (e.g. on DCs) but also in potential disease-worsening effects (e.g. on CD8$^+$ cells). Therefore, our results indicate that dissecting positive and negative IFN effects via cell-specific targeting may result in better and safer IFN-based MS therapy.

Since SiglecH-AFN was as good as Clec9A-AFN to protect, at least during the initial phase of disease, we conclude that plasmacytoid DCs are critically involved. Later, conventional type I DC (Clec9A$^+$ and XCR1$^+$ but SiglecH$^-$) may contribute to protection. As most

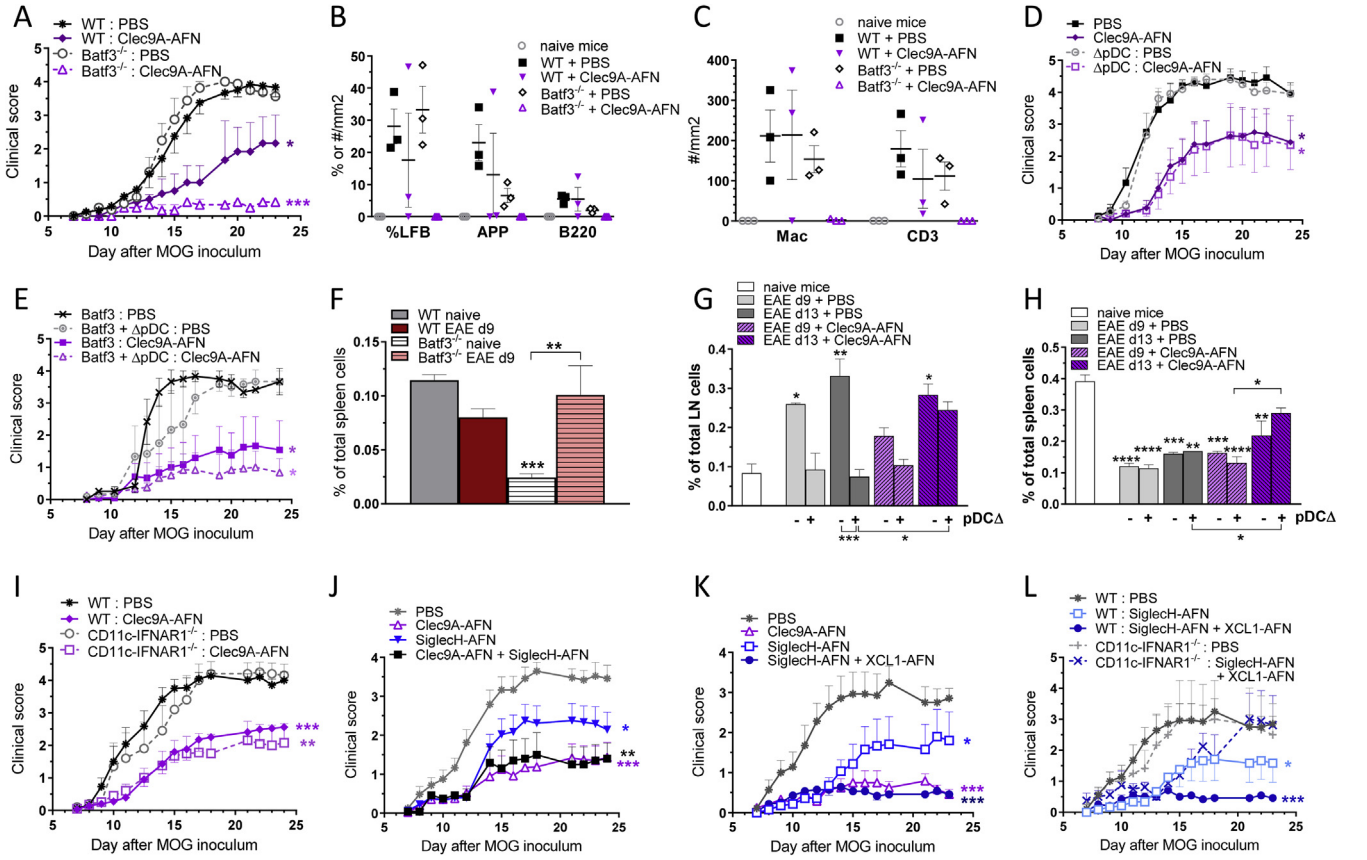


**Fig. 3. pDC rather than cDC1 targeting determines protection.** Clec9A-AFN protects in Batf3$^{-/-}$ (A–C), and in pDC-depleted mice (D,E). Shown are the clinical scores, n = 5 (A,D,E) and spinal cord analysis, n = 3 (B,C) to evaluate demyelination (LFB), axonal damage (APP), B cells (B220), macrophages (Mac) and T cells (CD3). However, EAE in Batf3$^{-/-}$ causes compensatory cDC1 development (F, n = 4), and pDC depletion in LNs is not evident in case of Clec9A-AFN therapy (G) or in spleens (H), n = 3. (G,H) Plus and minus signs under the X axis indicate pDC depletion treatments (+) or not (−). CD11c-IFNAR1$^{-/-}$ are still protected by Clec9A-AFN (I). Selective pDC targeting with SiglecH-AFN is as effective as Clec9A-AFN during initial EAE phase (J, n = 6) and aided by XCL1-AFN later (K, n = 6), but not in CD11c-IFNAR1$^{-/-}$ (L, n = 6). Shown are representative experiments (A-H, J-L), or pooled results from 3 independent experiments (I, n = 10). Differences were assessed using one-way or two-way ANOVA followed by Tukey's multiple-comparison test; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with PBS treated animals, unless otherwise indicated.

autoimmune diseases, MS is the result of incorrectly balanced immunogenic versus tolerogenic responses. DCs are generally acknowledged as key to launch adequate and specific immune responses required to fight infections and cancer [35]. However, DCs, as well as other regulatory cell types such as regulatory T cells (Tregs) and B cells (Bregs), can also dampen T cell responses. While IFN is known to help mature cDC1 to orchestrate an attack on virally infected cells and tumors, IFN has also been shown to induce tolerance, both in *ex vivo* generated DC [36,37] and in isolated pDC [38,39]. In addition, IFN stimulates pDC migration and lymph node accumulation *in vivo*, where Treg expansion may ensue [40,41]. Last but not least, MS patients show a reversed ratio of two pDC types (pDC1/pDC2), favoring effector T cell (Teff) over Treg development, and IFN therapy restores this inverted ratio [42]. Using our DC-targeted AFNs, we found that splenic pDC numbers were increased and their tolerogenic nature was enhanced, as evidenced by augmented TGFβ and IDO expression. In addition, Treg numbers were increased, and they produced more immunosuppressive IL-10 and TGFβ. Although Breg numbers were not increased, they also produced significantly more IL-10 and TGFβ. Together, these data indicate a strong and systemic tolerizing effect of DC-targeted AFNs (Fig. 5).

The use of autologous, *ex vivo* generated tolerogenic DCs (tolDCs) was recently advocated as a promising novel therapy for MS and other autoimmune diseases, and clinical trials are being set up [34,43,44]. In addition, recent evidence suggests that pDCs, *ex vivo* differentiated from bone marrow derived cells, induce recovery in EAE mice [45]. Needless to say, these therapeutic cell-based strategies are extremely laborious, time-consuming, and entirely personalized, and challenging obstacles and pitfalls are associated with the *ex vivo* generation of tolDCs [46]. Next to *ex vivo* vitamin D3 treatment, also other factors, including IFN, have been suggested for generating tolDCs [47]. Hence, we speculate that targeting IFN activity to DCs in patients using AFNs may induce systemic tolerization and thus provide an *in vivo,* generic, safe and easy means to dampen MS, in contrast to cell-based DC transduction.

We have recently shown that Clec9A-AFN reduces tumor growth in various mouse and humanized mice cancer models [9]. We now provide evidence for the same DC-targeted AFN to downmodulate auto-immune effects in EAE. This may seem contradictory. However, as an anti-cancer treatment, the effect of Clec9A-AFN crucially depended on IFN signaling in cDC1 [9]. In the EAE model discussed here, this is not the case. Moreover, in EAE we showed that pDC rather than cDC1 are the principal responsible cells for the tolerizing Clec9A-AFN effect, reproducing the protection with pDC-targeted SiglecH-AFN, especially during the induction phase of the disease. Our results are supported by literature. Indeed, while pDC depletion exacerbates EAE from the onset on [10], cDC depletion worsens disease during the later effector phase [48]. Combined, these results suggest that the effect of type I IFN on pDC during auto-immune EAE development is tolerizing, while their influence on conventional DCs is more complex and context dependent, provoking anti-tumor efficacy in a tumor context, while inducing tolerance in the auto-immune EAE setting. Of note, the activation state of the immune system in a tumor setting is completely different than in

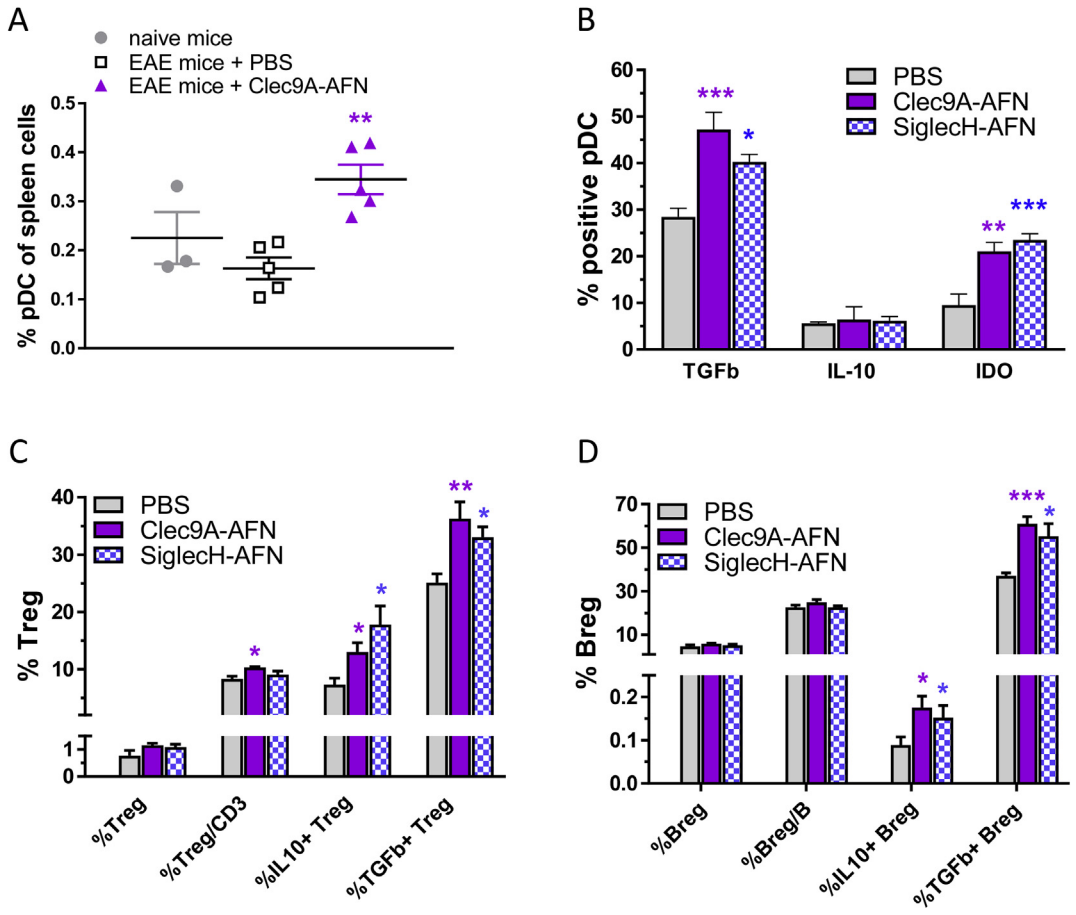


**Fig. 4. Protective treatments produce tolerogenic pDC, Tregs and Bregs.** Amounts of splenic pDC were increased on d12 (A), and more of them produced TFGb and IDO, but not IL-10 (B, n = 4–5). Tregs were increased by Clec9A-AFN if counted within the $CD3^+$ population (C, n = 5). Both in the Treg and Breg population, the % of IL-10 or TGFβ producing cells increased (C,D, n = 5). Differences were assessed using one-way ANOVA followed by Dunnett's multiple-comparison test; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with PBS treated animals.

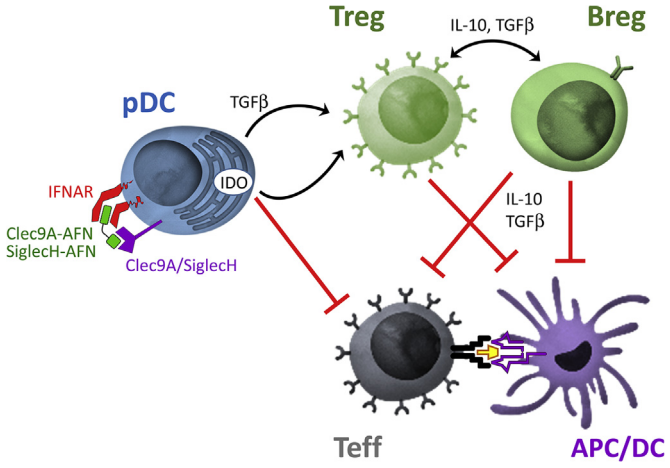


**Fig. 5. Schematic of AFN targeting and proposed tolerizing effects during EAE.** Targeting AFN via Clec9A or SiglecH to pDC tolerizes them, increasing their TGFβ and IDO expression. TGFβ and IDO-induced kynurenine synthesis are known to induce Tregs, while IDO-induced tryptophan catabolism inhibits Teff. Tregs and Bregs may increase each other's immunosuppressive effects via IL-10 and TGFβ; the latter will also inhibit immunogenic Teff and self-antigen presenting cells such as DC.

the EAE setting. Comparable differential, seemingly conflicting effects have already been described with regard to regulatory T cells. While type I IFN signaling attenuates Treg function in the tumor microenvironment or during viral infection, IFNAR signaling promotes Treg function in autoimmunity, including EAE [49–51].

## 5. Conclusions

The use of autologous, *ex vivo* generated and manipulated tolDCs has been promoted as a new therapy for MS and other autoimmune diseases, and clinical trials are underway [34,43,44]. Type I Interferon (IFN) remains a first line treatment to reduce attacks and lesions in MS patients, but its many different side effects are dose- and time-limiting. In this study, we provide evidence for a novel, generic and safe strategy for the *in vivo* tolerization of DCs as a successful means to control autoimmunity. Indeed, targeting IFN activity specifically to pDC (and later also cDC1) during EAE induced a strong tolerogenic phenotype, evident in both pDC, Treg and Breg, that efficiently dampened EAE disease progression without toxic side effects. DC-specific targeting of IFN activity not only abolished all systemic adverse effects and toxicities, it also dissected positive from negative IFN effects and may thus represent a better and harmless MS therapy.

## Author contributions

AC and JT conceived and designed the research. AC conducted the mouse experiments. SVL performed the immune cell analysis. DC, ER and AV provided technical support, including constructions, purifications and help with animal work and FACS analyses. FP aided with AFN designs. SP and MP analyzed spinal cords. NK and GU helped with experimental designs. AC and JT wrote the manuscript.

## Conflicts of interest

JT and NK are affiliated with Orionis Biosciences (as scientific advisor and/or employee) and hold equity interests in Orionis Bioscience. JT received financial research support from Orionis Biosciences NV. All the other authors declare no conflict of interest.

## Acknowledgments

We thank Reza Hassanzadeh Ghassabeh (VIB Nanobody Core) for the selection of the sdAbs, Geert Van Loo and Sofie Voet for teaching us the EAE model, Veronique Flamand for the Batf3$^{-/-}$ mice, Ulrich Kalinke for CD11c-IFNAR1-deficient mice. JT and NK are affiliated with Orionis Biosciences (as scientific advisor and/or employee) and hold equity interests in Orionis Bioscience. JT received financial research support from Orionis Biosciences NV. This work was supported by UGent Methusalem and Advanced ERC (CYRE, N° 340941) grants to JT; grants from LabEx MabImprove, Institut Carnot CALYM, the Canceropôle - Institut National du Cancer (INCa) to GU; and by Orionis Biosciences.

## References

[1] K. Rejdak, S. Jackson, G. Giovannoni, Multiple sclerosis: a practical overview for clinicians, Br. Med. Bull. 95 (2010) 79–104.
[2] D.M. Wingerchuk, B.G. Weinshenker, Disease modifying therapies for relapsing multiple sclerosis, BMJ 354 (2016) i3518.
[3] A. Westad, A. Venugopal, E. Snyder, The multiple sclerosis market, Nat. Rev. Drug Discov. 16 (2017) 675–676.
[4] L. Durelli, E. Verdun, P. Barbero, M. Bergui, E. Versino, A. Ghezzi, et al., Every-other-day interferon beta-1b versus once-weekly interferon beta-1a for multiple sclerosis: results of a 2-year prospective randomised multicentre study (INCOMIN), Lancet 359 (2002) 1453–1460.
[5] A. Lugaresi, M. di Ioia, D. Travaglini, E. Pietrolongo, E. Pucci, M. Onofrj, Risk-benefit considerations in the treatment of relapsing-remitting multiple sclerosis, Neuropsychiatric Dis. Treat. 9 (2013) 893–914.
[6] R.C. Axtell, C. Raman, Janus-like effects of type I interferon in autoimmune diseases, Immunol. Rev. 248 (2012) 23–35.
[7] C.L. Verweij, S. Vosslamber, Relevance of the type I interferon signature in multiple sclerosis towards a personalized medicine approach for interferon-beta therapy, Discov. Med. 15 (2013) 51–60.
[8] A. Cauwels, S. Van Lint, G. Garcin, J. Bultinck, F. Paul, S. Gerlo, et al., A safe and highly efficient tumor-targeted type I interferon immunotherapy depends on the tumor microenvironment, OncoImmunology 7 (2018) e1398876.
[9] A. Cauwels, S. Van Lint, F. Paul, G. Garcin, S. De Koker, A. Van Parys, et al., Delivering type I interferon to dendritic cells empowers tumor eradication and immune combination treatments, Cancer Res. 78 (2018) 463–474.
[10] S.L. Bailey-Bucktrout, S.C. Caulkins, G. Goings, J.A. Fischer, A. Dzionek, S.D. Miller, Cutting edge: central nervous system plasmacytoid dendritic cells regulate the severity of relapsing experimental autoimmune encephalomyelitis, J. Immunol. 180 (2008) 6457–6461.
[11] M. Isaksson, B. Ardesjo, L. Ronnblom, O. Kampe, H. Lassmann, M.L. Eloranta, et al., Plasmacytoid DC promote priming of autoimmune Th17 cells and EAE, Eur. J. Immunol. 39 (2009) 2925–2935.
[12] R.L. Tuinstra, F.C. Peterson, E.S. Elgin, A.J. Pelzek, B.F. Volkman, An engineered second disulfide bond restricts lymphotactin/XCL1 to a chemokine-like conformation with XCR1 agonist activity, Biochemistry 46 (2007) 2564–2573.
[13] A.L. Blasius, M. Colonna, Sampling and signaling in plasmacytoid dendritic cells: the potential roles of Siglec-H, Trends Immunol. 27 (2006) 255–260.
[14] A.P. Robinson, C.T. Harp, A. Noronha, S.D. Miller, The experimental autoimmune encephalomyelitis (EAE) model of MS: utility for understanding disease pathophysiology and treatment, Handb. Clin. Neurol. 122 (2014) 173–189.
[15] R.C. Axtell, B.A. de Jong, K. Boniface, L.F. van der Voort, R. Bhat, P. De Sarno, et al., T helper type 1 and 17 cells determine efficacy of interferon-beta in multiple sclerosis and experimental encephalomyelitis, Nat. Med. 16 (2010) 406–412.
[16] R.D. Schubert, Y. Hu, G. Kumar, S. Szeto, P. Abraham, J. Winderl, et al., IFN-beta treatment requires B cells for efficacy in neuroautoimmunity, J. Immunol. 194 (2015) 2110–2116.
[17] S. Zhornitsky, T.A. Johnson, L.M. Metz, S. Weiss, V.W. Yong, Prolactin in combination with interferon-beta reduces disease severity in an animal model of multiple sclerosis, J. Neuroinflammation 12 (2015) 55.
[18] C.S. Constantinescu, N. Farooqi, K. O'Brien, B. Gran, Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS), Br. J. Pharmacol. 164 (2011) 1079–1106.
[19] A. Denic, A.J. Johnson, A.J. Bieber, A.E. Warrington, M. Rodriguez, I. Pirko, The relevance of animal models in multiple sclerosis research, Pathophysiology 18 (2011) 21–29.
[20] S. Sinha, F.R. Itani, N.J. Karandikar, Immune regulation of multiple sclerosis by CD8+ T cells, Immunol. Res. 59 (2014) 254–265.
[21] A. Javed, A.T. Reder, Therapeutic role of beta-interferons in multiple sclerosis, Pharmacol. Ther. 110 (2006) 35–56.
[22] B. Schreiner, M. Mitsdoerffer, B.C. Kieseier, L. Chen, H.P. Hartung, M. Weller, et al., Interferon-beta enhances monocyte and dendritic cell expression of B7-H1 (PD-L1), a strong inhibitor of autologous T-cell activation: relevance for the immune modulatory effect in multiple sclerosis, J. Neuroimmunol. 155 (2004) 172–182.
[23] N.J. Karandikar, C.L. Vanderlugt, T.L. Walunas, S.D. Miller, J.A. Bluestone, CTLA-4: a negative regulator of autoimmune disease, J. Exp. Med. 184 (1996) 783–788.
[24] P.J. Perrin, J.H. Maldonado, T.A. Davis, C.H. June, M.K. Racke, CTLA-4 blockade enhances clinical disease and cytokine production during experimental allergic encephalomyelitis, J. Immunol. 157 (1996) 1333–1336.
[25] A.A. Hurwitz, T.J. Sullivan, R.A. Sobel, J.P. Allison, Cytotoxic T lymphocyte antigen-4 (CTLA-4) limits the expansion of encephalitogenic T cells in experimental autoimmune encephalomyelitis (EAE)-resistant BALB/c mice, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 3013–3017.
[26] I. Caminschi, A.I. Proietto, F. Ahmet, S. Kitsoulis, J. Shin Teh, J.C. Lo, et al., The dendritic cell subtype-restricted C-type lectin Clec9A is a target for vaccine enhancement, Blood 112 (2008) 3264–3273.
[27] D. Sancho, D. Mourao-Sa, O.P. Joffre, O. Schulz, N.C. Rogers, D.J. Pennington, et al., Tumor therapy in mice via antigen targeting to a novel, DC-restricted C-type lectin, J. Clin. Invest. 118 (2008) 2098–2110.
[28] K. Hildner, B.T. Edelson, W.E. Purtha, M. Diamond, H. Matsushita, M. Kohyama, et al., Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity, Science 322 (2008) 1097–1100.
[29] R. Tussiwand, W.L. Lee, T.L. Murphy, M. Mashayekhi, W. Kc, J.C. Albring, et al., Compensatory dendritic cell development mediated by BATF-IRF interactions, Nature 490 (2012) 502–507.
[30] M.S. Diamond, M. Kinder, H. Matsushita, M. Mashayekhi, G.P. Dunn, J.M. Archambault, et al., Type I interferon is selectively required by dendritic cells for immune rejection of tumors, J. Exp. Med. 208 (2011) 1989–2003.
[31] J. Zhang, A. Raper, N. Sugita, R. Hingorani, M. Salio, M.J. Palmowski, et al., Characterization of Siglec-H as a novel endocytic receptor expressed on murine plasmacytoid dendritic cell precursors, Blood 107 (2006) 3600–3608.
[32] M.P. Domogalla, P.V. Rostan, V.K. Raker, K. Steinbrink, Tolerance through education: how tolerogenic dendritic cells shape immunity, Front. Immunol. 8 (2017) 1764.
[33] V.K. Raker, M.P. Domogalla, K. Steinbrink, Tolerogenic dendritic cells for regulatory T cell induction in man, Front. Immunol. 6 (2015) 569.
[34] C. Horton, K. Shanmugarajah, P.J. Fairchild, Harnessing the properties of dendritic cells in the pursuit of immunological tolerance, Biomed. J. 40 (2017) 80–93.
[35] G. Schiavoni, F. Mattei, L. Gabriele, Type I interferons as stimulators of DC-mediated cross-priming: impact on anti-tumor response, Front. Immunol. 4 (2013) 483.
[36] C. Lippens, F.V. Duraes, J. Dubrot, D. Brighouse, M. Lacroix, M. Irla, et al., Ido-orchestrated crosstalk between pDCs and Tregs inhibits autoimmunity, J. Autoimmun. 75 (2016) 39–49.
[37] S. Rutella, G. Bonanno, L. Pierelli, A. Mariotti, E. Capoluongo, A.M. Contemi, et al., Granulocyte colony-stimulating factor promotes the generation of regulatory DC through induction of IL-10 and IFN-alpha, Eur. J. Immunol. 34 (2004) 1291–1302.
[38] F. Fallarino, C. Asselin-Paturel, C. Vacca, R. Bianchi, S. Gizzi, M.C. Fioretti, et al., Murine plasmacytoid dendritic cells initiate the immunosuppressive pathway of tryptophan catabolism in response to CD200 receptor engagement, J. Immunol. 173 (2004) 3748–3754.
[39] C. Volpi, F. Fallarino, M.T. Pallotta, R. Bianchi, C. Vacca, M.L. Belladonna, et al., High doses of CpG oligodeoxynucleotides stimulate a tolerogenic TLR9-TRIF pathway, Nat. Commun. 4 (2013) 1852.
[40] C. Asselin-Paturel, G. Brizard, K. Chemin, A. Boonstra, A. O'Garra, A. Vicari, et al., Type I interferon dependence of plasmacytoid dendritic cell activation and migration, J. Exp. Med. 201 (2005) 1157–1167.
[41] Y. Gao, B. Majchrzak-Kita, E.N. Fish, J.L. Gommerman, Dynamic accumulation of plasmacytoid dendritic cells in lymph nodes is regulated by interferon-beta, Blood 114 (2009) 2623–2631.
[42] N. Schwab, A.L. Zozulya, B.C. Kieseier, K.V. Toyka, H. Wiendl, An imbalance of two functionally and phenotypically different subsets of plasmacytoid dendritic cells characterizes the dysfunctional immune regulation in multiple sclerosis, J. Immunol. 184 (2010) 5368–5374.
[43] N. Cools, W.P. Lee, M. De Laere, B. Willekens, J. Derdelinckx, G. Nijs, et al., Towards the clinical use of tolerogenic dendritic cells in multiple sclerosis by applying the immunomodulatory effects of 1,25-dihydroxyvitamin D3, Front. Aging Neurosci. (2016), https://doi.org/10.3389/conf.fnagi.2016.03.00045 Conference Abstract: 6th Belgian Brain Congress.
[44] S.H. Kim, H.H. Jung, C.K. Lee, Generation, characteristics and clinical trials of ex vivo generated tolerogenic dendritic cells, Yonsei Med. J. 59 (2018) 807–815.
[45] F.V. Duraes, C. Lippens, K. Steinbach, J. Dubrot, D. Brighouse, N. Bendriss-Vermare, et al., pDC therapy induces recovery from EAE by recruiting endogenous pDC to sites of CNS inflammation, J. Autoimmun. 67 (2016) 8–18.
[46] C.C. Gross, H. Jonuleit, H. Wiendl, Fulfilling the dream: tolerogenic dendritic cells to treat multiple sclerosis, Eur. J. Immunol. 42 (2012) 569–572.
[47] K. Schinnerling, L. Soto, P. Garcia-Gonzalez, D. Catalan, J.C. Aguillon, Skewing dendritic cell differentiation towards a tolerogenic state for recovery of tolerance in rheumatoid arthritis, Autoimmun. Rev. 14 (2015) 517–527.
[48] N. Yogev, F. Frommer, D. Lukas, K. Kautz-Neu, K. Karram, D. Ielo, et al., Dendritic cells ameliorate autoimmunity in the CNS by controlling the homeostasis of PD-1 receptor(+) regulatory T cells, Immunity 37 (2012) 264–275.
[49] A. Gangaplara, C. Martens, E. Dahlstrom, A. Metidji, A.S. Gokhale, D.D. Glass, et al., Type I interferon signaling attenuates regulatory T cell function in viral infection and in the tumor microenvironment, PLoS Pathog. 14 (2018) e1006985.
[50] A. Metidji, S.A. Rieder, D.D. Glass, I. Cremer, G.A. Punkosdy, E.M. Shevach, IFN-alpha/beta receptor signaling promotes regulatory T cell development and function under stress conditions, J. Immunol. 194 (2015) 4265–4276.
[51] A. Metidji, E.M. Shevach, Type I IFNs modulate T effector (Teff) and T regulatory (Treg) cell function during experimental autoimmune encephalomyelitis (EAE) (IRC4P.485), J. Immunol. (2014) 192 1 Supplement 60.12.