Published in final edited form as:

Semin Immunol. 2019 April ; 42: 101308. doi:10.1016/j.smim.2019.101308.

VISTA: A novel immunotherapy target for normalizing innate and adaptive immunity

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Abstract

V-domain Ig suppressor of T cell activation (VISTA) is a novel checkpoint regulator with limited homology to other B7 family members. The constitutive expression of VISTA on both the myeloid and T lymphocyte lineages coupled to its important role in regulating innate and adaptive immune responses, qualifies VISTA to be a promising target for immunotherapeutic intervention. Studies have shown differential impact of agonistic and antagonistic targeting of VISTA, providing a unique landscape for influencing the outcome of cancer and inflammatory diseases.

Keywords

VISTA; Cancer immunotherapy Autoimmunity; Immune checkpoint; Antagonist Agonist

1. Introduction

The discovery of immune checkpoints has led not only to new paradigms for defining regulatory networks in immune system design, but also to multiple breakthrough therapies that revolutionized the treatment of diseases such as cancer [1]. Numerous studies elucidated the mechanisms and therapeutic potential of cytotoxic T lymphocyte antigen 4 (CTLA-4) and Programmed Death-1 (PD-1) but with attention now shifting to combination therapies and newer targets with nonoverlapping expression and activity, significant interest is now in pursuing other negative checkpoint regulators [2,3]. V-domain Ig suppressor of T cell activation (VISTA) is a novel checkpoint regulator with limited homology to other B7 family members [4]. The VISTA gene *Vsir* is located far from other *Ig* superfamily members, within the intron of the *CDH23* gene on chromosome 10. Also, unlike other B7 family molecules, analysis of the VISTA gene sequence across species reveals a surprisingly high level of conservation, with 76% identity between mouse and human, especially in the cytoplasmic domain. Accordingly, this suggests a conserved signaling pathway and functional role [4,5]. As will be discussed in this review, antibodies targeting VISTA have been used in both cancer and autoimmune disease mouse models to either enhance or

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suppress immune responses, respectively. Although true agonism or antagonism has not been clearly shown, and actual signaling data is lacking, multiple *in vivo* systems show that antagonistic (immune enhancing) anti-VISTA antibodies, as with VISTA^{-/-} mice, enhance autoimmunity and agonistic (immune suppressive) anti-VISTA antibodies suppress autoimmunity. Understanding the contributions of the binding characteristics of each antibody to VISTA and the Fc regions which they bear will be useful in guiding antibody development for use in the clinic.

2. VISTA is a negative checkpoint

2.1. Expression of VISTA

VISTA is predominantly expressed within the hematopoietic compartment ([4]; Fig. 1). In leukocytes, the highest levels of VISTA protein expression are found in myeloid cells, particularly microglia and neutrophils [6], followed by monocytes, macrophages and dendritic cells. Within the T lymphocyte compartment, VISTA is most highly expressed on naïve CD4⁺ and Foxp3⁺ Regulatory T cells. Other T cell and NK cell subsets, including thymocytes, have detectable VISTA expression but at lower levels. It is intriguing to know that while B cells do not show any detectable VISTA levels, plasma cells have significant expression (Lines and ElTanbouly, Unpublished observation). Our analysis of VISTA expression on peritoneal macrophages reveals remarkable intracellular expression and significant surface punctate expression (Fig. 2A). Further analysis of the patterns of VISTA intracellular distribution revealed that VISTA is predominantly localized within endosomal compartments, as evidenced by its colocalization with Ras-related protein (Rab11) (Fig. 2B); a protein that associated mostly with recycling endosomes [7–9]. VISTA also colocalized with EEA-1 (Fig. 2C), a marker of early endosomes [10], but did not show colocalization with Rab4 (Fig. 2D) or TMEM165 (Fig. 2E). These data suggest that VISTA predominantly occupies early and recycling endosomes. Lee and colleagues identified VISTA as a direct transcriptional target of p53 in response to DNA damage, and showed significant VISTA upregulation in apoptotic cells [11]. Another group demonstrated intrinsic VISTA expression on tumor cells from human ovarian and endometrial cancers [12].

2.2. VISTA impact on innate immunity

The expression of VISTA on both the T cell and myeloid compartments makes it an exciting target for therapeutic intervention. Two different VISTA-deficient (VISTA^{-/-}) mouse strains have been reported, both on a C57BL/6 background [13,14]. In both strains, the percentage of antigen-experienced CD44^{hi} T cells increased with age suggesting higher levels of basal immune activation [13,14]. One of these strains developed autoimmune disease at 10–12 months, manifesting as severe skin inflammation and extensive immune infiltrates in the skin, lung, liver, and kidney [11]. While this phenotype was observed in the absence of intervention or crossing to a predisposing genetic background [11], a second VISTA^{-/-} strain showed no overt autoimmunity at steady state [14]. This strain did exhibit a dramatic enhancement in the incidence of spontaneous autoimmune encephalomyelitis (EAE) when crossed on to MOG-specific [15] TCR transgenic background [14]. The differences in sensitivity to autoimmunity may be a result of differences in the microbiome or environment

that the strains are maintained at, but VISTA deficiency clearly causes higher susceptibility to autoimmune induction.

As anticipated, a blocking (“antagonistic”) anti-VISTA antibody exacerbated EAE and increased the frequency of encephalitogenic Th17 cells *in vivo* [3]. Similarly, interbreeding VISTA^{-/-} mice with Sle1.Sle3 mice, a well-characterized model of systemic lupus erythematosus (SLE), results in a striking enhancement of lupus nephritis [16], with VISTA blockade recapitulating these effects [17]. In addition, VISTA^{-/-} mice had enhanced psoriasiform inflammation [18]. The authors attributed this to enhanced TLR7 signaling in VISTA^{-/-} DCs and hyperactivation in VISTA^{-/-} T cells. A more recent study revealed the unexpected finding that VISTA-deficiency or anti-VISTA treatment can profoundly ameliorate the induction of collagen-antibody induced arthritis in mice despite enhanced activation of VISTA^{-/-} myeloid cells [19]. The authors attributed this puzzling therapeutic effect to the interference with the C5a signaling pathway, where VISTA^{-/-} macrophages had reduced expression of C5aR1, and therefore signaling and migration. It is worth noting that C5a signaling is necessary for the development of CAIA and other models of arthritis including KB/xN [20,21]. Another study suggested a role for VISTA in regulating type II immunity by demonstrating that the absence of VISTA can exacerbate experimental asthma [22].

2.3. VISTA impact on adaptive immunity

The constitutive expression of VISTA on resting T cells suggests a unique non-redundant role for this molecule compared to CTLA-4 and PD-1; two checkpoints expressed after T cell activation occurs. Several studies demonstrate an important role for VISTA as an inhibitory receptor on CD4⁺ T cells. Chen and colleagues showed that an immunosuppressive agonistic anti-VISTA monoclonal antibody (mAb) completely prevented the induction of acute Graft-versus-Host disease (GVHD) in mice using multiple models [5]. They presented compelling evidence that direct antibody targeting of VISTA on T cells could suppress T cell activation which was not the result of antibody-dependent or complement-mediated killing. [6]. It is important that anti-VISTA only prevented GVHD induction if treatment was initiated -1 or 0 days prior to GVHD induction. After these timepoints, there was no therapeutic impact of anti-VISTA on GVHD outcome, suggesting that the impact of anti-VISTA was exerted on resting T cells. In a GVHD model, mice adoptively transferred with VISTA^{-/-} T cells had markedly exacerbated GVHD and reduced survival [6]. Purified VISTA^{-/-} CD4⁺ T cells exhibited enhanced proliferative and effector cytokine responses to antigenic stimulation [13]. *In vivo*, VISTA^{-/-} mice were markedly more susceptible to lethality and enhanced T-cell effector cytokines in a ConA-induced hepatitis model, which is largely dependent on CD4⁺ T cells. Wang and colleagues showed that mice double-deficient for VISTA and PD-1 exhibit enhanced spontaneous T cell activation and a chronic inflammatory phenotype than mice deficient for either molecule alone [23]. More recently, a role for VISTA in inducible Treg generation and stability was underscored, as genetic deficiency of VISTA on the T cells reduces iTreg differentiation [24]. It still remains unclear whether VISTA interferes with genetic programs underlying iTreg generation, or T cell polarization as a whole. However, these mechanistic studies raise important questions. First, is VISTA part of a coinhibitory module that is constitutively

expressed on T cells? Big data assessment of T cell inhibitory modules in cancer do not suggest this to be the case. Single-cell RNA-seq of exhausted T cells in multiple tumor models revealed multiple networks of T cell co-inhibition, with VISTA not being one of the differentially expressed candidates [25–28]. All of these studies addressed T cell exhaustion, however, which is a later state of T cell dysfunction. Alternatively, it is possible that VISTA activity is not primarily regulated at the level of transcription, but rather by post-translational modifications or trafficking. However, in a recent study, Marson and colleagues elegantly used CRISPR screens coupled to single-cell RNA-seq technology to profile the effects of CRISPR-mediated genetic knockout of select key target genes on human T cell stimulation programs. A shared transcriptional cell-state signature was observed for VISTA, 4–1BB and TMEM222 knockout T cells [25]. The constitutive expression of VISTA on multiple immune lineages combined with the inflammatory phenotypes of the VISTA-deficient mouse model establish VISTA as a homeostatic regulatory modality or “rheostat” that actively normalizes the immune responses by pruning excessive activation at the earliest stages of the immune response. This example is unique among negative checkpoint regulators, as most well-studied molecules (e.g. CTLA-4, PD-1, LAG-3) are induced after activation and not constitutively expressed.

3. VISTA in cancer

3.1. Innate and acquired resistance

There are two fundamental challenges for broad and successful checkpoint immunotherapy. The first challenge involves increasing the response rate of patients to initial checkpoint blockade – i.e. overcoming “innate resistance”. In mouse models, resistance of very large tumors to intervention with antibodies against PD-1 and CTLA-4 was coupled to the accumulation of granulocytic tumor myeloid-derived suppressor cells (MDSCs) [29]. The second vital challenge for therapy is improvement in the durability of initial response rates – i.e. circumventing relapse due to “acquired resistance”. Indeed, with anti-PD1 therapy, 43% of responding patients relapse within 3 years [30]. Many mechanisms have been proposed for acquired resistance, including the action of alternative immune checkpoints such as VISTA [31,32].

3.2. Activity of VISTA in cancer

With an established role for VISTA as a negative checkpoint regulator, much interest has centered on the role of VISTA in tumor immunity. Early on, several lines of evidence pointed towards a role for VISTA in restraining anti-tumor responses. VISTA-deficient^{−/−} mice show resistance to GL261 glioma, and this protection is dependent on CD4⁺ T cells [13]. As discussed in Section 1, while some clones of anti-VISTA are suppressive, clones with immuno-stimulatory activity have been successfully used to impede tumor growth. 13F3 was shown to suppress growth of CT26 colon cancer, B16OVA melanoma, MB49 bladder carcinoma, B16BL6 melanoma and importantly, an inducible melanoma model [23,33]. Efficacy was associated with increased CD45⁺ immune infiltration, and T cell cytokine responses and in some models a reduction in tumor MDSCs [33]. Interestingly, a role for VISTA in mediating the suppressive activity of MDSCs generated in a LP-BM5 virus model was shown for B cells but not T cells [34]. Importantly, 13F3 treatment was

found to be non-redundant with anti-PD-L1 blockade [23], supportive of the future use of immunotherapy in combination with existing immunotherapies targeting the PD-1 pathway. In contrast, while the clone MIH63 did have some combination effect with CTLA-4 blockade in a squamous cell carcinoma (SCCVII) model, it was unable to further suppress tumor growth in combination with anti-PD-1 [35]. Even in a monotherapy regimen, where this clone could not delay growth of SCCVII, MIH63 did improve CD8⁺ T cell responses as indicated by expression of IFN γ , Eomes and Ki67. The authors point to the high activity of Tregs in this model, unrestrained by MIH63, as to why this improved CD8⁺ T cell response could not impact on tumor growth [35]. Le Mercier et al. demonstrated an impact of 13F3 on inhibiting conversion of OTII CD4⁺ T cells into Tregs adoptively transferred into B16OVA bearing mice [33], but did not report if Treg frequencies were altered in the models where they demonstrated an impact of 13F3 on tumor growth. Therefore, it remains unclear if Treg inhibition is part of the 13F3 mechanism of action. Altogether, these data substantiate VISTA as a negative checkpoint for tumor immunity, and warrant further investigation of mechanisms of action in migration, survival, proliferation, maturation, and activation of different immune subsets.

3.3. Expression of VISTA in the tumor microenvironment

Recently, an array of studies have examined VISTA expression in human tumors including Acute Myeloid Leukemia (AML) [36], pancreatic [37], non-small cell lung cancer [38], prostate cancer [39], cutaneous melanoma [40], metastatic melanoma [41], hepatocellular carcinoma (HCC; [42]), ovarian cancer [43], oral squamous cell carcinoma [44], gastric cancer [45,46], and colorectal cancer [47]. Across different human tumors, VISTA expression generally appears to increase with disease progression and is indicative of poor survival [38,40,43,48]. VISTA expression within human tumors may indicate poor prognosis for multiple reasons. For example, as discussed above, VISTA is able to suppress T cell responses, and may contribute to the immune suppressed nature of the tumor microenvironment [34,37,39,49]. Second, expression is highly associated with myeloid cell infiltration in multiple tumor types [39,40,48], and high myeloid infiltrate is generally correlated with poor prognosis [49]. Third, VISTA expression is induced by HIF-1 α which indicates hypoxia - itself an independent factor for poor prognosis [50]. A role for VISTA as a predictive factor was not observed for gastric cancer [46], and in oral squamous cell carcinoma, Cox multivariate analysis only showed significant association with survival when assessed in combination with CD8 expression, CD8-low and VISTA-high patients having the poorest prognosis [44].

A study that determined VISTA expression in metastatic melanoma patient biopsies taken prior to treatment and following their subsequent progression showed marked upregulation of VISTA and increased density of VISTA⁺ cells following progression [41]. VISTA was also upregulated in prostate cancer after ipilimumab treatment [39]. Interestingly, in this study, CD68⁺ macrophages were often either PD-L1⁺ (29.4%) or VISTA⁺ (26.5%), but rarely double positive (2%) [39], suggesting distinct suppressive subsets of macrophages that could potentially compensate for each other in antagonistic immunotherapy. These data suggest that VISTA may act as a compensatory inhibitory pathway in acquired resistance and support the use of VISTA immunotherapy following or in combination with other

immune therapies. More data is needed on the potential role of VISTA in determining the responsiveness of tumors to immune intervention.

3.4. Non-immune cell expression of VISTA in tumors

In murine tumor models, VISTA appears to have a hematopoietically restricted expression pattern, and is highly upregulated on T and myeloid cells in the tumor microenvironment (Fig. 1; [33]). This contrasts with the broader expression of PD-L1, which is often expressed on tumor cells, especially in instances of IFN γ expression and a ‘hot’ infiltrated tumor. Both PD-L1 and VISTA can be induced under hypoxic stress in tumors, and through HIF-1 α [48]. Additionally, VISTA blockade appears to be more effective in *in vitro* assays that are performed under hypoxic culture conditions [48]. While as a general rule VISTA is more consistently detected on immune cell infiltrates, some studies have shown tumor cell expression of human VISTA [38,42–44,47], and as compared to immune cells, in a particularly cytoplasmic pattern [44]. Mouse models have suggested that tumor cells may upregulate VISTA when they undergo apoptosis, or through a p53 activity [11]. Oliveira et al. suggested that VISTA mRNA may be controlled in gastric cancers through either miRNA125a or the methylation status of a CpG island at the 5′ end of the *vsir* gene [45]. A subsequent study did not find a role for miRNA125a in VISTA expression of endometrial and ovarian cancer cell lines, but did demonstrate that the DNA methylation status of *vsir* was correlated with VISTA expression in both clinical specimens and cell lines [12]. The authors also found that knock-down of VISTA on tumor cell lines reduced their suppressive activity *in vitro* to T cells from healthy donors [12]. In mouse models, overexpression of VISTA was able to release T cell-dependent control of tumor growth in MCA105 [4], ID8 and HM-1 tumor models [12]. These data suggest that there are multiple possible mechanisms that may allow for release of VISTA expression in tumorigenesis, and that this expression may be functionally relevant in suppressing anti-tumor immunity. Interestingly, in lung cancer and hepatocellular carcinoma, VISTA expression on the CD45-negative tumor cells was associated with improved survival [38,42]. This nonimmune cell expression of VISTA is not generally observed in normal tissues. It is tempting to speculate that tumor cell expression of VISTA may occur in ‘hot’ inflammatory tumors, and these may be more conducive to immune therapy, eliciting a survival advantage.

A key question for the future is how VISTA regulates the suppressive effects of MDSCs. Kuchroo, Regev, Anderson, Hacohen and other investigators have extensively investigated the coinhibitory program mediating T cell dysfunction in multiple cancer models and in humans [25–28]. However, we are relatively ignorant of the inhibitory modules and networks regulating myeloid cell suppressive activity. Could VISTA be part of a bigger regulatory network that regulates myeloid cell suppressive activity? If so, what aspect of myeloid function does VISTA regulate, and what other regulators act in concert?

4. The promise of VISTA targeting

4.1. VISTA antagonists in cancer immunotherapy

VISTA occupies a unique position as a candidate for cancer immunotherapy due to its expression pattern and activity. First, VISTA expression on infiltrating myeloid cells is

consistent across tumor types, making it relevant to a breadth of patients. Expression is further enhanced by hypoxia [50] and increases with cancer progression [39,41]. While most immunotherapy targets address either T cell or myeloid functions, VISTA has clear activity in regulating both arms (Table 1). VISTA is also a promising target for combinatorial approaches to immunotherapy. When T cell focused checkpoint therapy fails (anti-PD-1 and/or anti-CTLA-4), this can be a result of MDSC or TAM activity [29,51,52]. An ideal combination therapy should address both myeloid and T cell responses. Releasing suppression by myeloid cells by targeting VISTA would may improve T cell focused therapies like anti-PD1 and anti-CTLA4.

4.2. VISTA agonists

The presence of anti-VISTA antibodies that cause immune-enhancing or immunosuppressive effects propels VISTA targeting to a unique level. There are anti-VISTA agonists that can prevent GVHD development [5], ameliorate murine lupus (unpublished data), and experimental asthma [22] by suppressing immunity. On the other hand, there are anti-VISTA clones which enhance tumor immunity against multiple cancer models and enhance inflammatory disease [4,33]. Currently, to our knowledge, there are no other checkpoint agonistic antibodies in clinical development with the exception of VISTA. One could deduce from the studies combining VISTA blockade data and VISTA deficiency that blocking VISTA can phenocopy the gene deletion. However, the mechanistic basis of agonistic antibody activity is not yet clear. What distinguishes VISTA among other checkpoints for successful targeting by agonists? Two sets of investigations could answer this. One way would be to identify the signaling events mediated by VISTA in primary cells, which agonistic antibodies are expected to trigger. The second is to identify the natural VISTA ligand(s) *in vivo*, which may elucidate the cellular and molecular pathways regulated by VISTA.

4.3. Avenues for unique VISTA targeting

Two publications have shed the light on potential ligands. The first group suggested that VISTA mediates a homotypic interaction necessary for its function [14]. VISTA homotypic interactions have not yet been validated by independent studies. A more recent study identified VSIG-3 (known as IgSF11) as the ligand for VISTA demonstrating that VSIG3 can mediate functional inhibition of T cell activation [53]. However, VSIG3 expression is undetectable in the hematopoietic system, hindering validation of any potential function *in vivo*. A recent patent application filed by Bristol-Myers Squibb (BMS) revealed direct binding of VISTA to P-Selectin Glycoprotein Ligand-1 (PSGL-1; [54]). PSGL-1 has similar patterns of expression to VISTA and its role in T cell biology and as a newly identified immune checkpoint in cancer warrants interest [55–57]. It remains to be elucidated whether VISTA and PSGL-1 indeed interact *in vivo* and what consequences this would have on immune homeostasis. There are several novel aspects of the VISTA – PSGL-1 interaction. First, VISTA was reported to bind PSGL-1 only at lower pH values such as 6.5. The VISTA protein sequence has a uniquely high histidine content ([4], 8.5%), residues which undergo protonation at lower pH. From a disease perspective, the microenvironment of some tumor types have been reported to be as low as pH 6.44 [58,59], and the ionic environment in the TME can dictate the fate of anti-tumor T cell immunity [60]. The impact of low pH on

driving pathologic inflammation has been also been long appreciated [61–63]. The concept of immunoregulatory protein interactions being governed by the pH of the host tissue is a valuable one. Protonation can be considered as one of the important post-translational modifications that can change the outcome of immune-receptor binding to its ligand[s], and therefore the outcome and threshold of the immune response. Korman and colleagues also introduce pH-selective antibodies that bind VISTA only at lower pH, when residues critical for PSGL-1 binding are protonated, thus antagonizing this immunoregulatory interaction [54]. This further introduces a critical strategy for antibody engineering whereby the ligand is predominantly targeted at the sites of immune dysregulation (e.g. tumor or inflammatory site). This would allow regional normalization of immune responses with an anti-VISTA antibody, thereby limiting any potential adverse effects. This strategy is particularly favorable for VISTA targeting since VISTA-rich cells in the periphery and bone marrow may otherwise act as a ‘sink’ for VISTA antibody. A pH-dependent antibody likely would have an enhanced pharmacokinetic profile [54].

5. Conclusion

VISTA presents a new class of immune checkpoint inhibitory receptors with broad expression in the immune system at steady-state conditions, underlying a unique homeostatic role in normalizing immune responses.

The unique utility of targeting for both cancer and inflammatory diseases using anti-VISTA clones that enhance or suppress both innate and adaptive immune responses is an important advantage for therapeutic strategies. Mechanistic molecular understanding of the roles for VISTA on the innate versus adaptive immune system will yield potential therapeutic benefits, as well as greater insights into immune system homeostasis.

Acknowledgements

The authors acknowledge the following Shared Resource facilities at the Norris Cotton Cancer Center at Dartmouth College: Irradiation, Preclinical Imaging and Microscopy Resource (IPIMSR) with National Cancer Institute (NCI) Cancer Center Support Grant 5P30 CA023108-37 and NIH S10 SIG award S10OD21616.

Funding

This work was supported by the National Institutes of Health grants R01AR070760 and R01CA214062.

Abbreviations

AML	acute myeloid leukemia
CRISPR	clustered regularly interspaced short palindromic repeats
CTLA-4	cytotoxic T lymphocyte antigen 4
mAb	monoclonal antibody
MDSC	tumor myeloid-derived suppressor cells
TAM	tumor-associated macrophage

PD-1	programmed death-1
PD-L1	programmed death ligand-1
PSGL-1	P-selectin glycoprotein ligand-1
TCGA	the cancer genome atlas
VISTA	V-domain Ig suppressor of T cell activation

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Fig. 1.

Confocal microscopy reveals VISTA localization within endosomal compartments.

Thioglycolate-elicited peritoneal macrophages from 8-week old C57BL/6 mice were allowed to adhere on glass coverslips for 2h followed by fixing and staining. In all panels, VISTA was stained with an anti-VISTA antibody (MH5A at 1:100) or isotype control Ab, secondary staining with anti-hamster IgG-Biotin (1:250) and detected using Streptavidin DyLight 649 (1:250). VISTA KO peritoneal macrophages were used as a biological control to validate specific staining of VISTA. For all other proteins, secondary staining was performed using goat anti-rabbit IgG A488 (1:1000, green) A) VISTA staining of macrophages counterstained with DAPI (Blue), B) VISTA staining (violet) with Rab11 staining (green). A representative cell is presented singly-stained with either VISTA (red) or Rab11 (green 1:100) to compare overlapping patterns of expression C) VISTA staining (red) with EEA-1 (green, 1:100) counterstained with DAPI (blue) D) VISTA staining (red) with Rab4 (green 1:200) and E) VISTA (red) with TMEM165 (green).

Fig. 2.
VISTA is prominently expressed within myeloid and T cells in spleens and tumors.
Multiplex IHC performed with the Opal Multiplex Manual IHC Kit (PerkinElmer NEL811001KT) on spleen (A) and tumor (B) from mice bearing CT26 colon tumors.

Table 1

Impacts of Targeting VISTA on Innate and Adaptive Immunity.

VISTA-Deficiency	VISTA Antagonists	VISTA agonists
Exacerbated GVHD [13]	–	Prevention of GVHD induction [5]
Enhanced experimental asthma [22]	–	Ameliorates experimental asthma [22]
Reduced Collagen-antibody-induced arthritis (CAIA) [19]	–	Reduced Collagen-antibody-induced arthritis (CAIA) [16]
Increased leukocyte infiltration into non-lymphoid tissues [14,33]		–
Reduction in tumor MDSCs Reduced tumor growth [4,33]		–
Increased encephalitogenic T cell responses (Th ₁₇) in EAE models [14]		Reduced EAE induction [Unpublished]
Enhanced Lupus nephritis disease [16,17]		Ameliorate Lupus nephritis [Unpublished]
Enhanced T cell responses [13]		Reduced T cell responses [5,13]
Reduced iTreg differentiation [24,33]		–