Attempt at elucidating the role of Galectin-3 in CD8 T cell biology during viral infection using nanobodies

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Certificate of Examination

This is to certify that the dissertation titled "Attempt at elucidating Role of Galectin-3 in $CD8^+$ T cell biology during viral infections using nanobodies" submitted by Mr. Ayush Jain (MP18004) for the partial fulfilment of MS degree programme of the institute, has been examined by thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report is accepted.

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Dated: May 10, 2021

Declaration

I hereby declare that matter embodied in this thesis entitled **"Attempt at elucidating Role of Galectin-3 in CD8$^+$ T cell biology during viral infections using nanobodies"** is the result of investigations carried out by me under the supervision of **Dr. Sharvan Sehrawat** at the Department of Biological Sciences, Indian Institute of Science Education and Research (IISER) Mohali, SAS Nagar Mohali, India. This work has not been submitted in part or full for the award of any degree, a diploma, or a fellowship to any other university or Institute. Whatever contributions of others are involved, every effort is made to indicate it clearly with due acknowledgments. In keeping with the general practice of reporting scientific observations, acknowledgments have been made whenever the work was described based on the findings of other investigators. Any omission that might have occurred due to an oversight error in judgment is regretted. A complete bibliography of the books and journals referred to is given in the respective section of the thesis.

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In my capacity as the supervisor of the candidate's thesis work, I certify that the above statements by the candidate are true to the best of my knowledge.

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ABBREVIATIONS

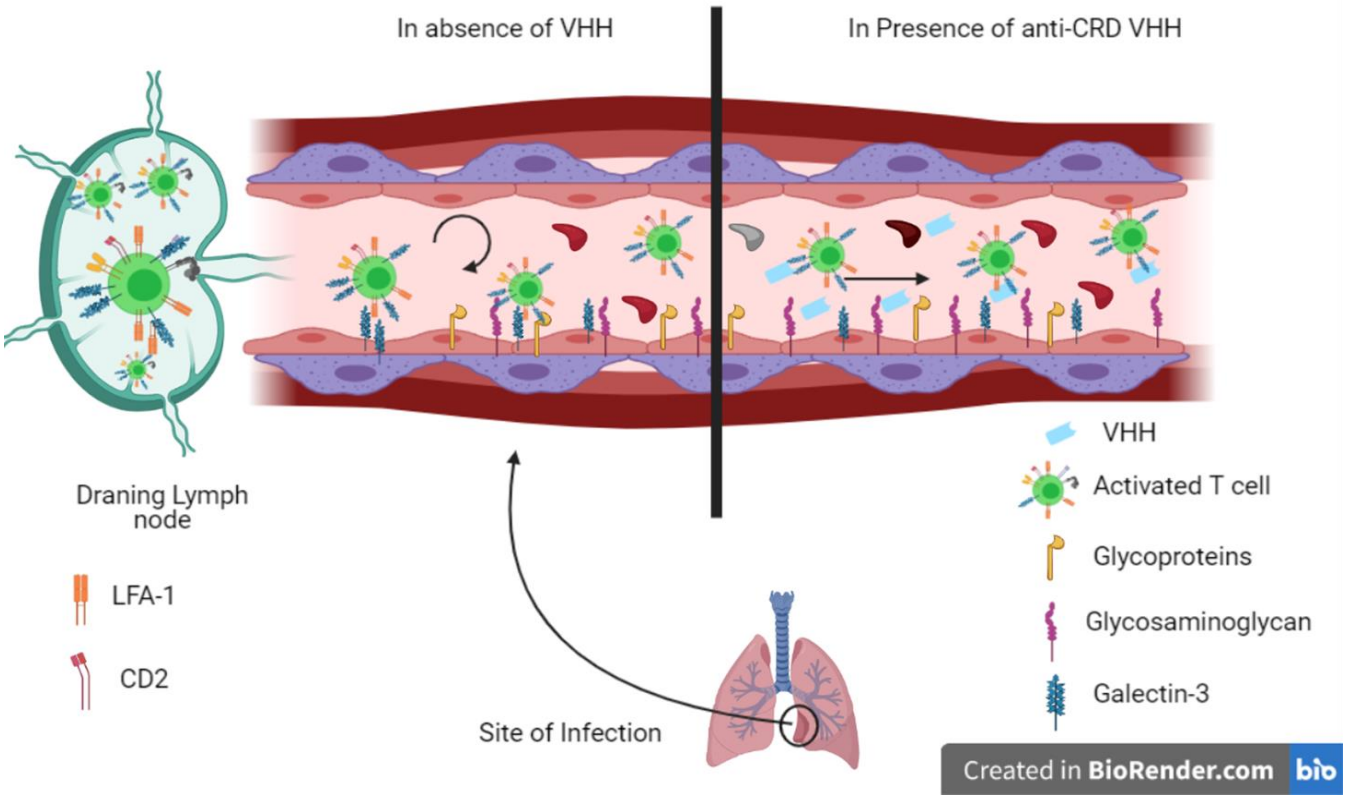
HCAb's	Heavy Chain Antibodies
FR	Framework Region
CDR	Complementarity Determining Region
VHH	Variable domain of heavy chain of heavy chain antibodies
VH	Variable domain of Conventional antibodies
SdAb's	Single domain antibodies
CRD	Carbohydrate Recognizing Domain
NTD	N- Terminal Domain
WSN-SIIN	Influenza virus expressing SIINFEKL epitope
WT	Wild Type
Ni-NTA	Nickel Nitrilotriacetic Acid
RT	Room temperature
IFNγ	Interferon Gamma
TNFα	Tumour necrosis factor
LTR's	Long Terminal Repeats
ICCS	Intracellular Cytokine Staining
ORF	Open Reading Frame
FACS	Fluorescence-Activated Cell Sorting
KO	Knock Out
IL-2	Interleukin-2
Dpi	Days post-infection
LN	Lymph Node
Gal-3	Galectin-3
IPTG	Isopropyl β-D-1 thiogalactopyranoside
FP	Forward Primer

RP	Reverse Primer
SDS	Sodium Dodecyl Sulfate
MLN	Mediastinal Lymph Node
In	Intranasal
Ip	Intraperitoneal
RPMI	Roswell Park Memorial Institute media
DMEM	Dulbecco's Modified Eagle Media
HRP	Horseradish Peroxidase
PAGE	Poly Acrylamide Gel Electrophoresis
RPM	Rounds Per Minute
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic Reticulum

Abstract

$CD8^+$ T cell-mediated immunity plays a pivotal role in the control of most intracellular infections. $CD8^+$ T cells exclusively exert their antiviral effect through close cellular interaction with the relevant virus-infected targets. The highly orchestrated response of anti-viral CD8+ T cells involves their activation, proliferation and their subsequent migration at the infected tissue sites. The interface between the circulating $CD8^+$ T cells and vascular endothelium along with extravascular chemokine gradients constitutes a major gatekeeper in regulating this $CD8^+$ T cell migration while the extravasation process involves complex interactions between adhesion molecules and their ligands present either on the T cell surface and vascular endothelium. This physical interaction is assisted via chemokines and their receptors which cause conformational changes in the integrins to increase their affinity for their ligands which is a prerequisite for firm adhesion and transmigration. We investigated the role of carbohydrate recognition domain (CRD) of extracellular and surface sugar-binding protein, galectin-3 in $CD8^+$ T cell migration during viral infection using in-house produced CRD-specific nanobodies (VHH). We show the critical role of the CRD in effecting the migration of activated $CD8^+$T cells to the site of infection. This can be corroborated with the previous observation that Galectin-3 through its CRD webs a reversible interaction with glycoconjugate ligands which advance $CD8^+$T to roll along the endothelial surface and thus creates the basis for firm adhesion and transmigration. Further to advance our understanding of various cellular functions of different domains of Gal-3, we further created a lentiviral mediated system for the production of intracellular nanobodies (intrabodies). A graphical abstract depicting the same is presented in Figure 1.

Figure 1. Graphical abstract of the study



Chapter 1

BACKGROUND AND OVERVIEW

1.1 Galectin-3 protein and its functions

Galectin-3 is a 35 kDa β-galactoside binding evolutionary conserved protein encoded by a single gene, LGALS3, located on chromosome 14. Identified in the year 1971 as an S-type lectin owing to its sulfhydryl dependency and solubility, it was renamed as galectin-3 after nomenclature systemization in the year 1994. The gene spans 17 Kb and contains 6 exons and 5 introns and has an open reading frame of 750 bp. Within a family of 15 proteins, galectin-3 is the most unique member, as it possesses along with its crown- jewel carbohydrate recognizing domain (CRD), an atypical N-terminal domain that is attributed to function independent of its CRD domain[1].

Owing to its wide range of functions, galectin-3 is present in a wide variety of tissues and cells. It can be found in the tissues of the digestive and urogenital tracts, eye, lungs, blood, kidney, prostate, and heart. Cells of the immune system are also not devoid of this molecule. There is a high expression of galectin-3 in myeloid cells like monocytes, macrophages, dendritic cells, neutrophils, eosinophils, and mast cells, and in lymphoid cells like T cells, B cells, and NK cells. Apart from this, galectin-3 is also found on epithelial and endothelial cells and is known to play a role in the migration of cancer cells. Besides its functions in adult life, it seems to play important role in embryogenesis, where it is more specifically expressed and located predominantly in the epithelia, kidney, chondrocytes, and liver. While its widespread distribution and multiple functions make one believes its indispensability to life, this notion is challenged by the observation that galectin-3-knockout mice are viable without abnormalities although with the exception of premature senescence[2].

Since its identification, galectin-3 has been one of the most studied molecules in terms of publication as per PubMed with the bulk of it contributed by basic biomedical research in the past 30 years. Galectin-3 has been attributed as a “molecule causing alphabet of diseases”, proving its importance as a viable target molecule by pharmaceutical companies either as a biomarker in diseases like coronary

heart diseases, renal cell carcinoma, chronic kidney diseases (CHD), thyroid carcinoma, and other multiple carcinomas or as a molecule to be targeted and eliminated therapeutically[2].

Structure of Galectin-3

Galectin-3 is classified as chimeric type galectin having a C-terminal domain (CTD) connected to a long, flexible N-terminal domain (NTD). The C-terminal domain is composed of about 130 amino acids that accommodate the whole carbohydrate-binding site and is, therefore responsible for the lectin activity of galectin-3. The CTD is folded into a typical β-sandwich (two anti-parallel β-sheets, composed of five and six β-strands) with a tryptophan core[3]. Galectin-3 protein contains multiple sites and motifs which assist it to perform a myriad of functions. The highly conserved NWGR sequence present on CRD is shown to be responsible for its anti-apoptotic activity similar to that achieved by Bcl-2 (Fig.1.1). It also confers the molecule the ability to self-associate in the absence of saccharide ligands with tryptophan being the kingpin of this interaction. The CRD also contains an F-site which is responsible for the interaction between the CRD and NTD of the same galectin-3 molecule[4]. Interestingly, CRD exhibits a stronger binding affinity for advanced glycation end-products (AGE) as compared to the full-length galectin-3 which suggests that the principal AGE-binding site present in CRD is sterically hindered by the NTD in the full-length galectin-3.

The flexible N-terminal domain of galectin-3 can vary between 110– 130 amino acids depending on the species and is sensitive to proteolysis by matrix metalloproteinases. It is composed of multiple homologous repeats (7–14), each of which includes a consensus sequence Pro–Gly–Ala–Tyr–Pro– Gly (PGAYPG), followed by three additional amino acids. It has two potential phosphorylation sites (residues 6 and 12). The ability of Galectin-3 to oligomerize or multimerize with other Galectin-3 molecules or to establish protein-protein interactions with other distinct proteins is attributed to glycine and proline-rich domains. The tip of the NTD contains IXTL and LI Motif which are nuclear localization signal (NLS) and nuclear exit signal (NES) respectively and are essential for the presence

of galectin-3 in the nuclear compartment. Interestingly, the protein sequence of Galectin-3 is approximately 25% homologous with some heterogeneous nuclear ribonucleoprotein (hn-RNP) complexes, suspecting its role in the splicing process. Galectin-3 also interacts with the endosomal sorting complex required for transport (ESCRT) through the P(S/T)AP motif present on the Base of NTD. While the NTD does not directly bind to carbohydrates recent studies have revealed its assistance (through Tyr 102) to CRD in Oligosaccharide binding[5]. It is also evident from previous reports that NTD plays an essential role in the full biological activity of galectin-3.

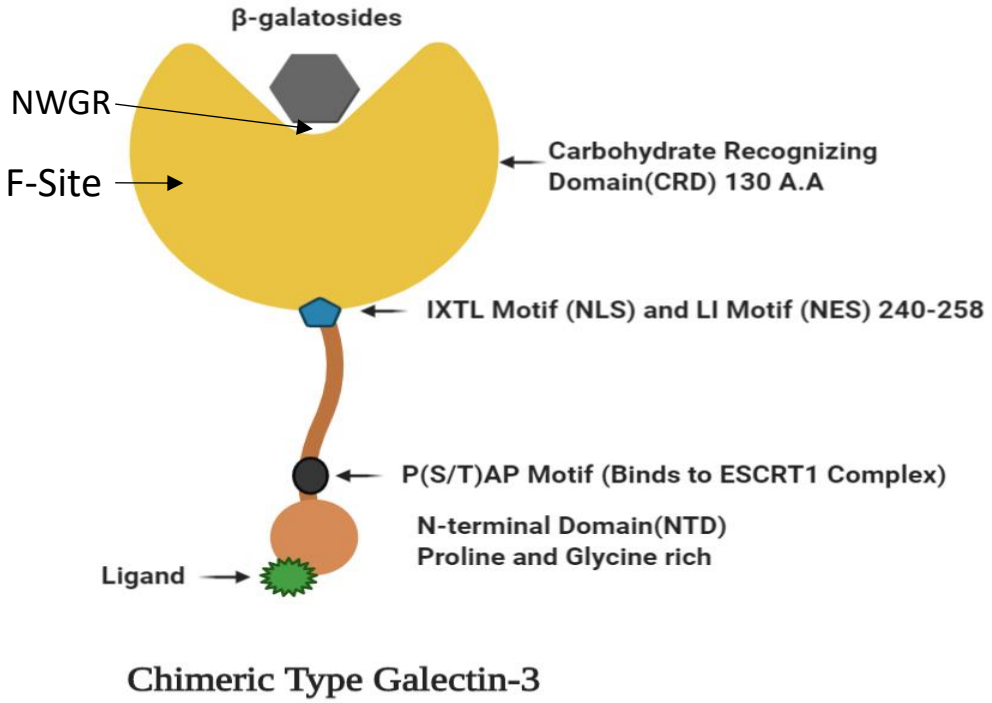


Figure 1.1 A schematic showing structural features of chimeric type Galectin-3 along with motifs.

Intra-cellular and extra-cellular functions of galectin-3

Galectin-3 is predominantly located in the cytoplasm and shuttles between the nucleus and mitochondria. Moreover, its localization affects its biological functions, which are strongly dependent on the cell type, the proliferative status of the cell, and growth conditions of the cell. Galectin-3 is

found to be associated with numerous cytosolic molecules as their ligand, which implies its involvement in multiple intracellular events. The various functions of intracellular galectin-3 are summarized in Table 1.1[1-12]. A thorough literature review points out that the majority of the nuclear functions of intracellular galectin-3 are mediated through NTD, while the major cytoplasmic functions are discharged through CRD, with some underlying exceptions.

Predominantly produced in the cytoplasm, galectin-3 is also exported outside the cell through a non-canonical secretory pathway i.e., not involving the standard Golgi/ER pathway. It is hypothesized that galectin-3 entry into the secretory pathway would disrupt the secretory pathway due to the binding of multiple glycoproteins to CRD of galectin-3. The non-canonical pathway involves the attachment of galectin-3 with multivesicular bodies (MVB) during their biogenesis, which then can recruit galectin-3 into exosomes. Galectin-3 is found on the cell surface as well as extracellular matrix and body fluids. After its secretion outside the cell, galectin-3 is mostly found in the vicinity of the cell, through its binding to cell surface glycoproteins, glycan ligands, and glycolipids or interacts with extracellular matrix glycoproteins surrounding the cell. Interestingly, extracellular galectin-3 was shown to mediate cell adhesion and signaling events that remain less well elucidated. Extracellular galectin-3 performs various functions through both the domains which are summarized in table 1.1[1-12]

A summary of the function	Ligand Involved	Localization of Galectin-3	Putative Galectin-3 domain Involved
Regulation of Apoptosis	Bcl-2	Cytoplasmic	CRD
Apoptotic signaling	CD-95 (Apo-1/Fas)	Cytoplasmic	CRD
Apoptotic signaling	Nucling	Cytoplasmic	CRD
Regulation of Apoptosis	Alix/AIP1	Cytoplasmic	CRD
Proliferation, differentiation, survival, and death	Activated K-Ras (K-Ras-GTP)	Cytoplasm and plasma membrane	CRD
Regulation of Apoptosis	Synexin (annexin VII)	Mitochondrial	CRD
Cell mechanical stability	Cytokeratin	Cytoplasmic	CRD
Nucleus associated functions	Chrp proteins	Cytoplasmic and Nuclear	CRD Independent
Splicing	Gemin 4, hn-RNPA2B1	Nuclear	CRD
Hedgehog signaling pathway	SuFu	Nuclear and Cytoplasmic	Not known
Regulation of Gene transcription	CREB and Sp1	Nuclear	CRD
Cell division	TTF-1	Nuclear	CRD
Wnt/B-Catenin signaling	Axin	Nuclear	CRD
Cell cycle regulation	P21,p27, Cyclins E,A,D	Nuclear	Not known
Mitotic spindle arrangement	NuMa	cytoplasmic	Not known
Cell adhesion	α1β1, αMβ1 integrin	Extracellular	CRD
Cell adhesion	Lamp1 and 2	Extracellular	CRD

Table 1.1 Domain-specific intracellular and extracellular functions of galectin-3

Immunological functions of galectin-3

Galectin-3 has been associated with complex Immunological functions. It affects the modulation of the immune system owing to both cis and trans effects on immune cells. It can have both pro and anti-inflammatory activities in the innate immune system, whereas it shows predominantly immunosuppressive effects in the cells of adaptive immunity. Major immunological functions of galectin-3 are summarised in table 1.2 [10-25].

Immune setting	Functions/ effects of Galectin-3	Outcome of effect
Pathogenic Infections and Inflammation	Negatively Regulates TCR expression in both CD4+ and CD8+ T cells	Increased viral infection
	Gal-3 enhanced the secretion of IL-10 by monocytes in TB	Anti-inflammatory
	Gal-3 also diminished the ability of monocytes to differentiate into DCs in mycobacterial infection	Increased infection
	Chemoattractant for Monocytes and Macrophages	Initiation of Inflammation
	Viral attachment to host cells in SARS-CoV-2	Increased viral infection
	Expression is upregulated *by Neisseria meningitis and Candida albicans*	Anti-inflammatory
	Upregulated expression by Helicobacter pylori in gastric epithelial cells	Anti-inflammatory
	Acts as PRR and DAMP to activate innate immune cells	Pro-inflammatory
	chemoattractant for monocytes and macrophages	Clearing of infection
	recognition and endocytosis of bacteria, mycobacteria, and fungi	Clearing of infection
	Increased expression in small airways of patients with severe chronic obstructive pulmonary disease	Anti-inflammatory
Cancers	Negatively Regulates the anti-tumor activity of cytotoxic T cells	The restricted killing of target tumor cells
	Captures IFNγ in tumor matrix to reduce chemokine gradient potential	Reduced T- cell tumor infiltration
	Cancer progression and metastasis	Pro-cancerous
	Responsible for Cancer stemness	Pro-cancerous
Regulatory functions and Autoimmunity	Prevents T cell proliferation and Th1 differentiation and induces T cell apoptosis	Prevents Autoimmunity
	Promotes phagocytosis of apoptotic cells	Prevents Autoimmunity
	Promotes B cell deletion and Anergy	Prevents Autoimmunity
	Overexpressed in synovial membrane during RA	Auto-inflammatory

Table 1.2 Various immunological roles of galectin-3

1.2 Nanobodies and their advantages

All camelids produce a unique class of antibodies along with classical immunoglobulins in their sera, a serendipitous discovery made in the year 1993. These antibodies were called 'heavy chain only antibodies' as they are devoid of light chains found in classical antibodies. The heavy chains of these antibodies contained canonical variable domains and it was soon realized that these variable domains could be expressed as independent and stable protein fragments. These independent variable domain fragments or VHH (Variable domain of heavy chain of heavy chain only antibody) could retain full antigen-binding capacity. Owing to their smaller size (Diameter; 2.5nm, length; 4 nm, and 13-15 KDa) they are also called nanobodies or single-domain antibodies (sdAbs)[26].

SdAbs provides multiple advantages over conventional antibodies along with maintaining the diversity and specificity associated with conventional antibodies. Since the nanobodies only comprise a single domain making it easier for their genetic manipulation for increasing their affinity for a ligand. Additionally, they are easier to produce in multivalent formats due to more flexible linker designs. Structurally, they are robust and have high physiochemical stability and solubility due to the increased composition of hydrophilic residues. Most importantly they can recognize hidden antigenic sites and can rapidly penetrate tissues owing to their small size and extended flexible CDR3 region. These nanobodies can be produced in bulk amounts due to their expression in bacteria and yeast. They can be selected in high throughput manner against multiple antigens when used along with phage display technology. Other important advantages of nanobodies are, that the same nanobody can be used across multiple species unlike conventional antibodies since they lack an Fc region and thus do not cause an immune reaction or antibody-dependent enhancement (ADE) during viral infections. Overall, these nanobodies confer multiple advantages due to their small size and high specificity and are increasingly becoming popular for therapeutic usage as well as a research tool.

Structural features of Nanobodies

Nanobodies show unique features as compared to their conventional counterparts. Nanobodies have a prolate-shaped paratope made up of 3 looped CDRs named CDR1, CDR2, CDR3 which are interspersed by four framework regions (FR). The VHH show more than 80% sequence homology with human VH domains. Interestingly, the VHHs show presence of amino acid substitutions at four FR2 positions (positions 37, 44, 45, and 47; Kabat numbering) which are conserved in conventional VH domains and forms the hydrophobic interface with VL domains. The single-domain nature of VHH is attributed to the presence of hydrophilic residue (mostly arginine) at position 103 instead of a conserved hydrophobic residue (tryptophan) that contacts VL in conventional antibodies. Most

importantly the CDRs of VHHs contains some characteristic features such as their lengthy structures with additional 2-4 amino acids as compared to the canonical antibodies to compensate for their reduced number of CDRs forming paratope, the N-terminal part of CDR1 is more variable, while the extended CDR3 is often stabilized by an additional disulfide bond with a cysteine in CDR1 or FR2, that results in the proper folding of the CDR3 loop[21]. Additionally, the extended CDR3 is stabilized by an extra disulfide bond with a cysteine at position 50 in FR2. In general, nanobodies assume a strict secondary structure with the majority of anti-parallel β–sheets interspersed by loops and disulfide bonds which gives them a typical CD signature.

Use of single-domain antibody fragments to study multi-domain proteins

Studying the functions of a single domain of multidomain proteins usually requires either domain deletion mutants, point mutants, or domain-specific chemical inhibitors. These techniques have several disadvantages as deletion mutants may hinder the functions of any unknown cryptic sites associated with the deleted domain of the protein. For example IXTL motif, LI Motif, F-site present in the CRD of galectin-3. Also, it can affect the stability and stability-mediated functionality of these proteins. Using specific chemical inhibitors can cause toxicity or unbalanced osmotic regulation during cellular and in-vivo experiments. For example, β–galactose if used to inhibit galectin-3 can cause osmolarity problems. Understanding the functions of these different domains using point mutations or alanine scan can eliminate the above-mentioned problems, but can significantly increase the duration and complexity of the study. Using sdAbs fragments to specifically study the function of individual domains of proteins provides significant advantages above all methods. Since these sdAbs can be selected against any domain and can be produced in larger quantities using bacterial or yeast expression systems they automatically qualify as the prime choice for such studies.

1.3 Intrabodies and their advantages

Intrabodies are intracellular antibodies that can target selected proteins inside the cell. The inherent stability of sdAb's makes them the most suited candidates for production in the intracellular environment as compared to other Single chain variable fragments (scfvs). The single-domain nature of VHHs makes them a better choice to select and engineer for targeting cryptic epitopes. These intrabodies can be conjugated with fluorescent markers like e-GFP, RFP, m-cherry, e-BFP, etc. to study the cellular localization of target proteins using confocal microscopy in primary cells without going through tedious and costly immune-staining procedures. Also, various cell organelle localization tags like ER retention tags (KDEL tags), NLS- tags, or mitochondrial localization tags can be used to

target specific proteins into the various organelle, to study the effect of their disruption in primary cells. Also, the ability of intrabodies to bind cleft-like structures makes them the ideal choice to disrupt their functions by binding to active sites of enzymes. Intrabody technology opens a new range of potential therapeutic targets, which were previously inaccessible to a traditional monoclonal antibody (mAb). Apart from this intrabodies can be used for proteasome-mediated selective degradation of target proteins in the lumen of ER.

Types of intrabodies and their comparisons

Intrabodies can be broadly divided into 2 subgroups based on their mechanism of action. Cytoplasmic intrabodies are produced in the cytoplasm and organelle-specific intrabodies, which are produced or targeted in different organelles. While different methods used to produce these intrabodies have been discussed in the next section. It is important to understand functional variations among these 2 kinds of intrabodies. Cytoplasmic intrabodies can attach to or neutralize targets in the reducing environment of cytoplasm. They can detect cellular components in living cells. Cytoplasmic intrabodies have been shown to successfully function against EFGR, NSP-5 protein of rotavirus, HBV antigen, HIV-1 protein, VP 35 protein of Zaire Ebola virus, DF-B8 protein of *Clostridium botulinum*, and many others [21,22,23]. The organelle-specific intrabodies can be of multiple types ex: ER intrabody, nuclear intrabody, mitochondrial intrabody, etc. The ER intrabodies are major organelle intrabodies that can functionally knockdown targeted proteins in the secretory pathway even without the requirement for antibody mediated neutralization. ER, intrabodies are targeted to the lumen of ER through the fusion of SKDEL signal to the C-terminus, which prevents its secretion along with the target protein it is bound. This triggers an ER-associated degradation pathway that can be proteasome-dependent or independent. Nuclear intrabodies can block the function of protein in the nucleus and similarly mitochondrial intrabody in mitochondria. Further, a schematic depicting types of intrabodies are shown in figure 1.2

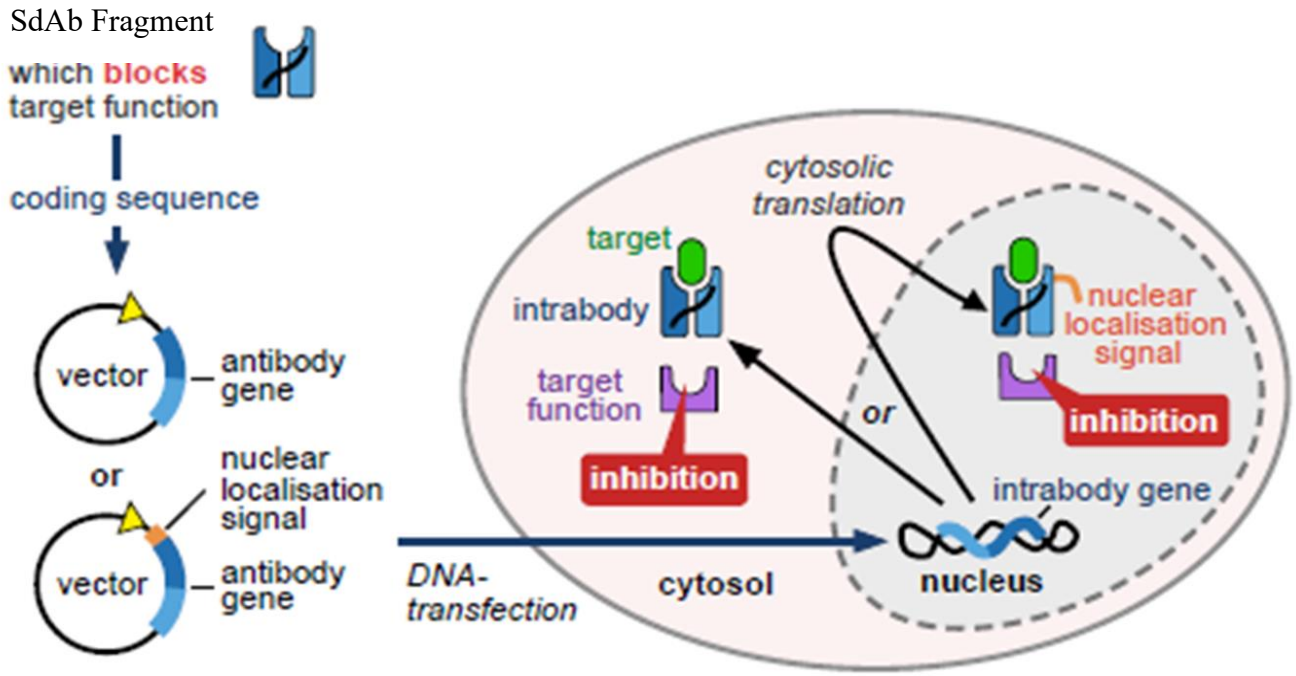


Fig 1.2 Types of intrabodies and their production process

Different methods to synthesize intrabodies

Intrabodies are generated by cloning the in-vitro selected (by phage display) VHH clone into a cytoplasmic or ER targeting vector. The intrabody gene is expressed inside the target cell after transfection in HEK-293 cells with a mammalian expression vector. Further to produce intrabodies in primary cells, retroviral or lentiviral vectors can be used that can produce intrabody laced recombinant lentiviruses which can further be transduced into primary cells to produce intrabodies in primary cells. Successful transfection and subsequent intrabody production can be analyzed through western blotting. While the localization of organelle intrabodies can be ascertained by co-staining with different organelle markers like calnexin for ER, DAPI for nucleus, etc. Apart from vector-based intracellular production of intrabodies, new techniques have been identified, where intrabodies are synthesized outside the cell and then delivered into the cell. Techniques such as microinjection, electroporation, nano-particle based photoporation, and polycationic resurfacing can be implemented although each of these techniques has its disadvantages when compared to the viral transduction method.

Intrabodies to target wide-spread intra-cellular and extracellular proteins

Attaching various signal sequences to intrabody fragments allows compartment-specific targeting of different domains of intracellular proteins. While ER targeting allows for the interference with the

function of membrane and secretome, which serves differently when compared to cytoplasmic and nuclear intrabodies. These strategies have allowed in-vitro as well as in-vivo knockdown of proteins in mice models for example intrabodies against VEGFR-2 in xenograft mice models[30], use of ER intrabodies against amyloid-β in a mouse model of Alzheimer's disease[31]. Cytoplasmic intrabodies have also been used in plants to disrupt cell-pathogen interaction. Intrabodies can also be tagged with secretion signals for their extracellular transport to target extracellular proteins. Furthermore using inducible and cell or tissue-specific promoters can facilitate the functional analysis of target proteins and their domains in tissue or cell spatial and temporal fashion.

Advantages of intrabodies and their emerging therapeutic applications

Intrabody technology is an attractive alternative to strategies like gene-targeted knockout animals and other knockdown techniques such as RNAi, mi-RNA, shRNA, etc. due to various advantages. These include 1. High specificity and less off-target effects owing to antigen-antibody interactions. 2. Possibility for a functional knockdown of several protein isoforms (splice variants and post-translation modifications) by single intrabody. 3.Intrabodies can be engineered to target proteins at specific cell compartments and ER intrabodies can result in functional knockdown of the proteins. 4. Intrabodies with high affinity can also efficiently recognize linear epitopes in partially folded proteins and can accelerate their degradation. 5. As compared to pharmacological agents for protein inhibition intrabodies can be easily identified and can be rationally and systematically generated in quick time. 6. ER Intrabodies have potentially longer half-life when compared to RNA- molecules commonly used in RNAi. 7. Intrabodies do not trigger interferon and pro-inflammatory cytokine responses which are evident during RNAi and siRNA mediated knockdown and. 8. Intrabodies can be used to study the functions of embryonically lethal genes as intrabodies can be engineered for only partial functional knockdown these proteins. 9. Intrabodies can be generated against bio-informatically predicted ORF proteins of unknown function without the availability of unknown target protein[34].

Intrabodies are emerging as therapeutic agents as they can be engineered in various formats for optimal pharmacokinetic properties. In 2018 first VHH based therapeutic, caplacizumab was approved for a rare blood clotting disease. Several other clinical trials are currently ongoing. Some of the known therapeutic applications of intrabodies are 1. Intrabodies or VHHs have the potential to outperform mAbs for the treatment of solid tumors, owing to their small size and more tissue penetration. 2. ER intrabody-based functional degradation can act as a therapeutic solution for diseases like Parkinson’s and Alzheimer’s which are caused due to protein misfolding. 3. Intrabodies can act as a potential therapeutic option against emerging viruses like Coronaviruses and other zoonotic and live-stock

viruses. 4. Intrabodies against common surface glycoproteins of pathogens can help prevent broad-scale infections and associated co-morbidities[35]. In a nutshell, curiosity and creativity are the only limitations in optimal utilization of sdAbs for a myriad of applications.

Chapter 2 Materials and Methods

2.1 Selection and synthesis of single-domain antibody fragments

Single domain antibody fragments or VHH (Variable region of the heavy chain of heavy chain only antibody) were biopanned against the CRD and NTD domains of galectin-3 from the VHH library and were subcloned into bacterial expression plasmid vector yBNT and transformed into *E. coli* strain, Origami, and selected by ampicillin. These selected colonies were then subjected to colony PCR to identify recombinant clones over self-ligated vectors.

After cloning and sequence verification, selected clones were cultured at 37^0C after IPTG induction for 4 hrs, and the culture was pelleted at 8000 rpm for 10 mins. The recombinant proteins were extracted from the inclusion bodies as described elsewhere **( )**. Briefly, the culture pellets were subjected to sonication in lysis buffer (100mM Tris Base, 10mM EDTA, pH 8.0) for 8 cycles followed by high-speed centrifugation at 4^0C (15000 RPM). The supernatant was discarded and the pellet was treated with wash buffer A (100mM Tris Base, 10mM EDTA, 1% v/v Triton 100 X, pH 8.0) and sonicated which then followed by high-speed centrifugation. This step was repeated multiple times until the pellet showed a milky white appearance. The collected pellet was then treated two times with Wash buffer B (100mM Tris Base, 10mM EDTA, 1M NaCl, pH 8.0) followed by high-speed centrifugation to remove detergent. The inclusion body containing pellet was then subjected to solubilization by treating it for 16hrs on Rota spin with a denaturing buffer (100mM NaH_2PO_4, 10mM Tris base, 8M Urea, pH 8.0).

2.2 Purification of single-domain antibody fragment

The soluble proteins dissolved in denaturing buffer were purified using a 2-step purification process. In step 1, the His-tagged VHH was separated from other soluble proteins present in denaturing buffer, by binding it on a Ni-NTA column. Loosely bound non-targeted proteins were washed out using 20mM imidazole-containing denaturing buffer. The His-tagged containing recombinant protein was eluted using Elution buffer (400mM Imidazole, 100mM NaH_2PO_4, 10mM Tris base, 8M Urea, pH 8.0). The eluted protein was then run using a 15% SDS-PAGE gel to check for purity and was subjected to Bradford estimation to check the concentration of the purified protein.

2.3 In-vitro refolding of single-domain antibody fragments

The purified single-domain antibody fragments or VHH were subjected to in vitro refolding at 4^0C in refolding buffer [10mM Tris Base, 1mM EDTA, 1mM GSH(Reduced), 0.1mM GSH(Oxidized), 400mM L. Arginine]. For a 50ml refolding reaction, 3mg of purified protein was added in three injections of 1mg each at an interval of 9hrs and the reaction mixture was stir at low rpm for 24 hrs after the 3rd injection. The refolding solution containing refolded protein was spun and concentrated using a 3KDa molecular weight cut-off filter. After concentrating the purified protein to 1ml, it was collected and subjected to size exclusion chromatography to separate folded fractions from the unfolded fractions based on their hydrodynamic volume. Based on the UV 280 signal on the chromatogram, different eluted fractions were collected, and their purity was checked by running them on an SDS-PAGE gel, while their folding status was checked using Circular Dichroism (CD). The complete process of VHH identification and functional protein production is summarized in figure 2.1

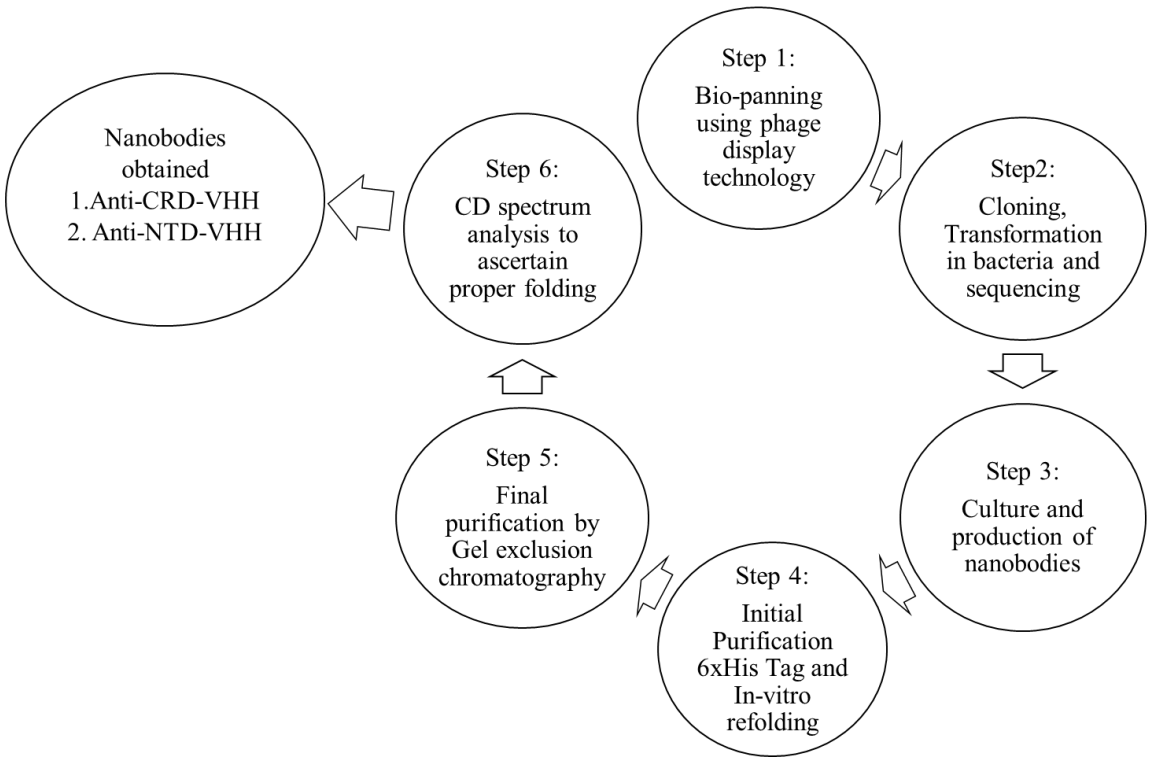


Fig. 2.1 Process of VHH identification till functional protein production

2.4 Determining fine specificity of anti-CRD VHH using ELISA and computational molecular docking studies

Purified single-domain antibody fragments (VHH) biopanned against the CRD were subject to ELISA to establish its fine specificity. About 50µg/well of galectin-3 was incubated with different concentrations of lactose, a natural ligand of CRD of galectin-3, for 1 hr at 4C. This combination of galectin-3 and lactose was coated on the ELISA plates for 12hrs at 4°C. Following which the wells were washed with 0.05% PBST and were blocked in 5% BSA in PBST for 2hrs at RT followed by washing with PBST and incubation with biotinylated sdAb at 30 ug concentration for 1.5 hrs at RT. followed by washing and incubation with 1:50,000 conc. of Streptavidin conjugated-HRP for 1.5 hrs. The plate was washed and developed with 100µl of TMB substrate from Sigma Aldrich (1mg/ml) in hydrogen peroxide. 50µl of stopping solution (3M H_3PO_4) was added after the development of colour and absorbance was taken at 495nm.

Further computation docking studies were done to locate VHH binding groove in the CRD of galectin-3. i-Tasser online software was used to model the 3-D structure of VHH and further online molecular docking tool Clus Pro 2.0 was used for blind docking predictions for docking of predicted VHH structure with known Galectin-3 crystal structure (PDB-4R9C). The most stable predicted docked conformation was selected and analyzed using the UCSF Chimera tool.

2.5 Cell staining for flow cytometry using single-domain antibody fragments

The single-cell suspension was prepared from the secondary lymphoid organs of C57BL/6 mice and $CD8^+$ T cells were sorted using BD-FACS-Aria-III at the central flow cytometry facility at IISER, Mohali. Sorted $CD8^+$ T cells were incubated for 72 hrs with CD3 (1µg/ml) and CD28 (0.5µg/ml) at 37^0C for in-vitro activation. Activated T cells were washed with PBS and stained with anti-CD8-FITC (1:400) and Anti-CRD-VHH (1:100) dilution for 1 hr on ice followed by washing with PBS. The bound biotinylated VHH was then stained with Streptavidin-APC at 1:200 dilution. Proper controls were used and cells were acquired using a BD-Accuri flow cytometer.

2.6 Cell staining for confocal microscopy using single-domain antibody fragments

A-549 cells were fixed by 5% PFA on coverslips for 10 mins. After cells were fixed, they were permeabilized in permeabilization buffer (PHEM+0.2% Saponin + 5% FBS) for 30 mins. After which cells were stained with VHH in Blocking buffer (1:100) for 30 mins at RT followed by washing with 1X PBS and staining with anti-His-IgG mice antibody (2^0 antibodies for VHH) in 1:200 dilution for 2 hrs and washed with PBS. Following which the cells were stained with Alexa-flour Anti-mouse-488 antibody. Coverslips were analyzed under a confocal microscope and images were recorded and analyzed by ImageJ software.

2.7 Mice and Viruses

C57BL/6-OT1 mice were procured from Jackson Laboratory, USA, and bred in individual ventilated caging system at Small Animal Facility for Experimentation (SAFE), IISER, Mohali. Influenza virus was grown and titrated using MDCK cells (ATCC). Institutional Animal Ethics Committee (IAEC) for control and supervision of Experiments on Animals (CPCSEA), which is established under Chapter 4, section 15(1) of the prevention of Cruelty to Animals Act 1960 approved all the animal experimental protocols. All procedures were performed strictly according to the approved protocols.

2.8 Intracellular cytokine staining (ICCS) of CD8+T cells

Single-cell suspension was prepared from the infected organs and lymph nodes of WSN-SIINFEKL infected animals. $1x10^6$ cells were incubated with 1x brefeldin A and 20 ng/ml of IL-2 in the absence or the presence of cognate peptide (1μg/ml of SIINFEKL) for 4 hrs. After the incubation period was over, cells were surface stained with anti-CD8 and anti-CD44 antibodies. After 3 washes, cells were fixed in IC fixation buffer for 25 mins at room temperature and then permeabilized in 1X permeabilization buffer for 5 mins at room temperature (RT). After 2 more washing and Fc blocking, cells were stained with anti-IFN-γ and anti-TNF-α antibodies for 25 mins in dark at RT. To remove unbound antibodies, cells were washed thrice and acquired using a flow cytometer.

2.9 Treatment of mice with galectin-3 and anti-galectin-3 sdAbs to measure its effect on $CD8^+$ T cell migration during viral infection

Sex matched C57BL/6 mice infected with WSN-SIINFEKL intranasally were divided into three groups designated as VHH, galectin-3, and Sham. Mice in groups of three were treated intraperitoneally with anti-CRD-VHH (10mg/Kg body weight) and galectin-3 (10mg/Kg body weight) at 4^{th} dpi to determine the change in the migration pattern of $CD8^+$ T cells owing to different treatments. These mice were continuously monitored for loss in weight and blood was collected through the retro-orbital plexus until 8dpi, after which mice were sacrificed and perfused with PBS. Lymphoid organs such as the draining lymph node (Mediastinal Lymph node) and Infected organs (Lungs) and Spleen were collected in 10% RPMI on Ice. Following which single-cell suspension was prepared. Cells were washed with PBS and stained using antibodies against CD8, CD4, CD44 along with Kb-SIINFEKL-tetramers in required dilutions. A slightly modified staining procedure was used for Blood Staining. Cells were then analysed using a BD-FACS-Aria-III flow cytometer and analyzed by Flow-Jo software.

2.10 In-vitro culture of Tregs from mice in the presence of an anti-CRD galectin-3 sdAbs

Splenocytes were collected from naive Foxp3-GFP knock-in mice and single-cell suspensions were made, which were then stained with anti-CD4 antibody and were 2 way sorted using BD-FACS-Aria-III using CD4 and GFP markers. $CD4^{+ve}$ and GFP^{-ve} cells were then *in vitro* stimulated with anti-CD3 and anti-CD28 for 16 hrs and thereafter were pulsed with different concentrations (0.2µg,0.5µg, 2.5µg, 5.0µg, 6.25µg, 12.5µg, 25µg) of anti-CRD-VHH. These pulsed cells were then collected at different time intervals (12hrs, 16hrs, 24hrs, 36hrs, 48hrs, 72hrs) and analyzed in BD Accuri for their GFP expression.

2.11 Cloning of sdAbs into the retroviral and lentiviral vector

Identified domain-specific nanobody clones against different domains of galectin-3 were screened by colony PCR using VHH specific primers and were further PCR amplified using forward primer (FP)

of VHH FR1 and reverse primer (RP) of VHH FR4 along with the addition of 3'Myc-tag. The amplicon and a modified retroviral vector pMKO.1 GFP (pMAD) vector were then digested with Age1 and Eco RI and ligated in vector to insert ratio of 1:5. Clones with required inserts were identified through double digestion. The clones were grown in LB-amp for plasmid isolation and were then transduced into HEK 293T cells and their expression was monitored via GFP fluorescence. Since the retroviral vectors can only infect mitotically active cells that posed a limitation for infection of primary naïve cells. We then switched to the 3rd lentiviral vector pLKO.1 GFP system as lentiviruses are capable of infecting non-dividing and actively diving cell types. To produce intrabodies in primary cells using a lentiviral vector, identified VHHs against galectin-3 were cloned as per the following cloning strategy, 5'- CMV promoter-VHH - c-Myc-tag - e-GFP -3'. The selected VHH were amplified using the following primers FP VHH FR1 pLenti (Common primer for producing Intrabodies from any VHH) 5'-GCATTCTAGAGGCACCCCGGGATGGCCGATGTTCAACTGCAGGAG-3' and RP VHH FR4 C-Myc pLenti (Primer with C-Myc tag attached to 3' end of VHH) 5'-GCTAGGATCCTGCAGATCCTCTTCAGAGATGAGTTTCTGCTCTGTGGAGACGGTGACCT G- 3'. The amplicon along with pLKO.1 GFP vector was digested using BamHI and XmaI and ligated in a vector to insert ratio of 1:3 and transformed to Stbl3 *E. coli* strain. Colonies confirmed through colony PCR were re-confirmed by restriction digestion and further used for plasmid isolation and transfection of HEK293T cells. Two additional restriction sites (Xba-1 and Sma-1) were added at the 5' end of VHH to accommodate any organelle-specific tag for the production of organelle-specific intrabodies.

.2.12 Transfection of selected intrabody clones into HEK-293T cells to produce recombinant Lentiviruses.

Third-generation lentiviral packaging vectors, pCMVR.74 and pMD2.G (VSV-G envelop vector) were also used. pLenti-GFP (Core with 5' and 3' LTR) plasmid encoding intrabody sequence along with C-

Myc tag was used along with the plasmids containing Tat1b and Rev1b. The above mentioned 5 plasmids, pCMVR8.74 (9µg), pMD2.G (9ug), pLenti GFP VHH-C-Myc (10.8µg), Tat-containing plasmid (6µg) and Rev plasmid (6µg) were mixed with 1:3 of PEI (1µg/ml)43 in 5ml of serum-free DMEM/Petri-plate, followed by instant mixing by vortexing for 40 seconds. The above mixture was kept at RT for 15 minutes and co-transfected in HEK239T cells (Human Embryonic Kidney). The transfected HEK293T cells were used for generating replication-incompetent Intrabody laced recombinant lentivirus (LV). The cells were analyzed for GFP expression 72 hrs post-infection by fluorescence microscopy using Nikon Eclipse Ti and supernatants were collected at 72hrs post-transfection and centrifuged at 1000g to remove cell debris. The collected viral supernatant was concentrated with a solution, 1.2M NaCl and 40%(W/V) PEG-8000 on ice for 3-4 hrs followed by centrifugation at 3500g for 65 minutes. The precipitated virus pellets were dissolved in a serum-free medium, stored at 4°C, and used within a few days-time.

2.13 Transduction of Recombinant Lentiviral vector and checking the expression of Intrabodies in Transduced $CD8^+$ T cells

The concentrated intrabody laced recombinant lentiviruses were then added to MACS/FACS sorted primary $CD8^+$ T cells in RPMI Medium. Cells were then centrifuged at 800g (37^0C) for 1 hr for spin transduction. After resuspending cells, they are incubated at 37^0C, 5%CO_2. The cells were analyzed for GFP expression 72 hrs post-infection by fluorescence microscopy using Nikon Eclipse Ti. Further, the level of GFP expression in these cells is ascertained by Flow cytometry and at optimal GFP expression cells are sorted using BD-FACS-Aria-III under sterile conditions for further experimentation.

2.14 Statistical analysis

Graph pad prism software version 8 was used for statistical analysis. Data with similar variances and having Gaussian distribution were analyzed with two-tailed unpaired student's t-tests. Data not following Gaussian distribution were analyzed with two-tailed Mann-Whitney U tests. For multiple

comparisons, a two-way ANOVA test with Bonferroni post hoc analysis was used. The P-value below 0.05 is considered significant. * <0.05, ** <0.01 and *** <0.001.

Chapter 3 Results

3.1. Functional anti-CRD sdAb shows fine specificity for CRD of galectin-3

Using phage display technology sdAbs were successfully biopanned form the phage display library against galectin-3 protein. The selected phagemids were then PCR amplified using VHH specific primers and sub-cloned into yBNT vector and transformed into Origami expression strain of E. coli. Positive clones were identified using colony PCR as shown in Fig 3A. Further, the positive clones were sequenced and analyzed in comparison to known VHH sequences. The positive clones containing full-length VHH were cultured in LB media under Amp selection and induced using 1M IPTG for 4hrs at 37^0C as shown in Figure 3B. To extract VHH from the inclusion bodies of these bacteria's they were subjected to lysis, sonication, and washing, and finally purified inclusion bodies were dissolved into urea solution and purified using Ni-NTA resin as shown in figure 3C and 3D. To obtain the fully refolded and functional VHH, these purified proteins were subjected to in-vitro refolding under different conditions after which they were subjected to gel-filtration chromatography to separate impurities and other partially folded products from refolded VHH as shown in Figure. 3E also the Secondary structure of these refolded VHH was ascertained by Circular dichroism (CD) plots which were analyzed in comparison to typical VHH CD signature as shown in 3F and 3G. These refolded VHH's were there subjected to ELISA to ascertain their fine specificity against different domains of Galectin-3, which showed a significant difference among the lactose (natural ligand for CRD of galectin-3) bound and unbound Galectin-3 when detected through anti-CRD VHH as shown in Figure 4A. In addition to ELISA, computational and molecular docking studies were performed which revealed that anti-CRD VHH specifically interacts with the CRD of galectin-3 as more interactions (51 interactions) with amino acid residues present in sparsely conserved galectin-3 specific D subsite were observed in the carbohydrate-binding region of galectin-3, whereas only 12 interactions with the widely conserved canonical C-subsite of galectin-3 CRD were observed. These results suggested that

binding of carbohydrate moieties can be inhibited by anti-CRD VHH (Figure. 4B). After ascertaining the fine specificity of anti-CRD VHH the functionality of these VHH was assessed using flow cytometry during which biotinylated VHH was conjugated with streptavidin PE and used for cell surface staining of FACS sorted, CD3/CD28 activated $CD8^+$ T cells as shown in the figure. 4C. Further, these VHH were used in immunostaining for confocal microscopy analysis as shown in the figure. 4D. Overall, these experiments suggest that a finely specific functional VHH against the CRD domain of Galectin-3 was obtained.

3.2. Anti-CRD single domain antibody fragment significantly hinders migration of $CD8^+$ T cells to effector site in virus-infected mice as compared to control.

To elucidate the role that galectin-3 might be playing in the shaping of anti-viral $CD8^+$ T cell dynamics *in vivo*, we employed the use of the panned single-domain antibody against the CRD of galectin-3. Figure 5A summarizes the scheme of the experiment. Briefly, naïve B6 mice have been intranasally infected with 200pfu of the WSN-SIINFEKL (Influenza A virus). These were then randomly divided into groups of three. At 4dpi, one group received an intraperitoneal injection of galectin-3 (100µg, Gal3 T_x) while another group was given a single dose of the anti-CRD VHH (100µg, VHH T_x) while the third group received the diluent (Sham T_x). Their weight loss was tracked throughout the course of infection. As is evident from figure 5B, the animals receiving exogenous galectin-3 as well as the VHH showed a higher loss of weight as compared to the control animals. Since this difference in weight was the maximum at 8dpi, which coincides with the peak of $CD8^+$ T cell response during an Influenza A virus infection (), we decided to perform cellular analysis at this time point. While the virus-specific K^b-SIINFEKL-Tet^+ cells increased in frequency in the circulation of the VHH treated animals (Figure 5D), their total counts were decreased in the peripheral circulation (Figure 5E) and site of infection, i.e., the lungs (Figure5 I-L). However, their frequency, as well as absolute count, was significantly higher in the draining mediastinal lymph nodes of the animals that received the VHH treatment (Figure 5F-H). This indicated the retention of the virus-specific cells in the group that had received the anti-

CRD VHH. Interestingly, the counts of the virus-specific cells in the mediastinal lymph node of the group that received an exogenous administration of Galectin-3 were severely less as compared to both the sham control and VHH treated animals. This however was not decreased in the lungs of the galectin-treated mice. These results suggested that Galectin-3 could be playing a role in the migration of the virus-specific cells to the site of infection during a local infection. Additionally, the attrition of virus-specific cells in the draining lymph nodes of the galectin-treated animals also hinted towards a role that Galectin-3 might be playing in the shaping of the dynamics of virus-specific cells during a local infection. Functionality-wise, the count of IFN-γ producing $CD8^+$ T cells was decreased in the lungs of the animals receiving exogenous galectin as well as the VHH (Figure 6A). The mediastinal lymph nodes of the Vhh treated mice however showed an increased count of IFN–γ producing $CD8^+$ T cells (Figure 6B), further showing that indeed a blockade of the CRD of endogenous galectin-3 was leading to the retention of virus-specific cells in the draining lymph nodes of treated animals.

3.3 Single-domain antibody fragment against CRD of galectin-3 does not upregulate foxp3 expression in mice Tregs.

Since galectin-3 has been shown to lead to immunosuppression by induction of $Foxp3^+$ Tregs during certain cancers, we tested whether the observed retention could be due to immunosuppression mediated by the induction of Tregs by the Vhh treatment. To this end, we first sorted $CD4^+FoxP3^-$ T cells from pooled lymph nodes of FoxP3-GFP knock-in mice. These sorted cells were then activated with anti-CD3 and anti-CD28 antibodies following which they were incubated with different concentrations of the Vhh. The cells were then flowed cytometrically analyzed for the induction of FoxP3 at 5 days post-incubation. As is seen in Figure 7A, we weren't able to get any induction of Tregs, suggesting that the retention of $CD8^+$ T cells could be due to a direct rather than indirect action of the Vhh on the virus-specific cells. Moreover, the phenomenon observed might be independent of the induction of Tregs.

3.4 Functional Intrabodies against Galectin-3 protein can be synthesized in mice $CD8^{+}$T cells using recombinant Lentiviruses

Using a 3rd generation lentiviral system Galectin-3 specific VHH were successfully cloned for intrabody production as shown in Figures 8A, 8B, and 8C. Further, these clones were used to produce intrabody laced recombinant lentiviruses were successfully produced in HEK293T cells which were evident through e-GFP expression visualized under a fluorescence microscope as shown in the figure. 8D. These recombinant lentiviruses were then concentrated and spin transduced into FACS sorted mice $CD8^{+}$ T cells to produced cytoplasmic intrabodies ascertained through e-GFP expression. These GFP+ cells were then FACS sorted as shown in figure 8E. to be further used in various experiments.

Fig. 3 Production of Functional Single domain Antibodies against CRD and NTD of Galectin-3.

After bio-panning of SdAb's against Gaelctin-3 protein the selected phagemids were PCR amplified and subcloned into yBNT vector and transformed into origami E. coli strain and positive clones were selected by colony PCR using insert specific primers to obtain 450 bp amplicon as shown in Fig. A. These clones were then cultured and subjected to IPTG induction, with prominent 17 kDa band as shown in Fig. B. Subsequently these induced cultures were pelleted down, lysed and purified using Ni-NTA resins as shown in Fig C for anti-CRD VHH and Fig. D for anti-NTD VHH. (FT- flow through, W1-Wash 1, W2-Wash 2, E1-Elution 1, B1- blank, Bd- Ni-NTA Beads, P- ladder). After purification, these VHH's were subjected to in-vitro refolding using different compositions of refolding buffer and then were subjected to Gel-filtration chromatography as shown in Fig. E. the larger peak corresponds to refolded VHH while other peaks correspond to misfolded or unfolded products which were ascertained by Circular Dichroism study of these different fractions eluted during chromatography as shown in Fig. F Misfolded product as it does not have characteristic VHH spectra while the spectra of properly folded VHH can be seen in Fig. G. as its spectra have a dip at 208 and 218 nm characteristic of properly folded VHH protein.

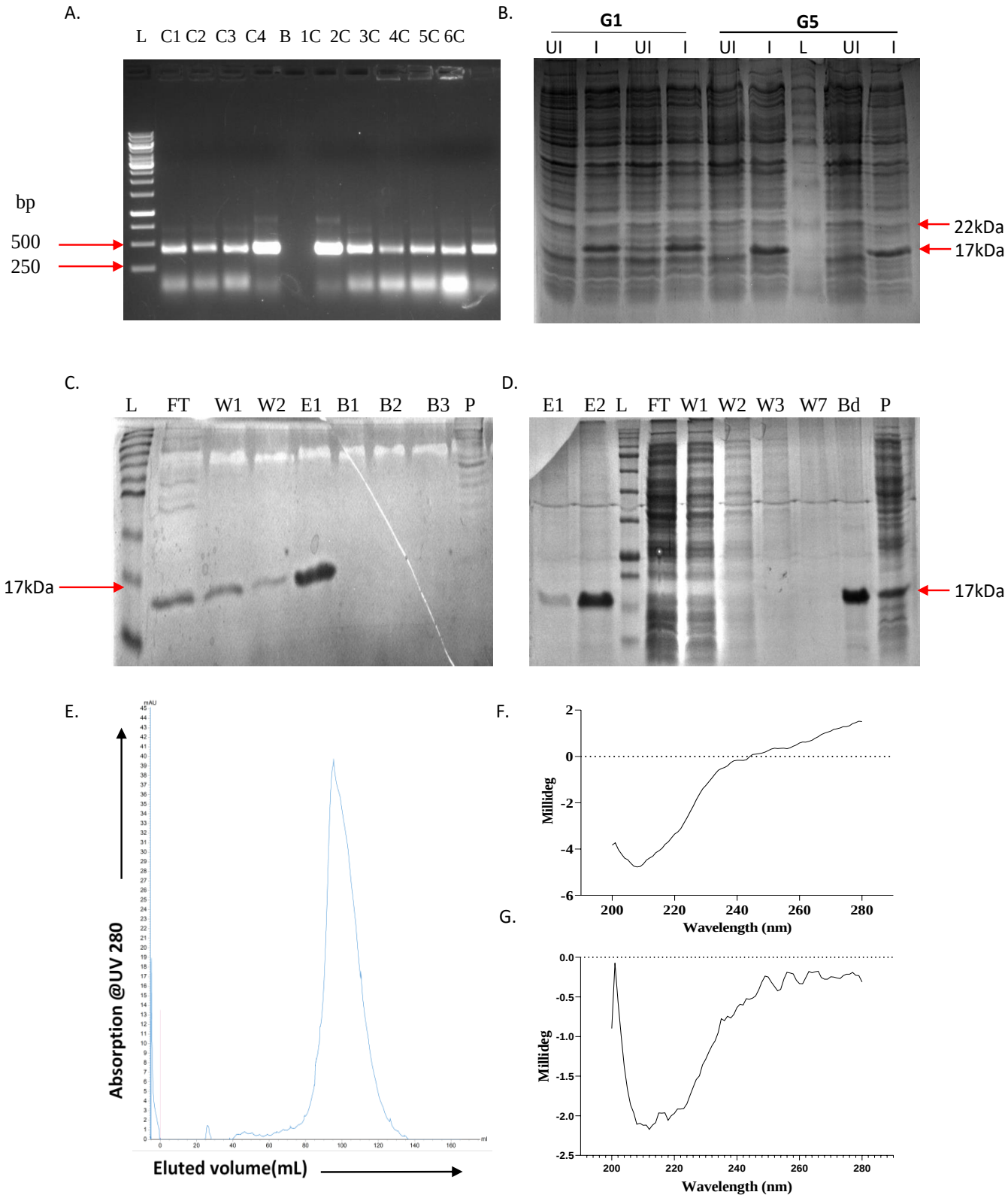
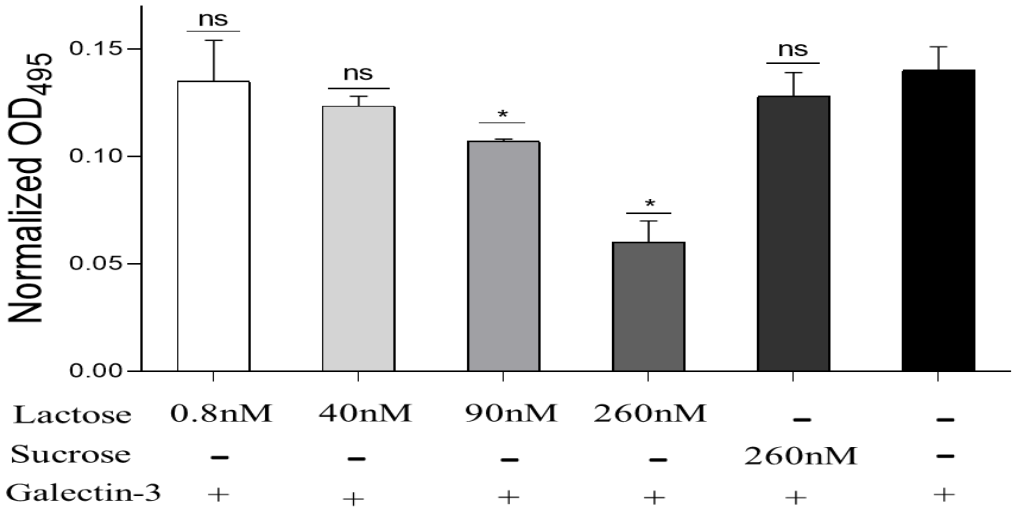

A.
L C1 C2 C3 C4 B 1C 2C 3C 4C 5C 6C
bp
500
250
B.
G1
G5
UI I UI I UI I L UI I
22kDa
17kDa
C.
L FT W1 W2 E1 B1 B2 B3 P
17kDa
D.
E1 E2 L FT W1 W2 W3 W7 Bd P
17kDa
E.
Absorption @UV 280
Eluted volume(mL)
F.
Millideg
Wavelength (nm)
G.
Millideg
Wavelength (nm)

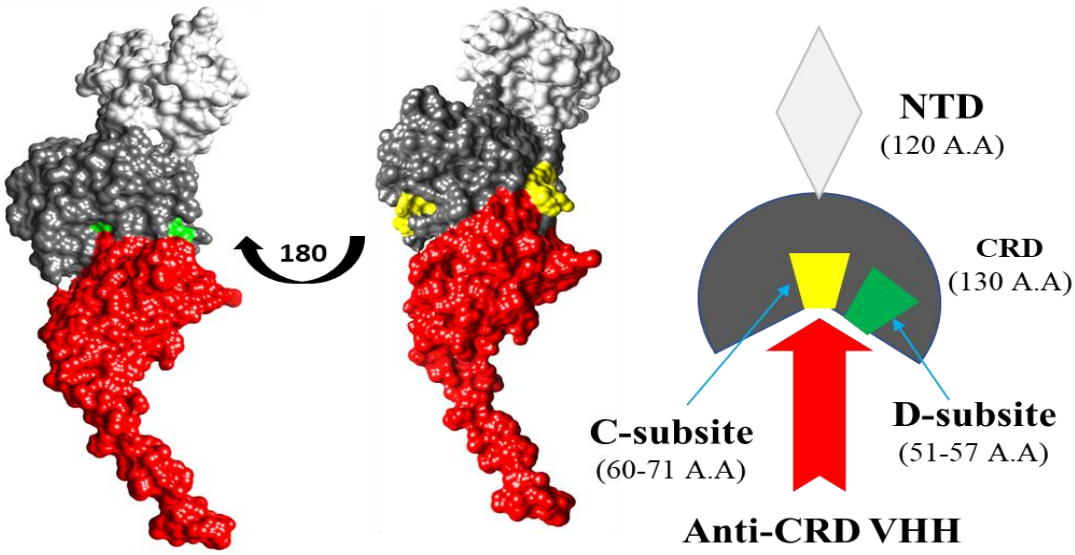
Fig. 4. Functional anti-CRD single domain antibody fragment shows fine specificity for CRD of galectin-3

To determine the fine specificity of anti-CRD VHH against gal-3 CRD domain, ELISA was performed in which 20ug Galectin-3 was coated in the wells along with the increasing concentration of lactose which is the natural ligand of CRD of Galectin-3, and non-ligand sucrose and free gal-3 were detected using anti-CRD VHH as shown in the Fig. A. with the increase in the concentration of lactose CRD of galectin-3 was saturated as gave low chemiluminescence using anti-CRD VHH. While the highest concern. Sucrose did not hinder the binding of anti-CRD VHH with Galectin-3. To further validate the fine specificity of anti-CRD VHH we performed computational molecular docking studies using i-Tasser and UCSF chimera which revealed that the conserved C-subsite of CRD is blocked by 12 interactions with anti-CRD VHH while the gal-3 specific and less conserved D-subsite showed 52 interactions, which confirms the specificity of anti-CRD as shown in Fig. B. After successful refolding of anti-CRD VHH, it was subjected to Flow cytometry staining of sorted and CD3/CD28 activated $CD8^{+}$T cells as shown in Fig. C. Were activated T cells show an increase in Galectin-3 surface expression as compared to inactivated cells. Further, these VHH were also used for immune-staining for confocal microscopy in A549 cells. Since 3 levels of staining were involved a control group without VHH was used. As shown in Fig. D. Anti-His panel show distinct and bright puncta in the cytosol in the VHH group as compared to the non-VHH group. DAPI was used as a marker to stain the nucleus.

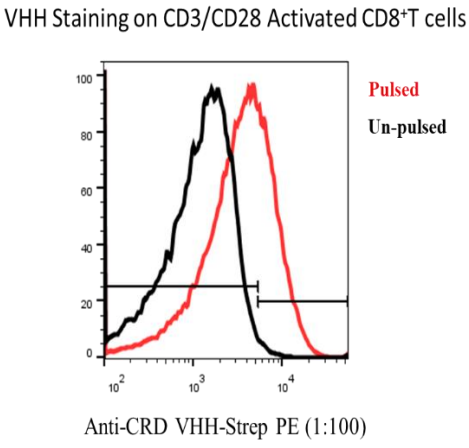
A.


Normalized OD495
0.15
0.10
0.05
0.00
ns
ns
*
*
ns
Lactose 0.8nM 40nM 90nM 260nM – –
Sucrose – – – – 260nM –
Galectin-3 + + + + + +

B.


180
NTD
(120 A.A)
CRD
(130 A.A)
C-subsite
(60-71 A.A)
D-subsite
(51-57 A.A)
Anti-CRD VHH

C.


VHH Staining on CD3/CD28 Activated CD8+T cells
Pulsed
Un-pulsed
Anti-CRD VHH-Strep PE (1:100)

D.

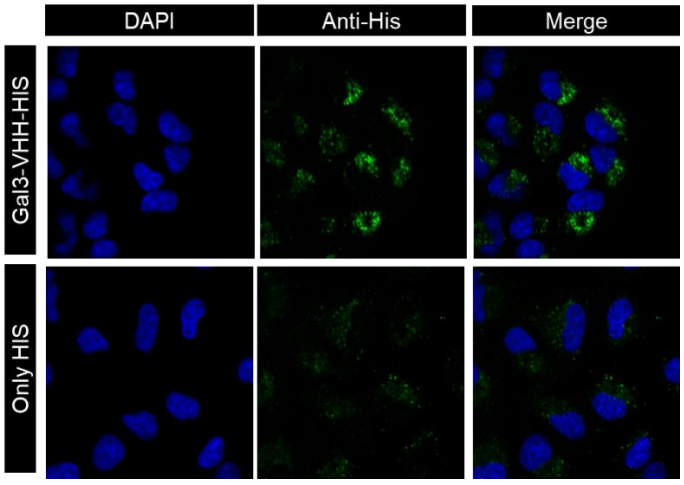
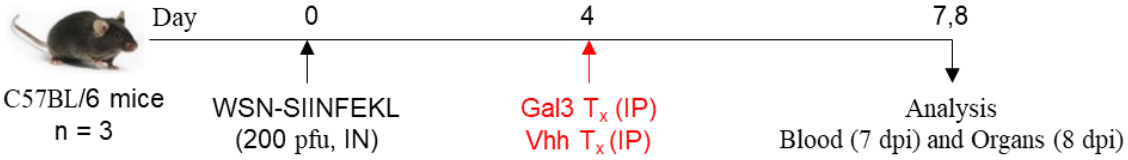

DAPI
Anti-His
Merge
Gal3-VHH-HIS
Only HIS

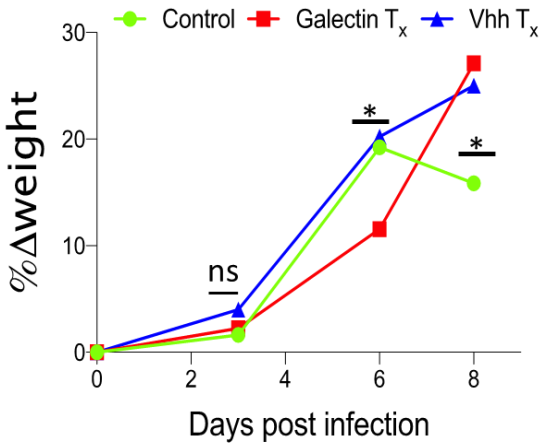
Fig 5. Galectin-3 affects the distribution of antigen reactive CTLs in influenza-infected mice.

A. A schematic of the experiments is shown. WT B6 mice were intranasally infected with WSN-SIINFEKL. Each group received the indicated dose of either the anti-CRD Vhh, Galectin-3 treatment, or diluent on 4dpi. Their weight loss was tracked. The frequency of virus-specific cells was checked in circulation at 7 dpi and the animals were sacrificed and analyzed for the virus-specific cells in various organs at 8 dpi. B. Cumulative plot summarizing the weight loss of animals in each group at different days post-infection (dpi) is shown. C. Representative images for organ size in each group is shown. D. Representative FACS plots showing the gating strategy for analysis in various organs are shown. E. Cumulative bar plots summarizing the frequency (Left row) and absolute count (right row) of virus-specific cells in the indicated compartments are shown. Data represents Mean ± SEM; ***$p<0.001$; **$p<0.005$; *$p<0.05$ and ns ($p>0.05$)- not significant (one-way ANOVA).

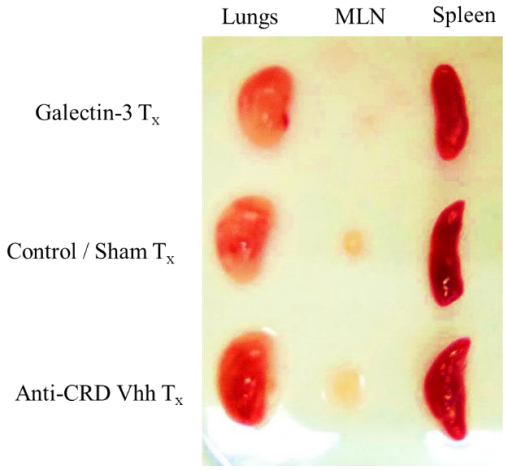
A.


Day
0
4
7,8
C57BL/6 mice
n = 3
WSN-SIINFEKL
(200 pfu, IN)
Gal3 Tx (IP)
Vhh Tx (IP)
Analysis
Blood (7 dpi) and Organs (8 dpi)

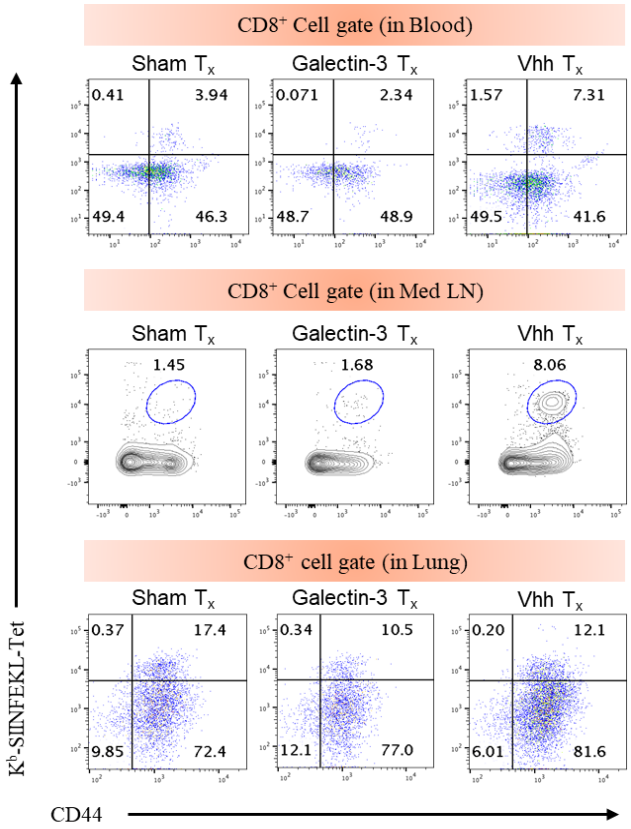
B.


Control
Galectin Tx
Vhh Tx
%Δweight
30
20
10
0
ns
*
*
0
2
4
6
8
Days post infection

C.


Lungs
MLN
Spleen
Galectin-3 Tx
Control / Sham Tx
Anti-CRD Vhh Tx

D.


CD8+ Cell gate (in Blood)
Sham Tx
Galectin-3 Tx
Vhh Tx
0.41
3.94
49.4
46.3
0.071
2.34
48.7
48.9
1.57
7.31
49.5
41.6
CD8+ Cell gate (in Med LN)
1.45
1.68
8.06
CD8+ cell gate (in Lung)
0.37
17.4
9.85
72.4
0.34
10.5
12.1
77.0
0.20
12.1
6.01
81.6
Kb-SIINFEKL-Tet
CD44

E.

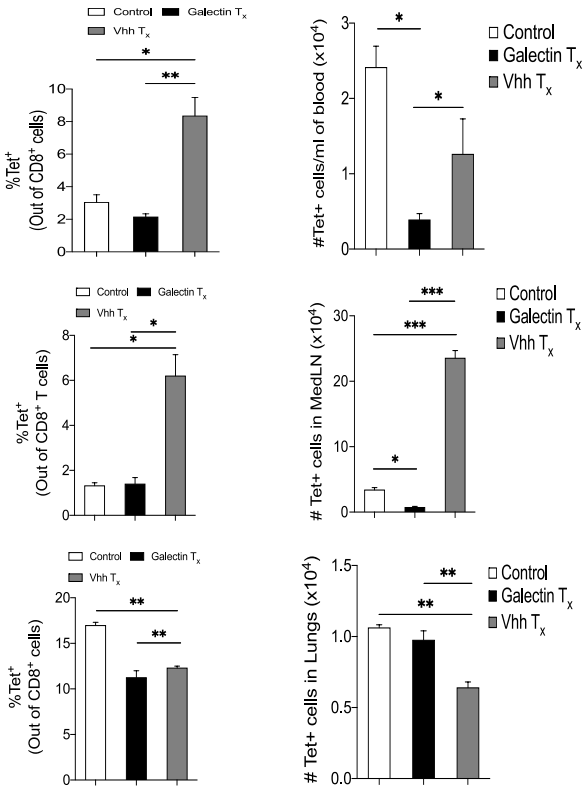

Control
Galectin Tx
Vhh Tx
%Tet+ (Out of CD8+ cells)
#Tet+ cells/ml of blood (x10⁴)
%Tet+ (Out of CD8+ T cells)
Tet+ cells in MedLN (x10⁴)
Tet+ cells in Lungs (x10⁴)

Fig 6. Anti-CRD VHH treatment affects the functionality of $CD8^+$ T cells

A. Representative FACS plots for the intracellular cytokine staining following peptide pulse on total CD8+ T cells in the various groups are shown. B. Cumulative bar diagrams summarising the count of IFN-g producing CD8+ T cells in the draining Mediastinal lymph node (Med LN, top panel) and Lungs (Bottom panel) are shown Data represents Mean ± SEM; ***p<0.001; **p<0.005; *p<0.05 and ns (p>0.05)- not significant (one-way ANOVA).

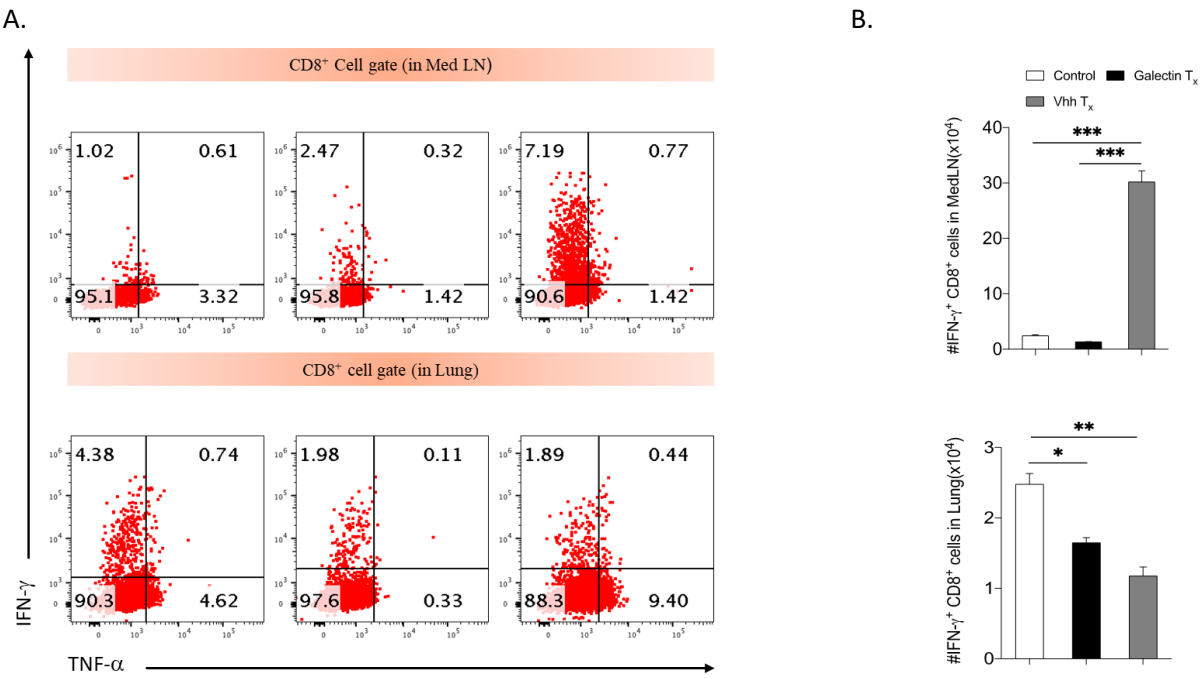


Fig 7. Galectin-3 CRD alters the quality of anti-viral T cell responses in a Treg independent manner.

A. Representative FACS plots for the experiment done to check the *in vitro* generation of Tregs at different concentrations of the anti-CRD VHH are shown

A.

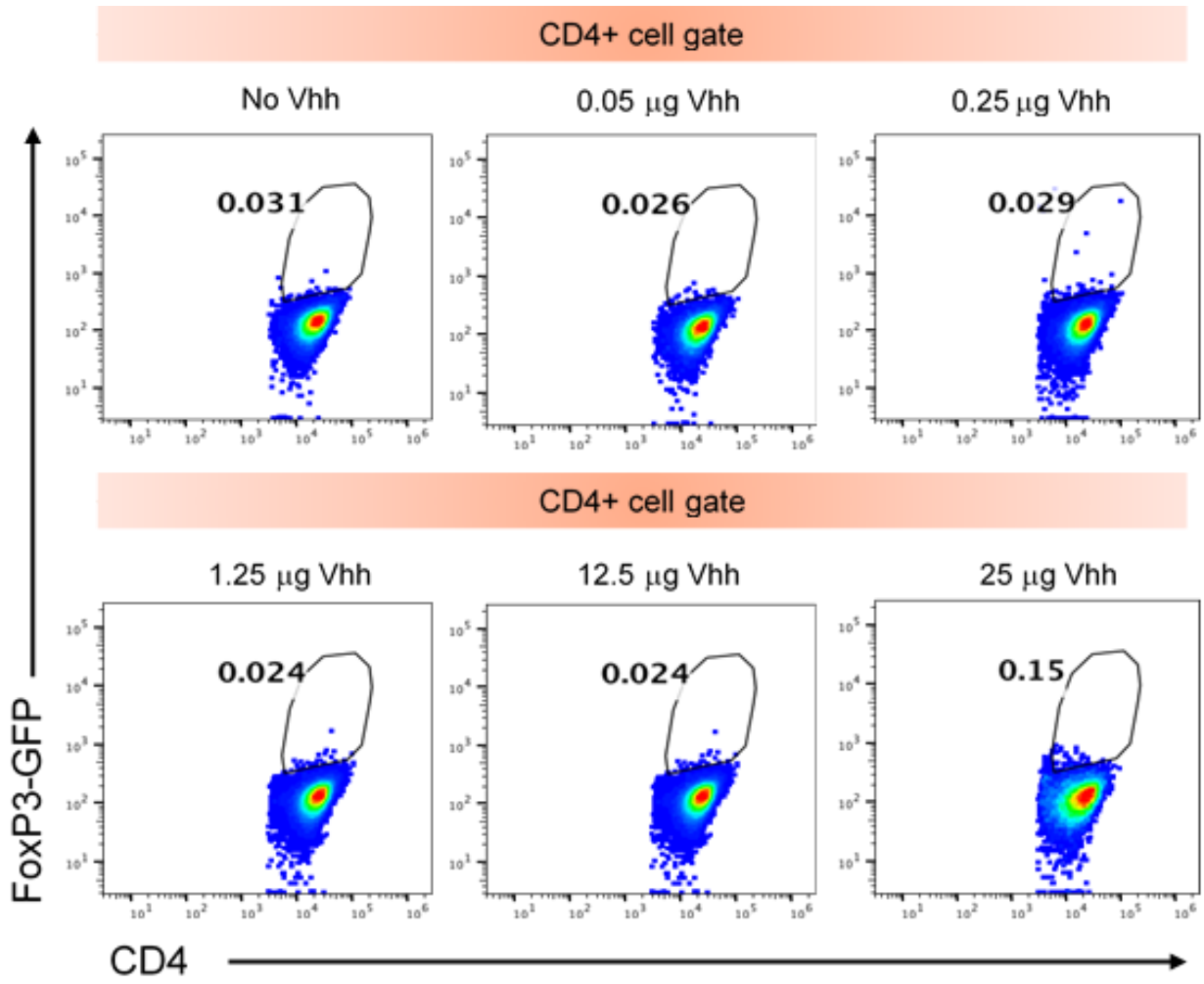
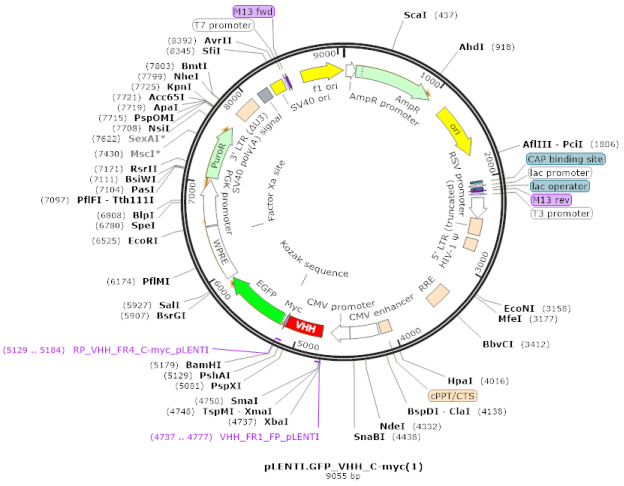


Fig 8. **Functional Intrabodies against Galectin-3 protein can be synthesized in mice CD8^{+}T cells using recombinant Lentiviruses.**

To produce functional cytoplasmic intrabodies against galectin-3 in mice CD8^{+}T cells Anti gal-3 VHH were cloned in Lentiviral vector pLenti.GFP as shown in the virtual map in Fig. A. The cloning strategy was simple was VHH along with C-Myc and e-GFP was cloned in-frame downstream of CMV promoter to produce GFP and C-Myc fused VHH as shown in Fig. B. Using VHH specific primers anti-Gal-3 VHH were PCR amplified and cloned into a lentiviral vector and positive colonies were confirmed by colony PCR and restriction digestion as shown in Fig. C. After successful cloning, VHH laced recombinant Lentiviruses were produced in HEK293T cells after PEI mediated successful transfection ascertained by GFP fluorescence as shown in Fig. D. Further this anti-gal-3 intrabody laced recombinant lentiviruses were spin transduced into sorted Mice CD8^{+}T cells and transduced cells were FACS sorted as shown in Fig. E. for further experimental use.

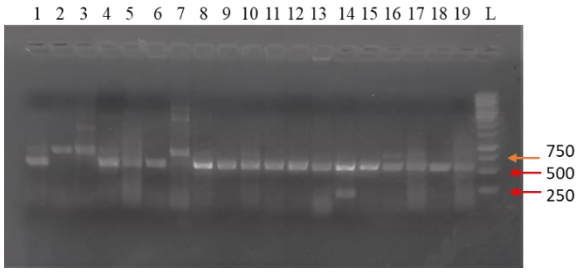
A.


M13 fwd
T7 promoter
ScaI (437)
AhdI (918)
AflIII - PciI (1806)
CAP binding site
lac promoter
lac operator
M13 rev
T3 promoter
EcoNI (3158)
MfeI (3177)
BbvCI (3412)
HpaI (4016)
cPPT/CTS
BspDI - ClaI (4138)
NdeI (4332)
SnaBI (4438)
(4737 .. 4777) VHH_FR1_FP_pLENTI
SmaI (4750)
TspMI - XmaI (4748)
XbaI (4737)
BamHI (5179)
PshAI (5129)
PspXI (5081)
(5129 .. 5184) RP_VHH_FR4_C-myc_pLENTI
PflMI (6174)
SalI (5927)
BsrGI (5907)
EcoRI (6525)
BlpI (6780)
SpeI (6808)
PflFI - Tth111I (7097)
PasI (7104)
BsiWI (7111)
RsrII (7171)
MscI* (7430)
SexAI* (7622)
NsiI (7708)
PspOMI (7715)
ApaI (7719)
Acc65I (7721)
KpnI (7725)
NheI (7799)
BmtI (7803)
AvrII (8345)
SfiI (8392)
f1 ori
SV40 ori
AmpR promoter
AmpR
ori
RSV promoter
5' LTR (truncated) HIV-1
RRE
CMV enhancer
CMV promoter
Kozak sequence
EGFP
Myc
VHH
WPRE
PGK promoter
Factor Xa site
SV40 poly(A) signal
3' LTR (ΔU3)
BsrGI
pLENTI.GFP_VHH_C-myc(1)
9055 bp

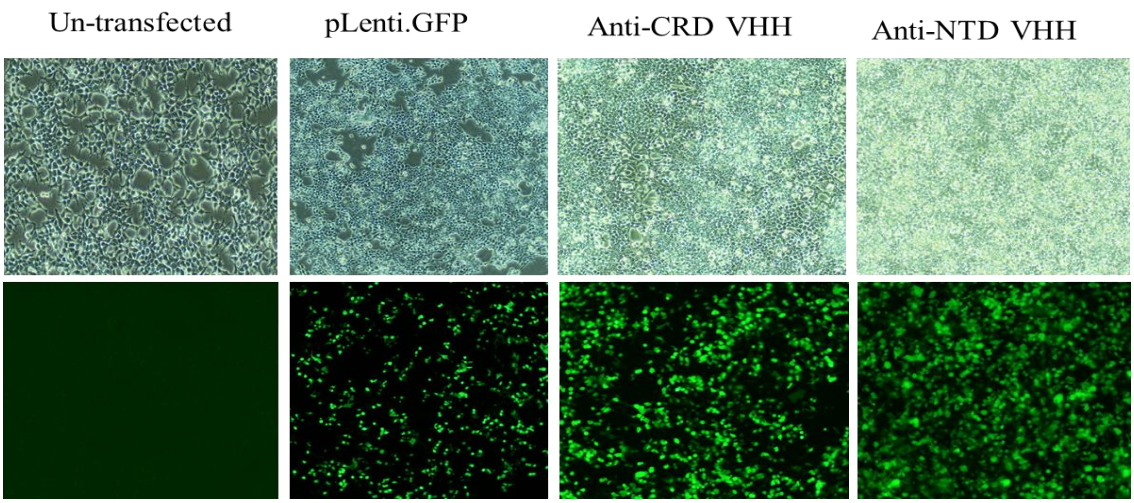
B.


CMV Promoter
VHH
C-Myc
e-GFP

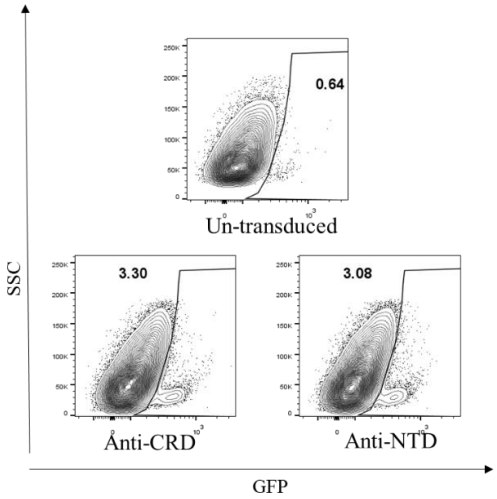
C.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 L
750
500
250

D.


Un-transfected
pLenti.GFP
Anti-CRD VHH
Anti-NTD VHH

E.


0.64
Un-transduced
3.30
Anti-CRD
3.08
Anti-NTD
SSC
GFP

3.4 Discussion

Galectin-3 has been attributed as a key regulator of T cell functions as both intracellular and extracellular galectin-3 modulates activation, proliferation, TCR downregulation, and apoptosis of T cells (). Previous studies in cancer models and in-vitro lateral migration studies have established the role of galectin-3 in the migration of cancer cells. While limited studies have been done to attribute the role of galectin-3 in lymphocyte migration. As per our present understanding activated T cells show upregulation of intracellular galectin-3 along with other cell surface adhesion markers like LFA-1, CD2, etc. Also, inflammation at the site of infections produces immunomodulatory cues, which upregulate glycoproteins like fibronectin, proteoglycans, and Glycosaminoglycans among others. These molecules display chemokines and cytokines, which attract activated T cells to the site of infections, and the presence of these glycoproteins and along with integrins and selectins lead to rolling adhesions and firm adhesions, final steps of lymphocyte egress into the ECF from the vascular network. Previous studies have attributed the role of galectin-3 in various immune cells and extracellular matrix interactions, [24,25,26] however our knowledge of galectin-3 in T cells migration and interaction has been largely premature. This study although indicates the role of galectin-3 in $CD8^+$ T cell migration by taking the advantage of VHH technology. Functional anti-gal-3 CRD VHH can specifically block the interaction of galectin-3 with its natural ligands present on the endothelium of blood vessels, this study by in-vivo blocking of CRD domain of Gal-3 opens multiple possibilities about these migration mechanisms. Our observations suggest multiple possible mechanisms like 1. Since the expression of intracellular galectin-3 is upregulated in activated $CD8^+$ T cells after viral infection and the surplus is readily transported on the cell surface and improves interstitial migration of $CD8^+$T cells. Since functional Anti-CRD Galectin-3 VHH increases the frequency of T cells in circulation and draining lymph node, while decrease at the site of infection. The decrease in the count of Galectin-3 injected mice can be attributed to the induction of apoptosis in activated T cells of these mice. 2. While another possibility can be which explains this observation is that the endothelial cells

at the inflamed tissue sites upregulate glycoproteins which help in cell adhesion and extravasation are saturated by the presence of Anti-CRD VHH, which results in less availability of docking site for T cell, required for extravasation. 3. Finally it is also possible that activated T cells upregulate LFA-1 which helps in its migration and adhesion interacts with galectin-3 present on endothelial cells of the infected site[33]. This interaction is blocked through anti-CRD galectin-3 antibody will lead to similar results as observed. Further experiments have to be performed to understand this complex phenomenon and the probable hypothesis behind it.

Conclusion

In this study, we successfully expressed and characterized VHH against CRD of Galectin-3 protein to elucidate the role of Galectin-3-CRD in the migration of virus-specific $CD8^{+}$T cells. Whereas, to study the role of different domains of intracellular Galectin-3 protein in $CD8^{+}$T cell dynamics during viral infection we successfully synthesized domain-specific anti-Galectin-3 cytoplasmic intrabodies in mice $CD8^{+}$T cells.

Future Prospects

To further improve our understanding of the possible role of galectin-3 in the migration of $CD8^{+}$T cells during viral infections molecular docking analysis of Anti-CRD VHH with different glycoproteins present in the endothelium cells to ascertain their interaction with Anti-CRD VHH has to be performed. While the acute stage mice studies have to be extended to the recovery phase (15 Days post-infection) to study the effect of Galectin-CRD blockade on the differentiation of virus-specific $CD8^{+}$T cells.

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