Antigen presentation on MHC class Ib-related molecules induces Aquaporin4-specific regulatory T cells in PBMC and prevents experimental autoimmune encephalomyelitis in mice

Shriya Mamatha Jayaram[1*], Valentin Bruttel[1,3*], Fadhil Ahsan[1,3], Heike Wecklein[1,3], Maja Heubner[1], Daniela Brünnert[1,3], Chi Wang Ip[2], Jörg Wischhusen[1,]

1: Department for Obstetrics & Gynecology, Section f. Experimental Tumor Immunology, 2: Department of Neurology, University Hospital Würzburg, Germany; 3: Institute of Pathology, University of Würzburg *equal contribution

Abstract

Background and Aims: Neuroinflammation and autoantibodies against ***Aquaporin-4 (AQP4)*** are highly characteristic for *neuromyelitis optica* spectrum disorders **(NMOSD)**. Here, we investigate whether novel antigen-specific tolerance-inducing biomolecules that present AQP4-peptide antigens on MHC class Ib-related molecules can induce AQP4-specific regulatory T cells. We further explore whether corresponding mouse-adapted molecules confer protection in murine neuroinflammatory disease models.

Methods: We generated single chain molecules termed **AutoImmunity Modifying Biologicals (AIM Bios)** comprising antigenic peptides, human or murine MHC class I α1–α2 antigen presenting domains, an HLA-G α3 domain and β2-macroglobulin. ELISpot was used to test if these molecules induce antigen-specific tolerogenic CD8⁺ T_{reg} cells both in human PBMCs *in vitro* and in mice. The therapeutic potential of AIM Bios was further analyzed in neuroinflammatory experimental autoimmune encephalomyelitis (EAE) mouse models & optic neuritis in 2D2 mice in vivo.

Results: AIM Bios selectively polarize cognate CD8⁺ T cells specific for the presented peptide towards a CD8⁺CD122⁺ IL-10 secreting T_{reg} phenotype. Such antigen-specific T_{reg} can also be induced with mouse-adapted molecules in mice *in vivo*. Here, *AIM Bios* prevent immune-mediated neuroinflammatory disease symptoms by either targeting autoreactive effector T cells, or conferring bystander protection via induction of tissue-specific regulatory T cells.

Conclusions: AIM Bios induce human AQP4-specific regulatory T cells *in vitro* and prevent neuroinflammatory disease in animal models. Aquaporin-4-specific *AIM Bios* may therefore be highly suitable for targeted immunosuppression in NMOSD.

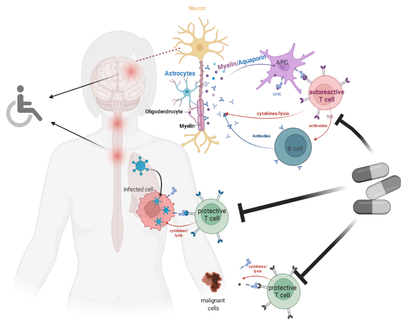
Introduction

➢ **Autoimmune Diseases**
- Driven by rare self-antigen-specific effector T cells (T_{eff})
- Target healthy organs, initiate inflammation, cause tissue destruction and disability.

➢ **NMOSD**
- Autoantigen: **AQP-4**, a water channel protein
- Predominantly expressed on **astrocytes** in brain, spinal cord and optic nerve

➢ **Pathological hallmarks:**
- Autoantibodies targeting Aquaporin-4 (AQP4) & myelin oligodendrocyte glycoprotein (MOG)
- Double-seronegative cases indicate T-cell involvement (1)
- **Prevalence:** 0.5–4/100,000 worldwide

➢ **Clinical symptoms:**
- Optic neuritis
- Transverse myelitis
- Lesions in spinal cord & optic nerve

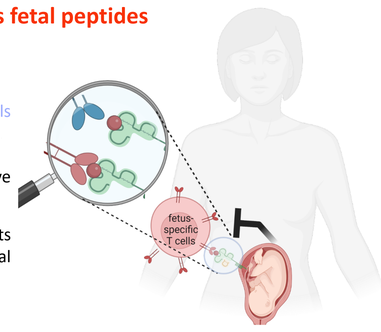


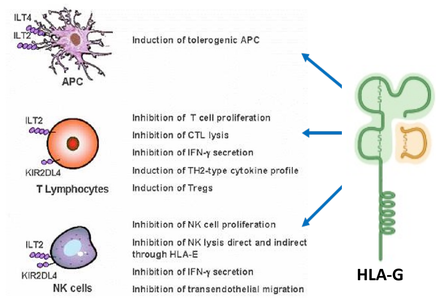
➢ **Current treatment options:**
- Anti-inflammatory drugs
- Plasmapheresis (PLEX)
- Immunotherapy: General B/T Cell inhibition

➢ **Drawbacks**
- Inhibit protective T_{eff}
- Increases risk for opportunistic infections & cancer
- Treatment of symptoms (2)

HLA-G Induces Tolerance towards fetal peptides

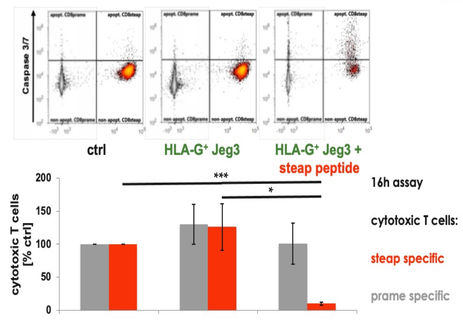
➢ **Selective Immunosuppression**
- Embryos selectively inhibit maternal T cells directed against paternal embryonic antigens.
- No clinically relevant side effects on protective $T_{eff.}$
- HLA-G expressed on extravillous trophoblasts in placenta contributes to feto-maternal tolerance.





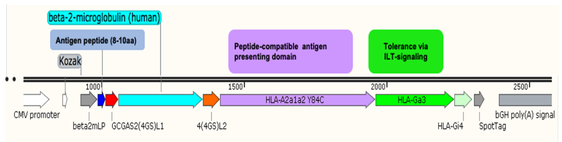
➢ **Known functions of HLA-G**
- HLA-G inhibit immune cell functions via ILT-2 and ILT-4 receptors (3).
- The initial characterization of HLA-G focused on its antigen-presenting capabilities (4).
- The role of peptide presentation on HLA-G is still enigmatic.

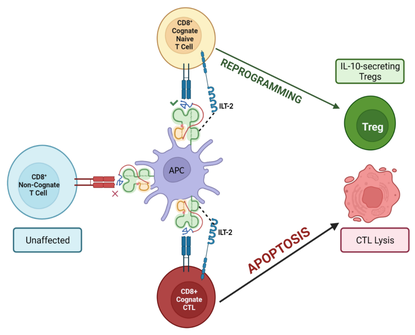
MHC class Ib molecules selectively inhibit defined immune responses

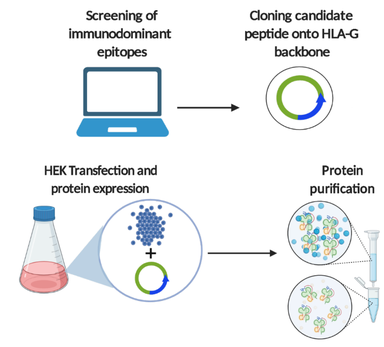


HLA-G-expressing cells loaded with peptide antigens induce apoptosis in cognate T cells.
- HLA-A2-restricted CD8⁺ T cell clones specific for STEAP1 or PRAME peptides were mixed and co-cultured with or without control JEG3 or STEAP1-peptide loaded JEG-3 cells.
- Within 16h, 90% of the targeted STEAP1 specific CD8⁺ T cells went into apoptosis.

Design, principle and production of AIM Biologicals





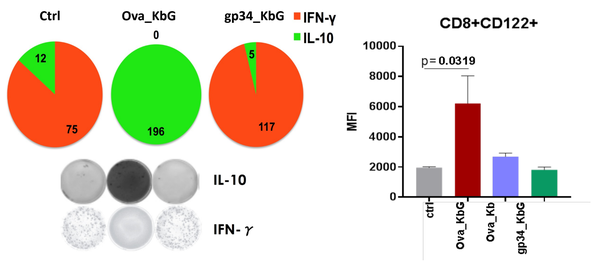


Design of single chain AIM Biologicals (AIM Bios)
- Linkers were introduced between the presented antigenic peptide, β2-microglobulin and HLA-G5 to improve stability & purification.
- The α1- & α2-domains of HLA-G can be replaced by other human or murine MHC class I antigen presenting domains.

Overview of effects of AIM Biologicals
- Binding of the peptide-MHC complex to its cognate TCR secures antigen-specificity.
- Engagement of the ILT2 receptor on T cells by HLA-G elicits immune tolerance.
- Non-cognate T cells are mostly unaffected.

Production and purification of AIM Bios
- Immunodominant MHC class-I-restricted epitopes were screened *in silico.*
- Peptides were cloned into respective MHC vector backbones with SPOT purification tag.
- Cloned plasmids were transfected into HEK293 cells for expression.
- AIM Bios were purified with affinity columns with SPOT-antibody.

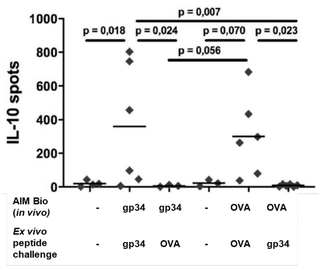
AIM Biologicals induce antigen-specific regulatory T cells



Mouse-adapted AIM Bios presented on bone marrow-derived dendritic cells (BMDC) induce murine Treg cells specific for the presented peptide.
- BMDC were loaded with 5 µg/ml murine AIM Bios comprising either

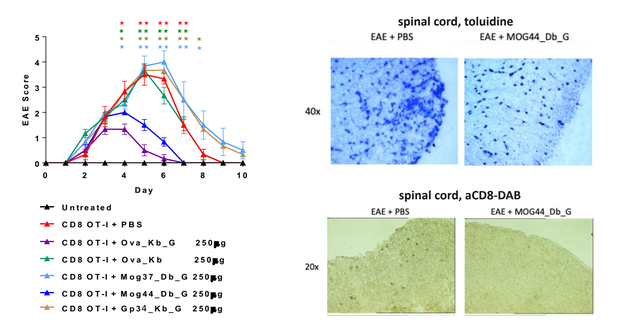
i. H-2K^b α1-3 domains and Ovalbumin peptide (Ova_Kb)

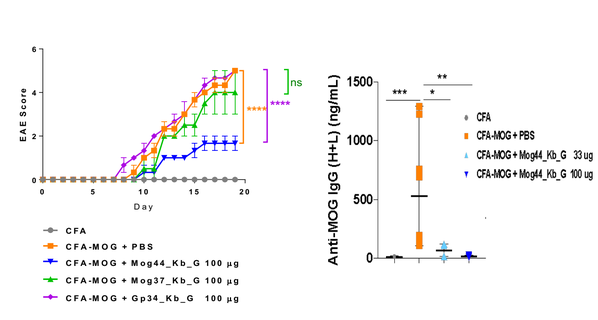
ii. H-2K^b α1+2 domain, HLA-G α3 domain and either an Ova (Ova_KbG) or a viral control peptide (gp34).
- Loaded DCs were co-cultured with Ova-specific OT-I CD8⁺ T cells.
- Cytokine quantification in ELISpot and flow cytometry analysis showed that AIM Biologicals induce CD122⁺, IL-10 secreting, antigen-specific $T_{reg.}$



Mouse-adapted AIM Bios selectively induce peptide-specific Treg that secrete IL-10 in *ex vivo* recall assays.
- 500 µg mouse-adapted AIM Bio molecules presenting gp34 or Ova257 were injected i.p. into C57BL/6J mice.
- Re-challenging harvested splenocytes after 2 weeks with peptide that had previously been presented on AIM Bios resulted in high (7/12 mice) or at least detectable (11/12 mice) numbers of IL-10 secreting T_{reg} .

Murine AIM Biologicals induce specific & bystander protection in EAE models and prevent autoantibody formation





Mouse-adapted AIM Biologicals presenting Ovalbumin- or Mog44-derived peptides selectively suppress OT-I-driven EAE in ODC-Ova mice models
- 250 µg mouse-adapted AIM Bio constructs were injected i.p. into ODC-Ova mice, which express ovalbumin in oligodendrocytes. In parallel, activated OT-I T cells were injected.
- AIM Bios inducing tolerance towards Ova257 or Mog44 selectively suppressed EAE symptoms and spinal cord inflammation. Virus-specific AIM Bios had no effect.

Mouse-adapted AIM Bios prevent MOG-induced EAE and autoantibody production
- In CD4⁺-driven MOG (35-55)-induced EAE model, 33-100µg of mouse-adapted AIM Bios inducing tolerance towards CD8⁺ MOG peptides selectively prevented EAE and anti-MOG autoantibodies.

Candidate AQP4 AIM Bios were prioritized and tested *in vitro* in PBMC from healthy donors

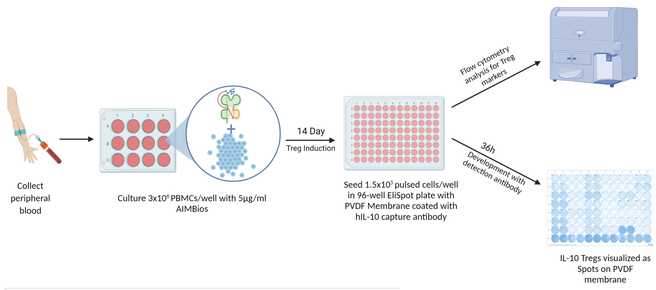
30 MHC-I restricted AQP4 peptides were prioritized *in silico*

19 AIM Bios were cloned and produced in mammalian cells

10 AIM Bios with high expression levels were selected for purification

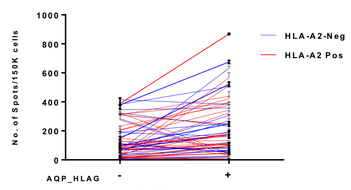
6 Purified AIM Bios were tested for T_{reg} induction *in vitro*

1 human AIM Bio successfully induced T_{reg} in healthy hPBMC

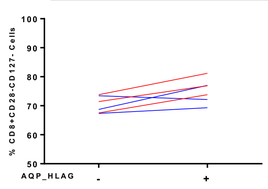


***In vitro* EliSpot assay with AIM Bios**
- PBMC from healthy, HLA-A2⁺ donors were cultured with or without AIM Bios (5µg/ml) for 14 days.
- 150K cells were seeded onto EliSpot plate & IL-10 secreting cells were captured after 36h.
- Remaining cells were characterized for T_{reg} markers using flow cytometry.

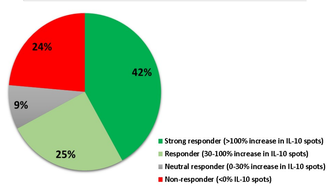
IL-10 induction in hPBMC treated with AQP_HLAG



Characterization of inducted T_{reg}



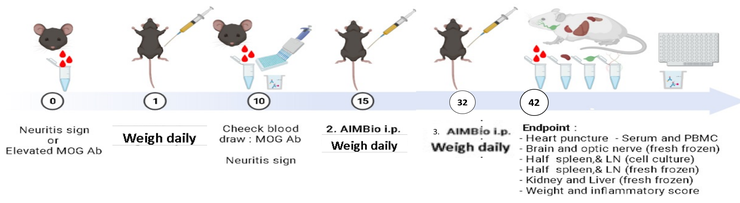
Donor response to AQP_HLAG

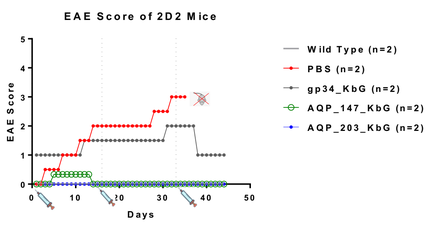


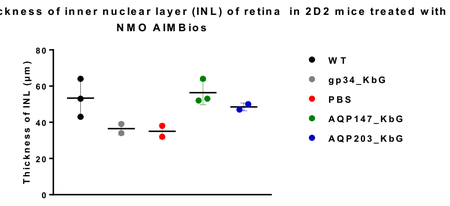
AQP_HLAG AIM Bios induce peptide-specific regulatory T cells
Treating PBMC from 55 healthy donors (HLA-A2$^{+/-}$) AIM Bios inducing tolerance towards AQP4 significantly increased the number of IL-10 secreting, CD8⁺CD28⁻CD127⁻ T_{reg} population (5) compared to untreated cells in 65% of tested donors.

Murine AIM Biologicals may induce protection in spontaneous optic neuritis models (Preliminary Data)

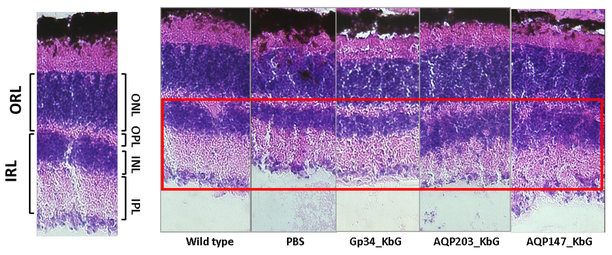
2D2 Spontaneous Optic neuritis model







Mouse-adapted AIM Biologicals presenting model AQP4 peptide may suppress EAE symptoms and optic neuritis in 2D2 mice
- 150 µg mouse-adapted AIM Bio molecules were injected i.p. into 2D2 mice (2 to 3 mice/group in pilot study).
- Preliminary results from this pilot study indicate that mouse-adapted AIM Bios inducing tolerance towards AQP4 selectively reduced EAE symptoms in treated mice.
- H&E staining of the eyes showed that AQP4 tolerance inducing AIM Bio preserved INL thickness in treated mice.
- A larger study will be needed to obtain robust data.



Retinal layer thickness of control & treated 2D2 mice was analyzed by H&E staining

IPL: Inner Plexiform layer — OPL: Outer Plexiform layer
INL: Inner nuclear layer — ONL: Outer nuclear layer
IRL: Inner retinal layer — ORL: Outer retinal layer

Conclusion

- Mouse-adapted AIM Biologicals selectively induce IL-10, inhibit IFN-γ secretion in cognate T cells, inhibit EAE symptoms and spinal cord inflammation and eliminate MOG-specific autoantibodies in CD4⁺-dependent MOG EAE models.
- AQP4_HLAG AIM Biologicals induce IL-10 secreting CD8⁺CD28⁻CD127⁻ T_{reg} in hPBMC.
- Preliminary data with mouse 2D2 models of spontaneous EAE indicate that mouse-adapted AIM Bios may reduce optic neuritis, loss of inner nuclear layer neurons and EAE symptoms. However, the data set is too small to be conclusive.

References

1. Jyh Yung Hor, Nasrin Asgari, Ichiro Nakashima, Simon A. Broadley, M. Isabel Leite, Najib Kissani et al (2020), Epidemiology of Neuromyelitis Optica Spectrum Disorder and Its Prevalence and Incidence Worldwide, Front. Neurol., 26 June 2020
2. Madina Tugizova, Luka Vlahovic, Anna Tomczak, Nora Sandrine Wetzel, May Htwe Han, New Therapeutic Landscape in Neuromyelitis Optica, Curr Treat Options Neurol (2021) 23:13
3. SulzerD, AlcalayRN, GarrettiF, CoteL,KanterE, AginUiebesJ, LiongC, McMurtreyC, etal. (2017), T cells from patients with Parkinson's disease recognize alpha-synuclein peptides. Nature546:656-661
4. Marianne Diehl, Christian Münz, Wieland Keilholz, Stefan Stevanović, Nicholas Holmes, Yung WaiLoke, Hans-Georg Rammensee,Non-classical HLA-G molecules are classical peptide presenters, Current Biology, Volume 6,Issue3,1996
5. Francesca Bernuzzi, Daniela Fenoglio, Florinda Battaglia, Marco Fravega, M. Eric Gershwin, Francesco Indiveri, Aftab A. Ansari, Mauro Podda, Pietro Invernizzi,a,d, Gilberto Filaci et al (2011), Phenotypical and functional alterations of CD8 regulatory T cells in primary biliary cirrhosis, J Autoimmun. 2010 Nov; 35(3): 176–180

Contact Information

Shriya Mamatha Jayaram
University Hospital Würzburg
mamatha_s@ukw.de

This work was generously supported by ÆTERNA ZENTARIS