Developing antibody discovery and recombination technology towards the goal of modulating cation channels

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Publication Date:
2016

Permanent Link:
https://doi.org/10.3929/ethz-a-010722799

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DEVELOPING ANTIBODY DISCOVERY AND RECOMBINATION TECHNOLOGY TOWARDS THE GOAL OF MODULATING CATION CHANNELS

A thesis submitted to attain the degree of DOCTOR OF SCIENCES of ETH ZURICH (Dr. sc. ETH Zurich)

presented by TIMOTHY JAMES EGAN
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born on 21.06.1985

accepted on the recommendation of Prof. Marcy Zenobi-Wong, examiner Prof. Hanns Ulrich Zeilhofer, co-examiner Dr. David Urech, co-examiner

2016
Acknowledgements

After 4½ years of work in support of this dissertation, there are many people who deserve my deepest thanks. In general, everyone who has supported and contributed to my work as well as those who have participated in my education and aided my personal growth have my sincerest gratitude. My efforts have required that I interact with multiple research groups and within three different laboratory settings. Exposure to such a diversity of perspectives and expertise has been an enriching and enlightening experience that I cannot imagine could have been replicated under any other conditions. Meanwhile, to benefit from such an experience while living in a country that is very foreign to me was truly special.

I must thank my thesis advisor, Prof. Marcy Zenobi-Wong, for extending me this opportunity to work as a member of her department at ETHZ. Marcy took a chance on an unknown, who would be required to work primarily in remote lab space under the auspices of a private industry collaborator. It is my sincere hope that she is both pleased with the output and happy to have overseen the work for this unusually-structured doctoral candidacy. I could not have completed this thesis in the absence of Marcy’s consistent support and flexibility.

I must also thank Prof. Hanns Ulrich Zeilhofer who generously accommodated me in his laboratory at UZH and whose input was absolutely vital to the completion of the now-provisionally accepted manuscript on TRPA1 glycosylation (Chapter 4 of this thesis). When beginning this project, I did not think I would have had the opportunity to learn techniques for completing whole-cell patch-clamp experiments. Under the incredibly valuable guidance of Prof. Zeilhofer and Mario Acuña, I was able to perform an electrophysiological analysis whose results were a critical component of our final manuscript. I am incredibly grateful to both Uli and Mario.

While the results are not described in this thesis, I spent nearly eight months of my time in Switzerland working with the Department of Organic Chemistry at ETHZ in the laboratory of Prof. Jeffrey Bode. It was there that we endeavored to create a pore mimetic for use as an immunogen in anti-hTRPA1 immunizations. Jeff was incredibly helpful during that time and made available all of his facilities for this project. Additionally, I was helped immensely by Dr. Maggie He and Dr. Vijay Pattabiraman. Being a complete novice in organic chemistry, Maggie and Vijay were tasked with instructing me, and I frequently relied on them for several steps of the synthesis. Our work led to the successful production of a tetravalent peptide display in which peptide N-termini were only 1-2 nm apart – effectively mimicking the outer pore of an ion channel. I still believe that this work can be of considerable value to the design of novel immunogens and in the selection of cation channel-reactive B-cells.

Meanwhile, the laboratory at Numab has been the venue for the lion’s share of the work described in the ensuing pages. I would like to thank all of the many Numab employees, past and present, who have helped me in more ways than can be adequately described here. At the laboratory level, Richard Weldon, Fabian Weiss, Dana Mahler, and Benjamin Küttner have been an absolute pleasure to work with and could be consistently relied upon for invaluable support. At the level of project management, Dr. Alexandre Simonin, Dr. Stefan Warmuth and Dr. Christian Hess provided considerable support and assistance, particularly with respect to troubleshooting biochemistry experiments as well as issues relating to antibody production, recombination and characterization. Among those in upper management, Dr. Tea Gunde was a constant source of helpful input, especially in the development and interpretation of functional assays.

There are many people who I worked with at Numab who deserve special thanks. First, Roland Helfenstein made my transition to Switzerland and navigating the logistics of my employment here much easier than it could have been. The company being as small as it is, no one has been asked to take on a more diverse set of responsibilities, and despite that, Roland always maintained a truly enviable demeanor,
attitude and work ethic. His assistance has been essential to the completion of my work and to making this whole experience manageable.

Tessa Neumann, Nadia Sawas, Sofia Arpagaus and Teddy Beltrametti were all labmates of mine when I initially joined Numab. When I was very green, they were the ones who were asked to step outside of the scope of their responsibilities and provide me with training and support. It is no overstatement to suggest that I am the scientist I am today largely because of them. It is also as accurate to say that I could not have asked for a better collection of minds and personalities to help prepare me for professional life after this thesis.

Dania Diem, meanwhile, also bears special acknowledgment here. I cannot imagine I will encounter anyone with a greater attention to detail or a work ethic as untiring as hers. Her contributions to the manuscript on the MATCH formats (Chapter 3 in this thesis) were huge. The project would not have progressed as smoothly or expediently in her absence, and there is no person in whom I place more faith to produce work of the utmost quality.

A special acknowledgment is owed, as well, to the head of Numab’s Biochemistry Group, Dr. Sebastian Meyer. Throughout my time here, no person has been more patient with my maturation as a scientist than he has, and that effectively means that no person has been as helpful during the work for this thesis. Sebastian was willing to communicate at length about anything I would come to him with, ranging from designing or troubleshooting experiments and clarifying topics in scientific literature to political arguments and recitation of dialogue from TV and film. Often times, answering my questions required that Sebastian venture outside of his immediate expertise, which he was always willing to do. It should be noted, however, that Sebastian often claimed minimal expertise in subjects he was remarkably conversant in.

This whole project began, in effect, after a conversation with Dr. David Urech, Numab’s co-CEO and CSO. I cannot say with certainty what he anticipated from me after that discussion, but I certainly hope that I have fulfilled his expectations. David provided me with a truly remarkable opportunity – to obtain a doctorate while working for a start-up company employing cutting-edge technology in the applied sciences. David was always excited about, and supportive of, projects with some potential to revolutionize drug discovery or patient treatment. I spent years during this doctoral candidacy working on projects that perhaps provided a low probability of success but whose impact could be substantial. David was always willing to provide intellectual, material and financial support to projects of this kind, wherever the work took me, always with an eye towards paradigm-shifting innovation. Whether or not one considers the final output of my work underwhelming by that standard, I am very grateful to David, and my experiences with both him and the others acknowledged above render me very optimistic that the scientific community is occupied by ambitious, hard-working visionaries.

I feel compelled to deeply thank the people in my personal life who have seen me through this challenging, but rewarding, time. To all my friends in Switzerland and in America, your presence and communications have been incredibly meaningful, often in ways you likely did not imagine. I also want to thank my brothers, Mike and Matt, and my parents, who have always been eager to support me in all ways possible. Finally, Helga, no person has vicariously endured the peaks and troughs of my experience quite as intensely as you; simply put, my efforts would have been made immensely more difficult were I not able to rely on your presence, strength and warmth, and I cannot thank you enough for being so generous with all of those things. I count myself very lucky at the end of this time, and I look forward to what's next.
Abstract

The growth of monoclonal antibody (mAb)-based therapeutics on the market and in the pipelines of pharmaceutical companies owes itself largely to the remarkable target-selectivity that these proteins exhibit and the increasing sophistication of antibody engineering methods. The capacity of mAbs to selectively identify discrete protein isoforms, even ones occupying protein subfamilies with considerable interprotein homology, is thought to underlie their high clinical trial success rate vis-à-vis small molecules, due to their limited off-target interactions and thus side-effects. Furthermore, the antigen-binding region of mAbs can accommodate recombination to generate reactive molecules of various sizes, specificities and valences without compromising the target-selectivity they possess in full-length form.

Meanwhile, cation channels have long been considered attractive drug targets, particularly for indications such as pain and cancer, because their sensitization or dysregulation can result in, or contribute to, various pathologies and/or their symptoms. While ligand- and voltage-gated ion channels represent ~13% of targets among clinically approved pharmaceuticals, the drugs targeting them are typically small molecules that need to be locally administered and do not target a single ion channel discretely. Additionally, the majority of such molecules were approved more than a decade ago. Because individual cation channel isoforms share considerable sequence homology with related proteins that are critical to normal physiological functioning, safe pharmacological modulation of such proteins requires utilizing molecules with high target-affinity and minimal off-target interactions.

Researchers have long been engaged in the effort to produce therapeutic mAbs that act by inhibiting cation channels. However, this effort has not yet produced a single cation channel-inhibiting mAb that has been approved for clinical use. The reasons for this are debatable, but the absence of such molecules bears initiating a discussion that focuses on the ostensibly unique challenges inherent to raising cation channel-inhibiting mAbs. Using natural cation channel inhibitors as a guide, and considering the biological mechanisms governing antigen internalization, processing and presentation by B-cells in vivo, it would seem that considerable strides could be made on the side of immunogen design. Ideally, immunogens
would display conformation-dependent, monovalent cation channel epitopes to the immune system in a format that is both immunogenic and would allow antigen uptake by B-cells upon specific recognition by a B-cell receptor. Meanwhile, antibody technology can likely be built upon to generate multi-reactive molecules, which, research suggests, may inhibit cation channels more effectively than mAbs. Finally, our knowledge of the structure and function of cation channels, including post-translational modifications, must improve to inform future cation channel-targeting immunization campaigns.

The original research presented in this thesis first focuses on the production of a novel multispecific antibody format. Multispecific antibody formats provide a promising platform for the development of novel therapeutic concepts, which could facilitate the generation of safer, more effective pharmaceuticals. However, the production and use of such antibody-based multispecifics is often made complicated by a) the instability of the antibody fragments of which they consist, b) undesired inter-subunit associations, and c) the need to include recombinant heterodimerization domains that confer distribution-impairing bulk and/or enhance immunogenicity. In Chapter 3, a broadly-applicable method for the stabilization of human or humanized antibody Fv fragments is described. It entails replacing framework region four of a V_{κ1}-V_{H3}-consensus Fv framework with the corresponding germ-line sequence of a λ-type V_{L} chain. This stable Fv framework was then used to generate a novel heterodimeric multispecific antibody format that assembles by cognate V_{L}/V_{H} associations between two split variable domains in the core of the complex. This multispecific format – termed the MATCH format – can be applied to produce highly stable antibody-derived molecules that simultaneously bind four distinct antigens and can easily be generated to a high purity. The heterodimeric design of the MATCH allows for efficient in-format screening of binding domain combinations that result in maximal cooperative activity. Such a format could theoretically be employed to target multiple domains/subunits of a cation channel simultaneously to inhibit protein function.

Subsequently, this thesis presents the results of experiments to characterize the function of two N-glycans displayed by wild-type TRPA1. Determining the functional significance of post-translational
modifications advances our understanding of many broadly-expressed proteins, and particularly ion channels. The respective enzymes that catalyze these modifications are often expressed in a cell-type specific manner, conferring remarkable structural diversity upon post-translationally modified proteins that are expressed across a variety of cell types. N-glycans attached to exogenous antigens have also been demonstrated to promote immune tolerance. TRP channels exhibit notoriously variable behavior between cell types in vitro and in vivo, and they are frequently modified with N-glycans that contribute to protein function. TRPA1 possesses two putative N-linked glycosylation sites at N747 and N753 that have not yet been studied in detail. As the original experimental results presented in Chapter 4 show, both of these sites can be modified with an N-glycan and the glycan at position N747 modulates agonist-sensitivity of TRPA1 in vitro. Additionally, these experiments found that N-glycosylation also modulates cooperative effects of temperature and the agonist cinnamaldehyde on TRPA1 channel activation. Collectively, the findings point to a dynamic role played by the N-glycosylation of human TRPA1. They also provide further evidence of the remarkable versatility of N-glycans and will assist in efforts to fully understand the complex regulation of TRPA1 activity.

This thesis focuses on the challenges of raising cation channel-inhibiting mAbs. The original work presented herein seeks to address some of these challenges by building on the versatility of antibody-based molecules and experimentally confirming and characterizing a functionally significant post-translational modification to a nociceptive cation channel, TRPA1. In addition, proposed future work is laid out that focuses on the development of rationally-designed immunogens for use in the generation of antibodies in vivo and their selection ex vivo.
Zusammenfassung


Das Bestreben der Medikamentenforschung therapeutische Antikörper zu generieren, die Kationenkanäle selektiv blockieren, war bisher nur bedingt erfolgreich und bis heute ist kein einziger zugelassener Kationenkanal-blockierender monoklonaler Antikörper erhältlich. Mögliche Gründe dafür

von verschiedenen Kombinationen von Bindungsdomänen, die eine hohe Aktivität durch Zusammenwirkung aufweisen.

Dieses multispezifische Antikörperformat, kurz MATCH-Format, kann zur Herstellung von stabilen antikörper-basierenden Molekülen verwendet werden, die gleichzeitig an vier verschiedenen Antigene binden können und aufgrund ihrer Stabilität eine hohe Reinheit aufweisen. Das heterodimere Design der MATCH-Moleküle erlaubt ein effektives Screening von verschiedenen Kombinationen unterschiedlicher Bindungsdomänen, wobei die maximale Bindungsaktivität aller Bindungsstellen stets erhalten bleibt.

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## Abbreviations

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<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>amino acid</td>
</tr>
<tr>
<td>ADC</td>
<td>antibody-drug conjugate</td>
</tr>
<tr>
<td>ADCC</td>
<td>antibody-dependent cell-mediated cytotoxicity</td>
</tr>
<tr>
<td>AITC</td>
<td>allyl isothiocyanate</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>apMATCH</td>
<td>antiparallel MATCH protein</td>
</tr>
<tr>
<td>apMATCH-diS</td>
<td>antiparallel MATCH protein with inter-Fv disulfide bridge</td>
</tr>
<tr>
<td>apMATCH-dis(L/H)</td>
<td>antiparallel MATCH protein with intra-Fv disulfide bridge</td>
</tr>
<tr>
<td>AU</td>
<td>absorbance unit</td>
</tr>
<tr>
<td>BCR</td>
<td>B-cell receptor</td>
</tr>
<tr>
<td>CA</td>
<td>cinnamaldehyde</td>
</tr>
<tr>
<td>Cav</td>
<td>voltage-gated calcium channel</td>
</tr>
<tr>
<td>CD3ε</td>
<td>cluster of differentiation 3 epsilon</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CDR</td>
<td>complementarity-determining region</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>CIP</td>
<td>channelopathy-associated indifference to pain</td>
</tr>
<tr>
<td>CL</td>
<td>(whole-)cell lysate</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>ConA</td>
<td>concavalin A</td>
</tr>
<tr>
<td>CS</td>
<td>cell surface</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>DI-DIV</td>
<td>domain I-domain IV</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglia</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>E1-E3</td>
<td>extracellular 1-extracellular 3</td>
</tr>
<tr>
<td>eGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>Fab</td>
<td>fragment antigen binding</td>
</tr>
<tr>
<td>Fc</td>
<td>fragment crystallizable</td>
</tr>
<tr>
<td>FcγR</td>
<td>fragment crystallizable gamma receptor</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>H chain</td>
<td>IgG heavy chain subunit</td>
</tr>
<tr>
<td>HER2</td>
<td>human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
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<tr>
<td>KLH</td>
<td>keyhole limpet hemocyanin</td>
</tr>
<tr>
<td>Kv</td>
<td>voltage-gated potassium channel</td>
</tr>
<tr>
<td>L chain</td>
<td>IgG light chain subunit</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MATCH</td>
<td>multispecific antibody-based therapeutics by cognate heterodimerization</td>
</tr>
<tr>
<td>MHC II</td>
<td>major histocompatibility complex class II</td>
</tr>
<tr>
<td>MWCO</td>
<td>molecular weight cut-off</td>
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<tr>
<td>Na_N</td>
<td>voltage-gated sodium channel</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>nNav1.5</td>
<td>neonatal splice-variant of the voltage-gated sodium channel 1.5</td>
</tr>
<tr>
<td>pAb</td>
<td>polyclonal antibody</td>
</tr>
<tr>
<td>PG</td>
<td>pore gateway</td>
</tr>
<tr>
<td>P-loop</td>
<td>pore-loop</td>
</tr>
<tr>
<td>pMATCH</td>
<td>parallel MATCH protein</td>
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<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>ProTx</td>
<td>protoxin</td>
</tr>
<tr>
<td>R&amp;D</td>
<td>research and development</td>
</tr>
<tr>
<td>RFU</td>
<td>relative fluorescence unit</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>RU</td>
<td>response unit</td>
</tr>
<tr>
<td>S1-S6</td>
<td>transmembrane region 1-transmembrane region 6</td>
</tr>
<tr>
<td>scDb</td>
<td>single-chain diabody</td>
</tr>
<tr>
<td>scFv</td>
<td>single-chain fragment variable</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate – polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SE-HPLC</td>
<td>size-exclusion high performance liquid chromatography</td>
</tr>
<tr>
<td>SIGLEC</td>
<td>sialic acid-binding Ig-like lectin</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering ribonucleic acid</td>
</tr>
<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>T\text{\textsubscript{H}}-cell</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TNF\text{\textalpha}</td>
<td>tumor necrosis factor alpha</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TRP</td>
<td>transient receptor potential</td>
</tr>
<tr>
<td>TRPA</td>
<td>transient receptor potential cation channel, “ankyrin”</td>
</tr>
<tr>
<td>TRPC</td>
<td>transient receptor potential cation channel, “classical”</td>
</tr>
<tr>
<td>TRPM</td>
<td>transient receptor potential cation channel, “melastatin”</td>
</tr>
<tr>
<td>TRPV</td>
<td>transient receptor potential cation channel, “vanilloid”</td>
</tr>
<tr>
<td>TTX</td>
<td>tetrodotoxin</td>
</tr>
<tr>
<td>TTX-r</td>
<td>tetrodotoxin-resistant</td>
</tr>
<tr>
<td>TTX-s</td>
<td>tetrodotoxin-sensitive</td>
</tr>
<tr>
<td>VGCC</td>
<td>voltage-gated calcium channel</td>
</tr>
<tr>
<td>VGSC</td>
<td>voltage-gated sodium channel</td>
</tr>
<tr>
<td>V_h</td>
<td>IgG variable heavy domain</td>
</tr>
<tr>
<td>V_h3</td>
<td>IgG variable heavy domain, human consensus sequence 3</td>
</tr>
<tr>
<td>V_l</td>
<td>IgG variable light domain</td>
</tr>
<tr>
<td>V_k1</td>
<td>IgG variable light domain, human consensus kappa 1</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
</tbody>
</table>
Chapter 1

Discovery and development of cation-channel modulating monoclonal antibodies:

The potential value, the progress made and the challenges remaining

A modified version of this chapter is being submitted to “Critical Reviews in Biotechnology” as a review article.
1 Therapeutic cation channel-modulating antibodies

Human proteins represent the overwhelming majority of drug targets among clinically approved pharmaceuticals (1–3). Among them, cell-surface proteins are considerably overrepresented (1,3), due largely to their accessibility, distribution patterns and involvement in various pathologies. Indeed, members of the G-protein coupled receptor (GPCR) family – complex, mult spanning integral membrane proteins – are estimated to be the target of ~30-40% of approved therapeutics despite comprising only 4% of the human genome (2,3). Ligand- and voltage-gated ion channels, meanwhile, are estimated to account for ~13% of all drug targets and only 1% of the human genome (1,4). The pharmacological modulation of cation channels has become a significant focus of R&D efforts within the pharmaceutical industry as evidence has emerged implicating them in the mediation of physiological functions ranging from nociception and/or inflammation in response to chemical, mechanical and thermal stimuli to tumor proliferation and metastasis (5–12). Additionally, channelopathies – pathologies caused by altered ion channel functionality – have supported not only the disease-causing potential of this protein family (13), but in the case of at least one channel, hint at a possible therapeutic benefit of complete functional ablation (7,14–17).

While there has been considerable progress in the production of molecules that selectively modulate cation channels, several obstacles to their downstream clinical development remain. A single ion channel gene is often capable of forming proteins that exhibit tremendous structural and functional diversity. In turn, they are often expressed across several different cell types, playing a crucial role in many disparate physiological functions. Therefore, it is possible that disease-management via even the highly selective pharmacological modulation of an ion channel may interfere with crucial, tightly-regulated processes involving the target channel in an off-target expression locus. Additionally, multi-subunit cation channels, e.g., transient receptor potential (TRP) channels, frequently display a capacity to heteromultimerize (18–22). Insofar as the preclinical analysis of therapeutic candidates that modulate multi-subunit ion channels tends to focus on the molecules’ effects on the activity of the protein’s homotetrameric form, this would imply some potential for drug cross-reactivity with non-target ion channels in vivo. The potential for in-
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ter-channel drug cross-reactivity is also a function of the unfortunately high sequence homology within, and even across, ion channel sub-families. Indeed, natural toxins that occlude ion channels by blocking their pore or interfering with voltage-sensitivity frequently do so with unacceptable levels of selectivity for clinical development.

The issues presently complicating the clinical development of cation channel modulators appear, collectively, to suggest a tremendous value of molecules that exhibit high specificity for functionally-critical, (possibly) inter-subunit sites that are unique to a single ion channel isoform. Additionally, for ion channel targets that are inaccessible to locally-/topically-administered molecules, a therapeutic exerting some level of control over its in vivo distribution (e.g., via a reactive domain that promotes local enrichment at the target site) would be hugely advantaged. The considerable functional diversity among ion channels would seem to indicate the presence of sequence differences that could theoretically be exploited by a molecule to highly selectively modify behavior. Monoclonal antibodies (mAbs) are capable of distinguishing between antigens with as little as 1 amino acid (AA) difference (23,24) and their use, therefore, presents an attractive strategy for selective cation channel modulation. Additionally, antibody recombination techniques can now accommodate the production of stable, monovalent mAb fragments – e.g., Fragment antigen-binding (Fab) and single-chain Fragment variable (scFv) molecules (25) – as well as multispecific formats (26,27). In other words, we can now produce high-affinity, highly selective biologics whose size, functionality, distribution patterns and valency can be readily modified.

The concept of generating therapeutic, cation channel-modulating antibodies is not new. The production and effects of several inhibitory mAbs and polyclonal antibodies (pAbs) targeting cation channels have been described (4,28–31). However, after roughly 20 years of research into anti-cation channel antibodies that bind to extracellular epitopes, and despite the aforementioned advantages of these molecules, the clinical approval of therapeutic ion channel-modulating mAbs has been strikingly elusive. Particularly in light of the growing number of antibody-based therapeutics entering the market and the considerable commercial success of their predecessors (32), the absence of anti-cation channel mAbs in the clinic bears
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thorough examination – and discussion – of the ostensibly unique challenges that cation channels present as targets for antibody-mediated modulation.

This introductory chapter to the doctoral thesis will focus initially on the unfulfilled therapeutic promise of cation channel modulation and discuss advantages of using antibodies, rather than small molecules, for such a purpose. Then, it will summarize the progress-to-date towards raising cation channel-modulating antibodies and outline some of the strategies employed by researchers to raise selective, modulating mAbs that target cation channels. Finally, it will present an outlook, beginning with the several potential challenges specific to cation channels that prevent the generation of selective, modulating antibodies, and it will offer possible solutions focused on immunogen design and antibody recombination strategies.
1.1 Cation channels as targets for therapeutics

Generally speaking, cation channels are multispansing integral membrane proteins whose functional structure is formed either by the tetramerization of individual subunits (e.g., voltage-gated potassium and TRP channels) or interactions between four domains (DI-DIV) encoded by a single concatemer (e.g., voltage-gated calcium and voltage-gated sodium channels). These subunits and domains, respectively, consist of intracellular N- and C-terminal regions, six integral membrane regions (S1-S6), two extracellular loops along the S1-S4 gating region (E1 and E2) and a membrane re-entrant pore-loop (P-loop) that – in this thesis – will be divided into a pre-pore, extracellular region (E3) and a solvent-exposed pore gateway (PG) region (Fig. 1.1). The pore is formed by the orderly convergence of the four S5-E3-PG-S6 regions produced in the tertiary structure. These pores possess a filter at their core that – through a combination of size-exclusion and electrochemical profile – are capable of conducting ions with remarkable selectivity, albeit nonselective cation conduction is a common feature among, e.g., TRP channels.

Once activated by a stimulus, these channels open and allow ions to flow across the cell membrane according to their electrochemical gradient. In the context of sensory afferent fibers of the peripheral nervous system (PNS), the activation of cation channels can initiate action potentials which ultimately communicate signals to the brain. This renders cation channels the initial gatekeepers for the perception of various stimuli, exposure to which humans associate with pain, irritation and discomfort. The sustenance of action potentials and their capacity to transmit to second-order neurons also requires the activation of voltage-gated ion channels expressed along, e.g., nodes of Ranvier and pre-synaptic axon terminals.

The now-widespread interest in modulating cation channels as a therapeutic strategy emanated from findings that implicate such channels in various pathologies. This is most obviously demonstrated in the case of channelopathies (13), which not only represent indications for which pharmacological cation channel modulation could be valuable, but also produce symptoms that often effectively betray crucial
roles of the respective ion channel in healthy individuals. This is perhaps most notable in the case of the voltage-gated sodium channel 1.7 (Na\textsubscript{v}1.7), whose loss-of-function results in the condition known as channelopathy-associated indifference to pain (CIP) (14–17). Conversely, gain-of-function Na\textsubscript{v}1.7 mutations result in the hyperexcitability of nociceptive dorsal root ganglia (DRG) neurons, resulting in chronic
pain states (33,34). These findings have earned NaV1.7 the moniker of “pain gatekeeper.” Despite the apparent inability of CIP sufferers to sense physical pain, the condition appears to be otherwise largely benign (excepting anosmia), suggesting that pharmacological inhibition of NaV1.7 could be a safe and highly-effective analgesic strategy. Indeed, many potent analgesics long in use – such as the local anesthetic lidocaine – act primarily by blocking NaV channels, including NaV1.7 (6,35,36).

While NaV1.7 appears to be a critical molecular component of nociception, in healthy individuals, its activation relies on membrane depolarization that is initiated by other cation channel receptors expressed on nociceptive afferent fibers in the peripheral nervous system (PNS). In particular, TRP channels of the vanilloid (TRPV) and ankyrin (TRPA) subfamilies are known to be directly activated by noxious mechanical (37–42), thermal (42–47) and chemical (42,48–54) stimuli as well as several mediators of inflammation (55). These likewise represent potential drug targets for pain management that have attracted considerable interest across the pharmaceutical industry (4,56).

Insofar as cellular cation influx via ion channels is crucial to establishing resting membrane potentials as well as to the initiation and propagation of action potentials in nociceptive neurons, it is unsurprising that altered expression and/or function of several different ion channels have been demonstrated to cause pain (6,34,55,57–62). Several research groups have endeavored to identify and develop cation channel blockers for therapeutic use in the treatment of pain, and in particular, animal venoms, composed of naturally-occurring peptide and small-molecule toxins, have been among the richest sources of such blockers available. Not only have these toxins been a useful tool in research settings, but recently, the FDA approved the clinical application of a synthetic ω-conotoxin peptide analgesic – Prialta® (a.k.a., ziconotide, Elan Pharmaceuticals) – that selectively blocks the N-type voltage-gated calcium channel 2.2 (CaV2.2) (63,64). This is in addition to the clinical application of natural analgesics such as menthol and capsaicin, which act by modulating ion channel receptors – TRPM8 and TRPV1, respectively – in the PNS (65–68).
Outside of the nervous system, cation channels are critical to maintaining cellular membrane potentials and participating in intracellular signaling and therefore are essential to regulating cellular homeostasis. It is in this context that ion channel dysregulation can often result in the development, progression, proliferation and metastasis of tumors (5,8–12). In fact, it is not uncommon for ion channels to be absent in tissue cells but present in tumor cells emanating therefrom. For example, the voltage-gated potassium channel 10.1 (K\textsubscript{v}10.1) is normally expressed solely in the brain, but its ectopic expression in tissues outside of the brain has been linked to the progression of several cancers (5,10,69–73). Indeed, selective blockade of K\textsubscript{v}10.1 was shown to reduce cancer cell growth in vitro and in vivo in a murine model (72).

In addition, the heightened expression of several ion channels has been linked to the progression of cancers. For example, the TRP cation channel “classical” 6 (TRPC6) has been shown to be overexpressed in human prostate cancers and positively associated with extraprostatic invasion (74,75). Additionally, blocking TRPC6 in prostate cancer cells in vitro suppressed hepatocyte growth factor (HGF)-induced proliferation (75). Similarly, TRPC6 is overexpressed in human glioblastoma multiforme, and its blockage suppressed proliferation of human glioma cells and enhanced the effects of radiation in vitro (76).

Finally, tumorigenic and pro-proliferative variants of native cation channels can arise from alternative splicing. This is the case for a splice variant of Na\textsubscript{v}1.5 which, in healthy individuals, is expressed in much greater abundance in neonates as opposed to adults (77). The neonatal splice variant of Na\textsubscript{v}1.5 (nNa\textsubscript{v}1.5) is, however, expressed at high levels in adults in breast cancer cell lines, where it potentiates metastasis (78). This nNa\textsubscript{v}1.5 variant fortunately differs sufficiently enough from other, functionally critical Na\textsubscript{v}1.5 variants to allow selective inhibition, in this case by a pAb (77). When applied to breast cancer cells in vitro, the nNa\textsubscript{v}1.5-inhibiting pAb was able to dose-dependently suppress cellular migration, mirroring the results obtained when using nNa\textsubscript{v}1.5-specific small interfering RNA (siRNA) (79).

The small-molecule cation channel modulators presently on the market are, unsurprisingly, primarily used to treat pathologies whose symptoms arise from malfunctioning electrical signaling, such as pain, arrhythmias, epilepsy, seizures (episodic), and hypertension (4). This is to say that the examples presented
above are far from a comprehensive picture of the potential therapeutic value of drugging this protein class. Rather, efforts to produce cation channel-modulating mAbs for clinical development have, to date, been primarily motivated by the goals of pain relief and cancer treatment. These efforts will be summarized in the next section.
1.2 Anti-cation channel antibodies

Researchers across private industry and in academia have long been engaged in the effort to generate antibodies that selectively bind cation channels for research, diagnostic and therapeutic purposes (28,29,31,56,80). The diversity and selectivity of antibodies make them attractive tools for the realization of therapeutic strategies that demand the modulation of highly conserved proteins, such as cation channels. Most commonly, efforts towards the development of anti-cation channel mAbs for therapeutic applications have been aimed at channel inhibition (56,80). Research focused on the development of inhibiting, anti-cation channel pAbs has been more consistently successful (29,77,81,82), but of course, clinical development of antibodies first requires the production and characterization of a mAb. This section will focus on the advantages inherent to mAbs versus small molecules for targeting cation channels and will recount the progress that has been made so far in raising cation channel-inhibiting mAbs.

1.2.1 Benefits of monoclonal antibodies for targeting human cation channels

Immunoglobulin G antibodies (IgGs) are ~150 kD multimeric proteins formed by the assembly of two identical light (L) and two identical heavy (H) chain subunits (Fig. 1.2). The N-terminal ends of both the L and H chains consist of ~13 kD globular domains that, through enzyme-induced gene recombination, nucleotide additions at gene segment junctions and somatic hypermutation, consist of AA sequences that vary considerably across B-cells in a given individual, hence their name: variable domains (V_L and V_H, respectively). The majority of this variability falls on the so-called complementarity determining regions (CDRs) (three per V domain), which are the regions of the antibody that collectively form the protein motif that recognizes the target antigen – the paratope. The tremendous diversity of IgG paratopes that naturally arise in vivo allows hosts to isolate and remove pathogenic organisms by recognizing exogenous antigens with exquisite selectivity. Additionally, IgGs are equipped with a functional Fragment crys-
tallizable (Fc) domain capable of recruiting immune-system effector cells by interacting with their cell-surface Fcγ receptor (FcγR).

Figure 1.2 | Basic structure of an IgG

A cartoon depiction of IgG structure including the V_H and V_L domains, which can be expressed in the absence of constant domains to form Fragment variable fragments (Fvs; shown in red), which possesses the full antigen-binding CDR. The other IgG fragments (rendered by protease digestion) include constant domains, and are known as Fragment antigen-binding (Fab), of which each IgGs possess two, rendering IgGs bivalent, and Fragment crystallizable (Fc), which engages Fc receptors (FcRs) on immune effector cells.

The acceleration of research into antibody-based therapeutics has been motivated by several factors, such as their often unique functionality and lengthy half-life relative to small molecules (26). Moreover, antibody recombination techniques can now accommodate production of antibody-based molecules with varying numbers of functionalities, specificities and/or valences, making antibodies a versatile platform upon which to develop multifunctional monotherapeutics with novel mechanisms of action (26,27). Another characteristic of antibodies that is particularly relevant to targeting ion channels is the size and conformation-dependent nature of the paratope/epitope interface, which often gives antibody/antigen interac-
tions a “lock-and-key” quality that yields superior drug selectivity – an ideal feature for minimizing off-target effects.

High target selectivity is always a desirable drug characteristic, but it is a feature that appears to be especially critical to targeting ion channels as a therapeutic strategy. Its significance is amply demonstrated by the many nonspecific toxins and small-molecules that potently block ion channels (83–89). As an example, the nine voltage-gated sodium channels (VGSCs) are divided into tetrodotoxin-sensitive (TTX-s) and TTX-resistant (TTX-r) subgroups on the basis of their susceptibility to pore-occlusion by TTX (64,85,90,91). The reason for the considerable cross-reactivity of TTX is that the residues it interacts with are highly conserved across TTX-s VGSCs (64,85,86) (Fig. 1.3). Therefore, applying a similar mechanism of action with a comparably-sized small-molecule inhibitor will inevitably suffer from the same cross-reactivity and therefore pose an unacceptable risk of dangerous off-target effects. Exploiting the modest inter-VGSC heterogeneity along the outer pore (Fig. 1.3) requires a much larger drug/target interface that only biologics seem poised to accommodate. Furthermore, membrane-impermeable small-molecule inhibitors tend to interact with the acidic side-chains of AAs along the outer pore – a cation-attracting, acidic ring that is a common feature among ion channels (86,92). By contrast, antibody/antigen engagement is more commonly mediated by interactions between hydrophobic residues, which are perhaps less likely to be conserved along the cation channel outer pore.

The FDA-approval of the Ca_{2.2}-inhibitor, ziconotide (a synthetic ω-conotoxin), somewhat bears out the idea that subtype selectivity among membrane-impermeable, pore-occluding molecules requires a bigger drug/receptor interface. As a 25 AA peptide, it is able to occlude a Ca_{2.2} pore with subtype selectivity at clinically acceptable levels (63). That said, ziconotide’s use is limited to patients suffering from severe pain, must be intrathecally administered and is associated with serious adverse events, particularly at higher doses (perhaps indicating cross-reactivity) (63). This suggests that targeting cation channels with biologics may be a good strategy for therapeutic development, but biologics with higher selectivity, such as antibodies, could potentially maximize effects and minimize cross-reactivity.
Figure 1.3 | Alignment of PG region of TTX-s NaV channels

This image presents a depiction of the inter-domain, TTX interaction site (hashed line) across the pore (gray/black colored square) of TTX-s human NaV channels. The residues highlighted in red are those that TTX interacts with specifically. The residues that are outlined in black are unique to NaV1.7.

The use of hydrophilic or lipophilic cation channel-modulating small molecules for therapeutic applications has been thoroughly covered in the review by Bagal et al. (4). In that review, the authors noted that while the number of clinically approved small molecules that modulate ion channels is somewhat large, the number of such drugs that targets discrete ion channels is quite small. Additionally, these drugs are often only topically or locally administered so as to avoid off-target effects, and most ion channel drugs were discovered over a decade ago, which has been followed by a long drought in market approvals (4). This speaks to the potential advantages not only of drugs that exhibit higher selectivity, but also ones
that can influence their own in vivo distribution. Newer multifunctional antibody-based monotherapeutics can be equipped with additional domains to enrich their presence in, e.g., areas of inflammation or even in the central nervous system (CNS) (93–96). The many advantageous features of antibody-based molecules explain the considerable research into their use as cation channel modulators.

1.2.2 Progress made in the development of cation channel-targeting antibodies

Starting around the mid-1990s, there has been an impressive acceleration of research focused on the production of ion channel-modulating antibodies (28–30). During that time, the overwhelming majority of cation channel-inhibiting antibodies that have been successfully raised have been pAbs targeting the E3 pre-pore loop (31,82,97–110). Additionally, two pAbs targeting the E1 and E2 regions, respectively, also showed an inhibiting effect (77,111). While pAbs are certainly a useful tool in research and preclinical settings, they are typically ill-suited for clinical applications. Moreover, pAb-mediated channel-inhibition does not necessarily imply that targeting a multi-epitope protein region with a single mAb will be effective. This was demonstrated by the work of Klionsky et al., who successfully developed a TRPV1-inhibiting pAb targeting the E3 loop but were unable to identify a single TRPV1-modulating mAb emanating therefrom, despite identifying multiple TRPV1-selective mAbs (82).

Presently, only four monoclonal antibodies that inhibit cell-surface, human cation channels in vitro have been described in the literature, all of which were raised in mice. These mAbs target: 1) the PG region of hKv10.1 (72); 2) the E2 region on DII of hNav1.7 (80); 3) an unknown region (antibodies were raised against full-length protein) on hTRPA1 (56); and 4) the E3 loop of hTRPM8 (81). Encouragingly, both the anti-hKv10.1 mAb and the anti-hNav1.7 mAb showed efficacy in vivo as well. In mouse xenograft models of breast and pancreatic cancer cells, the anti-hKv10.1 mAb slowed tumor growth (72). The anti-hNav1.7 mAb, meanwhile, was shown to reduce inflammatory and neuropathic pain in mice as well as itch (80).
In both cases, the mAb raised was cross-reactive with the orthologous mouse ion channel. This is not altogether surprising for Kv10.1 as its expression is confined to the immune-sequestered CNS, so it could likely tolerate the presence of self-reactive B-cells in the periphery. However, this is somewhat surprising for Na\textsubscript{v}1.7 which is expressed in the PNS and therefore accessible to circulating IgGs – further demonstrated by the fact that systemic administration of the mAb reduced pain \textit{in vivo} (80). Evidently, the B-cells that produced these IgGs were able to avoid peripheral deletion, which typically is the fate of autoreactive B-cells. Additionally, it is unclear why researchers in this case chose to demonstrate hNa\textsubscript{v}1.7 subtype selectivity by evaluating the mAb’s capacity to inhibit rNa\textsubscript{v}1.2, mNa\textsubscript{v}1.5, mNa\textsubscript{v}1.6 and rNa\textsubscript{v}1.8 rather than human Na\textsubscript{v} subtypes (80).

What is truly remarkable about the list of human cation channel-modulating mAbs is how short it is after two decades of research. At this moment, none appear poised to receive clinical approval in the near future. Intriguingly, the mAbs that have demonstrated efficacy \textit{in vitro} were generated by diverse means, suggesting that while there may be a one-size-fits-all approach, it is not necessary to achieve the goal of selective, antibody-mediated ion channel modulation. Simply expanding or sophisticating the existing toolkit could therefore be of considerable value. Additionally, the broader success of pAbs vs. mAbs in inhibiting cation channels may suggest that simultaneous binding at multiple sites could be an effective strategy, which could be accommodated by antibody cocktails or – more reliably – flexible multispecific antibodies.
1.3 Challenges associated with the generation of cation channel-modulating antibodies

In light of the considerable efforts described above, the complete absence of clinically available ion channel-modulating mAbs highlights the need to identify and examine the ostensibly unique challenges inherent to drugging this protein class with antibodies. There is a variety of ways to consider these issues, but it is perhaps best to focus on the most broadly used methods for therapeutic mAb generation and consider their possible strengths and weaknesses vis-à-vis the aim of ion channel modulation. For the purposes of such a review, we have several convenient frames of reference in the form of the various naturally-occurring toxins that potently block or otherwise modulate ion channels, revealing critical interaction sites and effective molecular mechanisms of antagonism. Additionally, since this thesis focuses exclusively on \textit{in vivo} immunizations, which remain a prolific method for the production of target-specific mAbs, it is important to consider the issue from an immunological perspective, factoring in the mechanisms governing B-cell activation, differentiation and proliferation.

1.3.1 What can ion channel-modulating toxins and peptides teach us?

As mentioned previously, the considerable research into the ion channel-modulating effects of naturally-occurring toxins is a useful starting point insofar as it reveals viable mechanisms ion channel antagonism in many cases (64,83,87–89,91,112,113). A goal, then, might be to capitalize on such information by designing immunogens or antibody-discovery platforms that generate or identify IgGs that bind the same domains with higher affinity and selectivity. Obviously, the usefulness of such research in the context of informing antibody discovery is largely limited to membrane-impermeable agonists and antagonists that interact with IgG-accessible protein domains. Excluding on the basis of this principle, there are essentially two antibody/channel interaction sites of particular relevance: 1) the E1/E2 loop(s) along the
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S1-S4 ion channel gating region, and 2) the outer pore formed by the convergence of the four PG regions in the cation channel’s functional structure.

The E1/E2 loops of voltage-gated and TRP channels have been demonstrated to be targets of peptides purified from the venoms of, for example, tarantulas and centipedes (83,87,89,112). The peptide toxins that bind to this region usually adversely affect gating of the ion channel, reducing its maximal activation and/or shifting its activation threshold towards more depolarized membrane potentials, thereby reducing cellular excitability. This mechanism of action was successfully exploited by Lee et al. (80) and Chioni et al. (77) in developing an inhibiting mAb and pAb targeting hNa\textsubscript{V}1.7 and nNa\textsubscript{V}1.5, respectively.

Meanwhile, several other ion channel-inhibiting toxins, such as TTX, conotoxins (CTXs) and saxitoxin (STX), act by blocking the pore – interacting with residues along the PG regions of multiple domains/subunits to effectively achieve pore-occlusion (85,86,88,91). Additionally, some membrane-impermeable small molecule inhibitors, such as the non-specific TRP channel antagonist, ruthenium red, act in a similar fashion (44,92). While mAbs specifically targeting the PG region of both human K\textsubscript{V}10.1 and rat L-type calcium channel partially reduced maximal activation of both (72,114), an important distinction between these and pore-occluding toxins is that the mAbs only interact with one of the channel’s repeat domains.

Interaction with any of these individual protein regions is far from a guarantee of antagonism. On the contrary, a bivalent peptide isolated from a tarantula venom interacts with the PG region of the nociceptive hTRPV1 and potently activates the channel (84). The reason for why this occurs lies in the peptide toxin’s structure – it consists of two identical reactive domains separated by a rigid linker domain. When the toxin encounters its ion channel target, this design putatively results in the outer pore of the homotetrameric channel being “pried” open, inducing cation flux. Additionally, the length and solvent-exposure of cation channel extracellular regions, and their heterogeneity vis-à-vis solvent-exposed regions of other proteins, varies considerably, suggesting a limited generalizability of targeting these sites for antibody-mediated, selective ion channel modulation.
Interestingly, there does not appear to be much evidence for ion channel antagonism via exclusive toxin interaction with the E3 pre-pore loop, despite this region being the one most commonly targeted for mAb-mediated ion channel inhibition. The reasons why this region is commonly targeted by researchers attempting to produce selective, inhibiting IgGs are clear: 1) the E3 loop tends to be lengthy, implying considerable solvent exposure and therefore IgG-accessibility; 2) The amino acid sequences among the many ion channel E3 regions appear to be sufficiently heterologous so as to confer channel-subtype specificity; and 3) the E3 loop’s proximity to the ion channel pore possibly suggests a critical role for this region in cation flux, which could be obstructed via binding by a bulky antibody or antibody fragment. As covered earlier, the approach of targeting the E3 loop of ion channels with antibodies has had some success. Inhibiting pAbs and mAbs have been developed against TRP and NaV channels by targeting this region.

In nearly all cases, however, antibody-mediated inhibition of ion channels is incomplete, in contrast to the complete blockade elicited by several small molecule and toxin inhibitors. What is clear from research into these many, potent toxins is that pore-occlusion is a fairly consistently effective strategy to achieve complete inhibition of ion flux. Such occlusion appears to require monovalent interaction with the PG regions of multiple subunits/domains, where they converge to form the outer pore. As will soon be described, developing antibody CDR sets in vivo that monovalently target a multi-subunit/-domain epitope is a challenging feat for a variety of reasons.

1.3.2 In Vivo antibody discovery

Antibody discovery methods that rely initially on the generation of antibodies in vivo in non-human animals begin, inevitably, with immunogen design. Generally, the immunogen used in immunizations will either be the full-length antigen, a recombinant (e.g., soluble) form of the antigen, or a derivative peptide conjugated to an immunoadjuvant (115). In each case, the goal is to raise IgGs that bind a solvent-
exposed epitope on the target antigen with high affinity. Over the course of an immunization campaign, the animal (e.g., mouse, rabbit, etc.) is routinely exposed to the immunogen via either repeat dosing, or – in the case of retroviral introduction of antigen-encoding cDNA into the host genome – constitutive ectopic expression. Antibody titers for the antigen are monitored for the duration of the campaign, and ultimately the animals are sacrificed. A splenectomy is then performed, and antibody-secreting B-cells are harvested and, most commonly, immortalized by hybridization with myeloma cells (forming hybridomas) prior to clonal selection. In addition to the antibody-containing hybridoma supernatants, isolated hybridomas are equipped with a B-cell receptor (BCR) – a membrane-anchored form of the cell-specific IgG – which means, in essence, that the cells themselves bind the antigen with the same affinity as the IgG they secrete.

Once the cells are isolated and cultivated, and their IgG/BCR is confirmed to bind the target antigen, a reverse transcriptase polymerase chain reaction (RT-PCR) can be performed to obtain the DNA sequence encoding the IgGs expressed by each relevant B-cell, allowing the generation of mAb-encoding cDNA for use in the large-scale production, in vitro, of mAbs in native or recombinant form using, for example, transformed E. coli or transfected mammalian cell lines. To develop non-human mAbs for human clinical use, humanization of the antibody is also normally required in order to prevent the development of anti-drug antibodies, which typically arise upon immune-system exposure to non-human biologics. This entails replacing part or all of the non-CDR IgG regions with the corresponding human IgG sequence. The humanization and/or recombination of mAbs can result in activity-compromising, or destabilizing, conformational change to the CDR sets. Generation of the antibodies in humanized form, therefore, requires repeated confirmation of antigen-binding, thorough pharmacokinetic analysis and characterization of the protein’s solubility and stability profile.

These steps are largely common to all in vivo antibody-discovery efforts. However, in the context of cation channels, perhaps the most critical step is in designing the immunization campaign. There are advantages and disadvantages inherent to each traditional approach to animal immunization, and such ap-
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proaches demand consideration of the features of the fully-assembled cation channel target in its native conformation, namely: 1) the level of the cation channel’s sequence conservation across species and its homology to related proteins; 2) the putative IgG-accessibility of its extracellular regions; 3) location of critical sites for channel gating and ion flux; 4) sites of post-translational modification, particularly glycosylation; and 5) the unique, functionally critical, epitopes that may emerge upon ion channel assembly into active form (as opposed to those that potentially lie along the basic, E1-E3 and PG regions in the protein’s known primary structure). It is equally important to consider the immunization campaign from an immunological perspective, keeping in mind the ways in which B-cell activation, differentiation and proliferation will theoretically proceed in vivo upon exposure to the immunogen.

1.3.2.1 B-cell activation and BCR/IgG generation

The production of antigen-specific IgGs in vivo after injection of an immunogen normally first requires the activation of mature, naïve B-cells whose BCRs (membrane-anchored form of IgM) bind an epitope on the immunogen. In the context of protein antigens, this activation is commonly T-cell-dependent, as is the differentiation into IgG-secreting plasma B-cells and memory B-cells. More specifically, B-cells typically are activated in the course of serving their function as antigen-presenting cells (APCs). This requires that the cells internalize and process the antigens to which they bind, and present derivative antigenic peptides resulting from proteolysis of the full-length antigen (Fig. 1.4).

These peptide antigens are presented as complexes with major histocompatibility complex class II (MHC II) molecules. If any of these MHC II:peptide complexes are, in turn, recognized by a T-cell receptor (TCR) on a CD4+ T helper cell (TH-cell) – i.e., they present a TH-cell epitope – co-stimulation between the respective cells occurs, with further ligand-receptor interactions and cytokine signaling inducing B-cell activation, antibody isotype-switching (e.g., IgM to IgG), and differentiation into secretory and memory B-cells. Additionally, B-cells and TH-cells interact in germinal centers, wherein B-cell antibodies undergo
somatic hypermutation, facilitating the development of higher-affinity immunobinders. B-cells exhibiting such heightened affinity are preferentially supported by T_{H}-cells for survival, in a process known as affinity maturation.

Figure 1.4 | Antigen internalization, processing and presentation by B-cells

B-cells bind and internalize an exogenous antigen – e.g., multiple copies of a peptide derived from an extracellular loop of an ion channel chemically ligated to the immunoadjuvant, keyhole limpet hemocyanin (KLH) – upon BCR cross-linking. The lower pH in endosomal/lysosomal compartments release the protein and induces protease activity that degrades the KLH-peptide conjugate into peptides for loading onto MHC class II molecules. The MHC class II:peptide complexes are then presented to CD4+ T_{H}-cells. TCR and CD4 recognition of the complex initiates costimulation of the cells.

The creation of antigenic peptides from internalized proteins in B-cells is catalyzed by luminal proteases in intracellular endosomes/lysosomes. It is also in these compartments where the peptides are loaded onto MHC II molecules, before the compartment fuses with the Golgi apparatus for delivery of the MHC II:peptide complex to the cell surface for binding by the TCR and CD4. While many algorithms have been developed to predict peptide binding to MHC II molecules, the restrictions that govern this process are still not entirely understood, nor is it a guarantee that MHC II:peptide complexes will produce a T_{H}-cell epitope (116–118). For this reason, conjugating antigens with an immunoadjuvant such as key-
hole limpet hemocyanin (KLH) is a common method employed for \textit{in vivo} antibody discovery campaigns, so as to ensure the presence of T\textsubscript{H}-cell epitopes after antigen uptake and processing by B-cells (Fig. 1.4).

The process of antigen internalization by B-cells and subsequent B-cell activation is influenced by whether the antigen is secreted or anchored to an insoluble carrier, such as an integral membrane protein in a lipid bilayer (119–121). In the case of soluble antigens, it is widely believed that BCRs are drawn into close proximity on the cell surface by simultaneous binding to the antigen, and this cross-linking of BCRs triggers the internalization of the antigen for processing and presentation (119). This would seem to imply that soluble antigens need to be multivalent, allowing simultaneous binding by multiple BCRs. By contrast, it seems that BCR-engagement of antigens on, for example, a lipid-bilayer does not require such cross-linking, as a carrier of sufficient size exerts the requisite mechanical force to trigger internalization in the absence of cross-linking-induced signaling (120,121) (Fig. 1.5). It is not altogether clear whether complex multispanners can be internalized by B-cells when presented in the lipid bilayers of large mammalian cells, but B-cells have been shown to internalize small liposomes with equal efficiency as soluble antigens (122).

All of these factors bear consideration when designing an immunization campaign. An additional issue complicating the generation of target-specific B-cells \textit{in vivo} arises when attempting to raise antibodies against human proteins that share considerable identity with the host’s orthologous protein. There are many biological mechanisms preventing the development of autoreactive antibodies – antibodies that recognize self-antigens. This includes the central tolerance established in the bone marrow, wherein maturing B-cells are exposed to self-antigens, with strong BCR/self-antigen interactions typically resulting in B-cell apoptosis or anergy (123). While there is a surprising quantity of self-reactive B-cells in the periphery, there are additional quality control mechanisms that facilitate peripheral B-cell deletion and down-regulation (123,124). For example B-cells express the inhibitory co-receptor, CD22, which recognizes terminal sialic acid residues commonly presented on glycans affixed to native glycoproteins (125–128).
1.3 Challenges associated with the generation of cation channel-modulating antibodies

The trans interaction between CD22 and an antigen simultaneously bound by a BCR produces a proximity between the two receptors that prevents BCR signaling.

Figure 1.5 | Capture and processing of membrane-bound antigens by B-cells

While it has been demonstrated that B-cells can internalize single-pass membrane proteins from APCs by “immune synapse” formation, it is unclear whether such a process can trigger internalization of complex multispanners, such as ion channels. Nonetheless, presentation of full-length ion channels in lipid bilayers is ostensibly the ideal way to develop conformation-dependent immunobinders – i.e., IgGs recognizing epitopes that are only present in the fully assembled protein. Evidence suggests that such antigen presentation, in the context of small lipoparticles, can induce internalization of the particle in the absence of BCR cross-linking. After internalization, processing and presentation proceeds normally.

When one considers the complexity of antigen capture, processing and presentation as well as the dynamic regulation of B-cell activation, differentiation and proliferation, the task of raising highly selective, modulating mAbs that target human ion channels – highly conserved integral membrane proteins with only limited IgG-accessible extracellular regions and even fewer of functional relevance – presents a daunting challenge.
1.3.2.2 Peptide immunization

One of the most widely-used methods to generate mAbs against human proteins is to first synthesize a peptide derived from an extracellular region of the protein’s primary structure, chemically ligate the peptide to an immunoadjuvant, and repeatedly dose an animal (e.g., mouse, rabbit, etc.) with the peptide-adjuvant conjugate. This has been, evidently, the most common method applied by researchers who have successfully raised anti-cation channel antibodies – utilizing synthetic peptides that mimic the E2 (77,80), E3 (31,81,82,97–110) and PG (114) regions, respectively, and attaching them to the immunoadjuvant carrier, KLH. It was this approach that gave rise to the several anti-E2, anti-E3 and anti-PG cation channel-inhibiting antibodies earlier described. It is likely the quickest, most-reliable means to generate at least modestly selective antibodies, although the mAbs emanating from such campaigns are often not inhibiting (82). Those that do inhibit the target cation channel, meanwhile, largely do so incompletely (56,80,81).

Targeting the extracellular sites along the S1-S4 gating region appears to be an inconsistently effective strategy to produce selective, cation channel-modulating mAbs. This is true for a variety of reasons. For one, these sites may not be critical to ion channel function in several instances, for example in the case of TRP channels. While protoxin-I (ProTx-I) – a 35 AA peptide isolated from the venom of the Peruvian green-velvet tarantula – was recently shown to inhibit TRPA1 function by interacting with the protein’s E1 region (87), toxin inhibition by S1-S4 region-interaction seems to be a relatively uncommon finding among proteins in this ion channel family. This is likely because there are multiple modes of activation of TRP channels, with disparate ion channel sites and molecular intermediaries playing roles.

An additional complication associated with E1 targeting by peptide immunization is that it is often a recipient site for post-translational modifications, such as N-glycosylation. This is a tightly regulated process that occurs during protein production and entails the covalent attachment of complex carbohydrate structures to extracellular protein regions. These structures can be quite large and can affect protein conformation considerably. They also can occlude antibody-binding sites, preventing antibody/antigen interactions (129,130). These structures are typically not replicated during peptide synthesis and, therefore,
such protein regions are not faithfully mimicked by derivative peptides used in the production of immunoadjuvant-peptide immunogens.

Toxin inhibition via E2 interaction is more commonly observed in voltage-gated channels, which is to be expected since the voltage-sensor paddle has been mapped to the C-terminally adjacent, S4 transmembrane helix of these ion channels. Although E2-targeting has been an effective strategy for raising inhibiting pAbs and one mAb, this region is occasionally too short to be recognized selectively by an antibody paratope. A short E2 region also suggests minimal solvent exposure and therefore lower IgG-accessibility. It also frequently means that there is not considerable inter-subtype sequence diversity at this position. This is certainly the case for hNa\textsubscript{V}1.7, wherein only 1 AA along the E2 region renders it unique among related VGSCs. While Lee at al. (80) have reported production of a subtype-selective, inhibiting mAb targeting this region, it appears as though selectivity for hNa\textsubscript{V}1.7 was only demonstrated \textit{in vitro} against three human subtypes from the same family. It remains unclear whether researchers can expect high cation channel selectivity \textit{in vivo} when using E2-derived peptides for immunizations.

Again, the most commonly used peptides for anti-cation channel immunization campaigns are derivatives of the extracellular region N-terminal to the ion channel pore – the E3 region. While this tends to be a lengthy and less-conserved region, it is also frequently modified with N-glycans among Na\textsubscript{V} and TRP channels, and the sequence diversity at this position may in fact betray a limited role in cation flux. To date, only two mAbs targeting this domain have shown an inhibitory effect, both of them raised against TRPM8 (81). As described earlier, in one case, successful generation of inhibiting pAb emanating from an E3-peptide immunization ultimately failed to yield inhibiting mAbs isolated from the same campaign (82), suggesting either an unlucky B-cell clonal selection or that channel inhibition was a function of simultaneous binding of several antibodies.

Meanwhile, peptide immunizations using PG-derived peptides are rare, likely because this region can be small, is fairly well-conserved and can even exhibit poor solubility. However, one such immunization did produce an inhibiting mAb against a rat VGCC (114). Intriguingly, this mAb only bound when
the cell was in a depolarized state, suggesting a dilation of the pore during channel activity that rendered the PG region more IgG-accessible. Although, a potential danger to targeting a single subunit/domain’s PG region is evidenced by the bivalent tarantula toxin described earlier, which engages this site on TRPV1 and activates the channel (84). Such activation might, then, be a consequence of engaging such channels similarly with a bivalent IgG.

Another, rather clever, immunization campaign did produce a PG-targeting mAb against Kv10.1 with an unconventional technique that bears describing here (72). Rather than employ a typical peptide-immunization strategy, the researchers in this case generated a fusion peptide consisting of E3 + PG extracellular regions while omitting the selectivity filter region that sits between these sites in the protein’s primary structure. Because the peptide loop between S5 and S6 transmembrane helices is membrane re-entrant, it folds in such a way whereby E3 and PG regions might be very adjacent in the channel’s native conformation, possibly resembling the E3-PG fusion peptide that was created. Additionally, these researchers fused a coiled-coil-generating protein motif – naturally present at the C-terminal end of the full-length Kv10.1 – to the C-terminal end of the E3-PG fusion peptide. This coiled-coil region then drove tetramerization of the peptide, essentially creating a recombinant ion channel pore mimetic. Due to its size, this multimeric, recombinant protein did not require any immunoadjuvant conjugation, and it effectively raised antibodies against both E3 and PG regions.

However, in some ways, this may have been an opportunity missed, because by administering this tetrameric fusion peptide as a soluble antigen in isolation rather than anchored to a carrier, and by selecting mAbs on the basis of affinity for peptide-derivatives of either of the respective protein regions (E3 and PG), no mAbs that monovalently interact with multiple peptides in the pore mimetic were discovered. Such a mAb would possibly have behaved similarly to the many exquisitely potent, pore-occluding toxins described earlier. Nonetheless, the creativity on display in the design of this immunization campaign inspires hope for the future of anti-cation channel mAb discovery, and to their immense credit, the researchers in this case produced selective, Kv10.1-inhibiting mAbs that were effective in vitro and in vivo.
1.3 Challenges associated with the generation of cation channel-modulating antibodies

Speaking from an immunological perspective, the success of KLH-peptide immunizations relies on the presence of BCRs that strongly interact with the peptide in question and that the B-cells bearing them can then be induced to differentiate and proliferate by T_{H}-cells. This likely means that, if the native protein is not confined to an immune-privileged location, such BCRs cannot cross-react with the animal’s orthologous protein, insofar as central or peripheral immune tolerance would likely prevent high levels of autoreactive B-cells from accumulating in the periphery. In practice, this means that the peptide used for immunization should not only differ from extracellular regions on related human subtypes to be adequately selective in humans, but the peptide should also possess a sequence that distinguishes it from the analogous region of the host’s related protein in order to stimulate a peptide-specific antibody response in the first place. This is often not the case, possibly because conservation of protein regions often suggests functional relevance.

Overall, the standard KLH-peptide immunization strategy has been useful in research settings and seems to be the most consistently effective strategy to raise anti-ion channel antibodies. However, the usefulness of this approach for generating therapeutically viable mAbs is not entirely clear. Particularly when considering the nature of toxin inhibitor/cation channel interactions, strategies that endeavor to present epitopes that arise only in the protein’s fully-assembled conformation – such as application of the recombinant immunogen designed by Gómez-Varela et al. (72) – seem better positioned to produce highly potent antibodies that possess a level of subtype selectivity in vivo that is absolutely crucial to clinical application. Typically, this is attempted by presenting the full-length protein to the immune system through either genetic immunization or protein immunization.

1.3.2.3 Genetic immunization

Another common approach to raising antibodies against human proteins is to immunize animals – by intramuscular or intradermal injection or epidermally with a gene gun – with an expression plasmid
possessing DNA encoding some form of the antigen (131). This procedure can be performed with conventional, recombinant expression vectors that simply induce transient expression of the full-length or recombinant antigen in successfully transfected cells. Alternatively, researchers have begun commonly utilizing retroviral vectors that introduce antigen DNA into the host’s genome (132). A genetic immunization applying a recombinant adenoviral plasmid (and subsequent boosts with a conventional expression vector and antigen-expressing mammalian cells) was successfully employed by Lee et al. to raise inhibiting mAbs against hTRPA1, but it is important to note that the researchers mentioned that their immunization strategy included an undisclosed modification to break immune-tolerance (56). This appears to be the only publicly documented occurrence of an inhibiting mAb targeting a human cation channel being produced via a genetic immunization. Without knowing further details, it is impossible to comment on the generalizability of such an approach, but the authors’ desire to withhold information highlights one of the biggest challenges associated with genetic immunizations against complex multispansing proteins – the unreliability of heterologous expression in vivo to induce an immune response against the target antigen.

The reasons why this seems to be the case are somewhat unclear, but the lack of immunogenicity elicited by heterologous expression of complex multispans on the cell surface of the host’s native cells must be due to issues with antigen presentation and/or immune-system processing. On the antigen presentation side, it is well documented that ion channel expression can be frustratingly low in heterologous expression systems in vitro (133,134). Conversely, the establishment of cell lines stably expressing non-native ion channels is notoriously challenging, as high expression of cation-permeable ion channels at the cell surface can produce unhealthy resting membrane potentials (134). This would suggest that genetic immunizations relying on induction of heterologous ion channel expression on the cell surface of host cells do not dependably result in sufficient antigen exposure to the immune-system to elicit a B-cell response, either because of low antigen expression or expression-induced death of successfully transfected cells. Importantly, BCR clustering has been shown to allow the internalization of membrane-bound, monovalent antigens by essentially forming a B-cell/APC synapse (120,121), so the absence of sufficient ex-
press on the APC could result in the absence of such clustering and uptake and therefore of a robust B-cell-mediated response.

Additionally, in any genetic immunization, there is likely some degree of plasmid uptake by non-APCs. In that case, the processing and presentation of heterologously expressed ion channels would result in a considerable CD8+, cytotoxic T lymphocyte (CTL)-mediated response which could compromise the level of B-cell mediated response desired. Furthermore, uptake and expression by, for example, dendritic cells (DCs), will likely still result in antigen processing, presentation and cross-presentation for a primarily T-cell-mediated immune response. In other words, the level of protein expression and balance of the immune response possibly results in limited B-cell exposure to, and internalization/processing of, the full-length antigen. Indeed, it has been documented that DNA plasmids tend to elicit a primarily T-cell-mediated response and only modest antibody responses (135).

Finally, the issue of central and peripheral B-cell immune tolerance remains. Some ion channels may be so tightly conserved so as to not produce any B- or T-cell epitopes. Also, because the expression of the ion channel will be occurring via the intracellular machinery of the host animal, it will be equipped with the animal’s native N-glycan structures. B-cells express members of a family of inhibitory coreceptors known as sialic acid-binding Ig-like lectins (SIGLECs), which includes CD22 (136–138). As their name implies, these receptors bind sialic acid, a carbohydrate building block present at the termini of glycoproteins such as cation channels. When a B-cell receptor pulls a glycoprotein antigen into close proximity, SIGLECs may engage in trans binding of the antigen’s glycan. The proximity of the trans bound SIGLEC and the BCR leads to a negative downregulation of B-cell signaling and cellular apoptosis or anergy (125–128,136–139). In such a case, even if the BCR selectively recognized the human protein, the native glycan structure may be enough to ensure B-cell clonal deletion, and therefore no antibody response. Pathogenic organisms have even evolved mechanisms of glycan mimicry that promote immune-evasion (138). Therefore, it may be advisable to remove glycosylation sites by nucleic acid mutations in order to mitigate the risk of downregulated B-cell signaling. However, the absence of glycans may have
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an impact on cation channel trafficking and/or conformation and will assuredly present unnatural antigen epitopes to the immune-system.

Genetic immunization has been a highly effective strategy for generating IgGs against secreted proteins, which is why a common strategy for the generation of IgGs targeting single-pass integral membrane proteins is to truncate the gene encoding it in order to produce a recombinant, secreted form in vivo (140). However, little progress appears to have been made in applying this strategy to the generation of mAbs targeting human complex multispanners, including ion channels. Although genetic immunization ensures some immune-system exposure of full-length antigen in its native conformation, protein expression levels and the potentially compromised health of successfully transfected cells may ensure that such exposure is limited. Additionally, high sequence conservation and the presence of glycan structures transferred by the host’s native transferases may suggest existing central B-cell tolerance or promote peripheral B-cell tolerance, respectively.

1.3.2.4 Protein Immunization

Another common immunization strategy entails exposing the immune-system to an antigen via direct protein injection. In the case of cation channels, this means that a recombinant and/or solubilized form of the protein is created, and/or it is presented in the lipid bilayer of exogenous mammalian cells (56), exosomes (141), virus-like particles (VLPs) (142), or other small liposomes (122). At present, there are no inhibiting mAbs targeting cation channels that have been raised by employing this strategy in isolation, unless one includes the tetrameric fusion peptide generated by Gómez-Varela et al. to produce anti-Kv10.1 mAbs (72). Additionally, the immunization strategy that gave rise to hTRPA1-inhibiting mAbs did include boosts with mammalian cells heterologously expressing a recombinant form of the antigen in addition to several rounds of genetic immunization (56).
1.3 Challenges associated with the generation of cation channel-modulating antibodies

The main advantages of protein immunization are clear: 1) it can ensure B-cell exposure to the full-length ion channel, putatively in its native conformation, providing a greater potential for the generation of conformation-dependent immunobinders; 2) the cells or vesicles used to display the ion channel can be enriched on the basis of antigen expression levels; and 3) the cells or vesicles can also carry additional immunogenic cargo to ensure a robust immune response. Smaller vesicles – VLPs, exosomes and proteoliposomes – can be internalized by B-cells upon the BCR engagement of surface antigens (122), which should theoretically accommodate the production of monovalent, IgG-binding of the kind of inter-subunit-domain regions that are targeted by potent toxins. It would seem, then, that such a strategy should be a very prolific method for anti-cation channel mAb production.

There is some overlap between the theoretical limitations of genetic and protein immunizations. Again, central and peripheral immune-tolerance still need to be overcome, and the possible absence of B-cell epitopes, the potential for BCR cross-reactivity with the orthologous protein, and/or the display of SIGLEC-engaging glycans along cation channel domains may result in a muted immune response. Additionally, low cation channel expression could remain problematic. However, there are also potential issues that are unique to protein immunization. Exogenous cells and the exosomes they secrete, as well as VLPs, possess a number of non-target proteins that can be bound by BCRs to induce B-cell internalization. Given that, the immune-system will likely not need to rely on any anti-cation channel BCRs and IgGs to efficiently clear the exogenous particles. The purification and reconstitution of ion channels in small proteoliposomes would theoretically be able to overcome such a limitation. However, methods for proteoliposome production typically rely on solubilization of the protein, which could compromise the structure of the ion channels that are reconstituted (143). Additionally, the process of reconstitution often results in roughly equal presentation of ion channels with an inverted orientation – with intracellular domains exposed to the extracellular space. The ion channel intracellular domains tend to be much lengthier, which means that they would be more accessible to IgGs and exhibit considerably more epitopes than the properly oriented channels.
It is intriguing that perhaps the most promising approach to generating cation channel-inhibiting antibodies \textit{in vivo} – presentation of a fully-assembled pore in a lipid bilayer – has not yet demonstrated any capacity to do so. This speaks to the challenges of manipulating these very large, complex proteins for presentation to the immune-system in a manner that is both sufficiently immunogenic and faithfully maintains the channel’s native conformation. Sophistication of this technology could allow the development of specific, conformation-dependent and pore-blocking IgGs that are notably absent from the literature.
1.4 Potential strategies to aid the discovery of anti-cation channel mAbs

The thorough characterizations of cation channel-blocking molecules – small-molecules, toxins and mAbs – available in the literature should inform future strategies concerning immunogen design, antibody selection and antibody recombination towards the aim of raising human cation channel-modulating mAbs. Based on a review of the literature, it appears as though the most consistently effective approach to potentially inhibit cation channels is to monovalently target inter-domain/subunit sites to occlude the pore. Raising mAbs in vivo that interact with ion channels in this fashion requires presentation to the immune-system of, at least, the natively conformed pore domain, or a pore-mimetic, anchored to a carrier or lipid bilayer. However, such strategies must contend with the molecular mechanisms promoting central and peripheral immune-tolerance while ensuring that clearance of the immunogen is at least partly driven by B-cell internalization triggered specifically by BCR/channel interactions. Theoretically, promising immunogens would present conformation-dependent ion channel pore epitopes without facilitating immune-mediated clearance via IgG and BCR interactions with non-target proteins, such as those present on VLPs. Additionally, promotion of ion channel immunogenicity may require protein modifications, such as deglycosylation or fusion with immune-stimulating ligands. B-cell selection pursuant to all such immunizations should then be performed to identify binding to the full-length protein.

A compelling immunization strategy that potentially addresses many of the aforementioned limitations was described in the paper by Delcayre et al. (141). The researchers produced and applied recombinant exosomes expressing human antigens to generate an antibody response in mice. In addition to HEK293 and CHO-K1 cells, the researchers used mouse DCs to produce these recombinant exosomes. What this suggests is that they could create small vesicles displaying full-length antigen in its native conformation and proper orientation, while all other displayed proteins are of the same animal origin as the host, theoretically resulting in modest off-target immunogenicity. This approach seems highly promising,
as it appears to circumvent many of the issues that plague other protein immunization strategies. However, it does not, in itself, ensure the immunogenicity of the target protein.

The issue of peripheral B-cell tolerance mediated by, for example, SIGLECs may require protein modification. Researchers have shown that deglycosylation of antigens – either by mutation or by treatment with glycosidases – can elicit a more robust antibody response (144,145). Perhaps applying such a modification to cation channels, which routinely exhibit numerous complex glycan structures, prior to protein immunization may help to boost the immune response by mitigating the risk of anergy or apoptosis of reactive B-cells. Additionally, researchers have shown that antibody responses to antigens can be considerably enhanced by fusion with recombinant CD40 ligand (CD40L), which promotes direct activation of B-cells (146,147). Meanwhile, another research group was able to express a functional TRPV5 variant which was fused to an additional N-terminal transmembrane helix, effectively producing a TRP channel with an extracellular N-terminus (148). Given that, one could envision such a fusion enabling the N-terminal presentation of reactive CD40L by recombinant cation channels.

Both deglycosylation and fusion to immune-stimulating proteins are potentially compatible with antigen presentation in a vesicle and therefore are compatible with the presentation of conformation-dependent epitopes. Additionally, protein recombination could be employed to enable targeting of the ion channel pore, specifically. Researchers have shown that – likely because the ion channel gating region and pore are evolutionarily distinct entities – pore-only constructs can be efficiently produced and reconstituted in functional form in proteoliposomes (149,150). These pore-only ion channels are unlikely to reach the cell surface when expressed in mammalian cells. However, insofar as exosomes seem to passively take up heterologously-expressed protein cargo (141), it is possible that such pore-only constructs could be expressed in the proper orientation and conformation in vesicular particles that can be purified for immunization.

On the other side of this equation, more can be done to address the current limitations inherent to antibodies. In particular, the production of flexible, antibody-based multispecifics could allow the simul-
1.4 Potential strategies to aid the discovery of anti-cation channel mAbs

taneous binding of multiple protein epitopes, which could better reproduce the effects of the many documented cation channel-inhibiting pAbs. Additionally, such multispecifics could replicate the inter-domain/subunit binding of toxins by combining multiple paratopes and adjusting the linker length between the reactive domains until the optimal rigidity is achieved to trigger channel inhibition. Unfortunately, conventional multispecific antibodies typically do not accommodate such flexible design, largely because they require the presence of bulk-conferring assembly domains or a pre-defined level of rigidity to prevent unwanted inter-domain associations. Addressing these issues would enhance the versatility of antibodies and could allow the development of highly selective, cation channel-inhibiting molecules that overcome the limitations of conventional immunization campaigns.

More generally, the scarcity of cation channel-modulating mAbs suggests that we can learn more about the structure and function of cation channels. This includes identifying the mechanisms that govern their expression, assembly, localization and activity. Characterizing functionally relevant protein sites and describing the production of functional, mutated variants could inform immunization strategies, particularly at the level of immunogen design. The work presented in this thesis will focus on these two elements: developing new antibody recombination strategies for the production of stable, flexible multispecific molecules and contributing to the functional characterization of the nociceptive human cation channel, hTRPA1.
Chapter 2

Scope of the thesis
2 Scope of the thesis

The work described in this doctoral thesis was undertaken pursuant to a collaboration agreement established between the Swiss biotechnology company, Numab AG (www.numab.com), and the Cartilage and Engineering Regeneration Lab (CERL) in the Department of Health, Science and Technology (DHEST) at the Eidgenössische Technische Hochschule Zürich (ETHZ), also known as the Swiss Federal Institute of Technology. The purpose of this work was to expand Numab’s technological capacity to target human cation channels with mAbs, specifically those involved in pain signaling. Effectively, this entailed contributing to the development of two of Numab’s proprietary technology platforms: their antibody fragment stabilization technology and their multispecific antibody technology. Also, research was performed to describe some of the structural and functional characteristics of a human cation channel pain target, hTRPA1, particularly with respect to N-glycosylation.

Numab specializes in the discovery and development of therapeutic mAbs, drawing primarily from the rabbit antibody repertoire as a source for antigen-specific CDR sets. Initial experiments performed for this thesis (not described in further detail in ensuing chapters) were performed with the aim of developing inhibiting rabbit mAbs targeting the human cation channels TRPV1 and TRPA1. To do so, standard peptide immunizations were performed by a contract research organization, ligating E3- (for TRPV1) or PG-derived (for TRPV1 and TRPA1) peptides to KLH and performing repeated injections of rabbits (2 animals per immunization arm) for ≥90 days. For the anti-TRPV1 immunization campaign, the exact immunogen described by Klionsky et al. (82) was developed and applied. During the campaigns, the anti-peptide titers in the animal sera were repeatedly monitored by a standard enzyme-linked immunosorbent assay (ELISA).

In all cases, the level of anti-peptide titers was insufficient – despite repeated boosts and extensions of immunization campaigns – to justify the advancement of these programs to B-cell selection stage.
This was especially surprising for the campaign that utilized the immunogen that, in other hands, was able to induce expression of TRPV1-modulating rabbit pAbs (82). However, we have found in many experiments since that development of antigen-specific titers can be remarkably variable on a rabbit-to-rabbit basis. The absence of anti-peptide titers in the TRPA1 immunization campaign was less of a surprise, because at the time, no modulating anti-TRPA1 pAbs or mAbs had been described in the literature.

We then chose to pursue an alternative immunization strategy for the generation of anti-TRPA1 antibodies, performing genetic immunizations in rabbits with a standard recombinant plasmid, pcDNA™5/FRT (Life Technologies), with cDNA encoding full-length hTRPA1 with an N-terminal FLAG tag. A similar injection and analysis schedule was applied. Anti-TRPA1 titers were monitored repeatedly by flow cytometry, but this once again revealed the absence of any specific titers by the end of the campaign. This then inspired attempts to create a novel immunogens for use in the development of pore-targeting anti-TRPA1 pAbs and mAbs.

It occurred to us that using a homotetrareactive chemical cross-linker to ligate the N-termini of four peptides derived from a cation channel’s PG region may produce a molecule that resembles a homotetrameric cation channel pore. Crystal structures of prokaryotic cation channels suggested that the diameter of the outer pore tends to be ~1 nm. This is the approximate reactive diameter of the outer rim of the macrocyclic chemical scaffold, calixarene, which can be modified to generate a homotetrareactive compound. In collaboration with the lab of Dr. Jeffrey Bode at the Department of Organic Chemistry at ETHZ, and utilizing their proprietary technology, we developed a hTRPA1 pore mimetic with a calixarene scaffold that was ultimately used to immunize mice. The tetravalent peptide display elicited a more robust immune response than the peptide on its own but substantially less than a KLH-peptide immunogen. Additionally, even after anti-pore-mimetic pAb affinity-purification, none of the animal sera exhibited any selective recognition of full-length hTRPA1 as determined by flow cytometry analysis.

Separately, several immunization strategies were employed to generate anti-hNa\textsubscript{v}1.7 rabbit antibodies: peptide-KLH immunizations using peptides derived from the sequences along DI E3, DIII E3 and
DIII PG; genetic immunizations; and hNaV1.7-displaying VLPs generated using insect cells. The peptide immunizations produced several selective mAbs, as determined by cell-based ELISA using permeabilized, HEK293 and CHO-K1 cells stably expressing hNaV1.7. However, the genetic immunizations failed to elicit a robust immune response, and the sera from VLP-immunized rabbits displayed considerable reactivity with non-hNaV1.7 protein expressed on the surface of VLPs, ultimately revealing no discernible hNaV1.7-specific antibody response.

Further development and epitope-mapping of the anti-hNaV1.7 mAbs raised by peptide immunization revealed that their paratopes collectively recognize four epitopes along the aforementioned protein regions. We determined that none of the antibodies were able to inhibit hNaV1.7 activation, whether the channel was induced by the selective agonist, veratridine (in fluorometric assays), or by electrode-induced cell depolarization (in automated and manual whole-cell patch-clamp). We also attempted applying antibody-drug conjugates (ADCs), fusing an E2-reactive NaV1.7 peptide inhibitor from centipede venom, Ssm6a (89), to the N-terminus of the H-chain of two of our antibodies (via a peptide linker) targeting the DI E3 and DIII PG regions, respectively. The ADCs also failed to inhibit hNaV1.7 in all functional experiments, although we were also unable to show that the peptide toxin, in isolation, could reduce maximal activation of hNaV1.7, despite applying similar methods to those described by the researchers who initially purified and characterized peptide (89).

These attempts to produce inhibiting, anti-cation channel mAbs could have failed for many of the reasons enumerated in the previous chapter. However, they also are a testament to the difficulty of raising such antibodies, particularly in light of the diversity of approaches we applied to targeting hNaV1.7 and TRPA1. This resulted in a pivot of the focus of the work for this thesis towards developing Numab’s antibody stabilization and recombination technology and generating potentially immunogenic TRPA1 variants whose expression patterns and functionality were also of interest to the research community. In other words, the focus shifted to the other side of the equation: enhancing the versatility of antibody-based therapeutics and thereby accommodating the production of more effective cation channel-modulators; and
performing original research characterizing TRPA1 structure and functionality, which also required production of non-glycosylated cation channel variants for potential use in immunization campaigns.

While the task of generating full-length, inhibiting mAbs against complex multispanners presents considerable challenges that bear addressing, it is equally critical that we fully develop the versatility of antibodies, which could potentially address the limitations inherent to current molecules developed for such applications. Antibody fragments that retain the full antigen-binding region, in particular, are a useful alternative to full-length IgGs. An immediate benefit to such fragments in the context of ion channel modulation as an analgesic strategy is that they omit the immune-effector domain of the full-length antibody. When an IgG is bound to a membrane-anchored antigen, the Fc region can recruit natural killer (NK) cells to induce antibody-dependent cell-mediated cytotoxicity (ADCC) of the antigen-bearing cell. If antibodies are targeting nociceptive ion channels expressed in the membrane of nociceptive neurons, ADCC could have devastating and enduring effects.

Additionally, antibody fragments are more broadly distributed in vivo compared to bulkier, full-length IgGs. While their smaller size also means they have a shorter half-life, perhaps the greatest benefit to antibody fragments is that they provide a convenient building block for the generation of multispecific molecules exhibiting multiple, antigen-specific CDR sets. This means that a single molecule containing multiple antibody fragments can accommodate the inclusion of domains that, for example, promote a lengthier serum half-life by binding to preservative serum proteins. Multispecificity could also be useful to elicit antibody-based inhibition of ion channels by binding multiple protein regions simultaneously, possibly exploiting the same mechanism of action that appears to make pAbs more consistently effective than mAbs in reducing ion channel activity. However, the length of the biological linker between reactive domains on such an ion channel-inhibiting multispecific antibody would ideally be of adjustable length, so as to accommodate the screening of several molecules with disparate flexibilities in order to promote multivalent channel-binding and, ultimately, the desired effect.
Unfortunately, the stability and production of antibody fragments is often compromised by the absence of IgG constant domains, which presents obstacles to the development of new multispecific designs. Numab has developed a broadly applicable, human scaffold for the humanization and/or stabilization of antibody fragments. In turn, they designed a new multispecific antibody concept that theoretically facilitates the production and screening of single molecules – consisting of heterodimerized polypeptide chains – that are capable of binding six different antigen targets with high affinity. Moreover, the design of this multispecific theoretically accommodates adjustable linker lengths on each subunit, providing a measure of flexibility that could allow the development of uniquely functional molecules from the same collection of reactive domains.

Chapter 3 of this thesis will focus on the development and characterization of Numab’s antibody fragment-stabilizing technology as well as its proprietary multispecific antibody format: the MATCH. The work described initially consists of the cloning and production of single-chain Fv (scFv) fragments with and without Numab’s stability-enhancing technology, which entails the use of an otherwise-conventional human antibody framework with a novel adjustment – the complete replacement of a framework region on the L chain with the analogous region from a human L chain originating from a different gene family. The novel framework was used to humanize both mouse and rabbit CDR sets. These scFvs were expressed in, and purified from, E. coli cells. They were tested for initial purity, and their antigen affinities were determined. Finally, their resistance to temperature-induced denaturation as well as their propensity to oligomerize during storage and/or concentration was analyzed. This analysis ultimately revealed that Numab’s technology was highly effective in stabilizing antibody scFvs without compromising reactivity.

The next component of the work required the cloning and production of four tetraspecific, proof-of-concept MATCH proteins. The CDR sets in the MATCH proteins were all of rabbit origin. The subunits forming the MATCH dimer were co-expressed in mammalian cell supernatants and purified. They were then analyzed in a fashion similar to the scFvs. In addition to the traditional, antigen-by-antigen af-
finity determinations, MATCH proteins were also screened for their ability to simultaneously bind all four of their target antigens. Importantly, the process by which MATCH subunits assemble could conceivably result in misassembled, inactive dimers of approximately the same size as functional MATCH proteins. For this reason, a series of experiments were carried out to determine to what extent this occurs. These experiments ultimately provided considerable evidence that functional assembly of MATCH proteins is highly favored, and such functional proteins were very stable and could be produced to a high purity with yields comparable to full-length IgGs.

Chapter 4 of this thesis focuses on the work performed on hTRPA1, which entailed characterizing the role of N-linked glycosylation of the protein. Most cation channels possess N-linked glycosylation consensus sequences, but the physical presence and function of N-glycans on many such channels has not been experimentally confirmed and characterized. To confirm N-glycosylation of hTRPA1, DNA encoding the wild-type (WT) protein as well as variants in which one or both of the two N-glycosylation sites were removed (by Asn-Gln mutations) was first amplified and cloned into expression plasmids. These plasmids were then used to induce heterologous expression of the various TRPA1 glycoforms in HEK293 cells. This enabled analysis of these proteins by Western Blot (WB), using different sample preparation and detection strategies to confirm the presence of glycans. This revealed that both sites were indeed modified with an N-glycan – the first experimental confirmation of this post-translational modification.

It was also critical to determine whether N-glycosylation affects the localization of proteins. This required the selective purification of hTRPA1 glycoforms located at the cell surface. Cell-surface biotinylations were performed, purifying membrane fractions with streptavidin beads and subjecting each sample to quantitative analysis by WB. These experiments revealed that the absence of one or both of the glycosylation sites does not prevent protein trafficking to the cell surface. Additionally, the results indicate that the removal of one glycosylation site reduces cell-surface expression of the ion channel, while the absence of the other enhances surface expression slightly.
Finally, the functional significance of the N-glycans was analyzed by producing glycoform-specific dose-response curves in fluorimetric and electrophysiological functional assays. These assays were performed using various hTRPA1-specific agonists and under different temperatures. This revealed that hTRPA1 is functional in the absence of one or both N-glycans, but one mutation causes significant loss of sensitivity to all agonists tested. Intriguingly, the impact of this mutation seemed to be affected by the temperature at which the assays were conducted, pointing to a potential cooperativity between temperature and agonist stimulation.

The thesis then concludes with an Outlook chapter (Chapter 5), focusing on a potential path forward for Numab’s multispecific antibody program and further experiments to be carried out to enhance Numab’s capacity to target multispanners with mAbs. This will include a proposal for further experiments to analyze the MATCH technology, including efforts to determine the extent to which efficient MATCH assembly is dependent on the CDR conformations of the dimer-forming Fvs. That is to say, it is critical to determine whether the remarkably efficient production observed with the proof-of-concept molecules can be reasonably anticipated with other combinations of CDR sets. Additionally, the Outlook chapter contains a proposal for an immunogen design for use in raising anti-cation channel, pore-occluding mAbs in rabbits as well as a potential multispecific design for blocking a cation channel target.
Chapter 3

Novel multispecific heterodimeric antibody format allowing modular assembly of variable domain fragments

This chapter, in manuscript form, has been submitted for publication in “mAbs” and is currently under review (as of September 2016). Timothy James Egan is first author on the manuscript, having written it and having designed, produced and purified scFvs and MATCH proteins, performed SDS-PAGE and SE-HPLC analysis, and established and performed the complexing analysis. Dania Diem and Richard Weldon assisted with several experiments.
3 The MATCH: a novel multispecific antibody format

Immunoglobulin G (IgG) recombination methods are rapidly growing in sophistication (151–160), and this in turn is allowing the development of diverse multispecific antibody formats (27,161–167). Relative to monospecific antibody-based therapeutics, multispecific antibodies enable the realization of entirely novel, potentially more effective and safer mechanisms of action. Furthermore, monotherapies that concomitantly engage multiple targets have been shown to synergistically interfere with signaling pathways in a manner superior to combination therapies consisting of monospecific antibodies targeting the same collection of antigens (165,168,169). Additionally, multispecific antibody therapeutics can site-specifically recruit effector cells (164,170) and can be endowed with enhanced pharmacokinetic properties such as prolonged serum half-life (171), improved tissue distribution (93–95), and local enrichment (96). Indeed, the therapeutic value of multispecifics has already been validated with the approval of the bispecific antibody drugs such as blinatumomab (Amgen) (164) and catumaxomab (Trion) (163).

Despite considerable progress in this area, the production and clinical development of multispecific antibody formats is frequently beset with complications, owing to methods of subunit assembly (172–177) and/or the stability of the recombinant antibodies, or fragments thereof (157,178–180), contained in a given format. After all, an antibody-based multispecific is ultimately only as stable as its least stable domain, and protein recombination techniques – such as those required to generate human or humanized antibody fragments – often result in compromised stability (157,179). This instability, in turn, can promote the formation of biological aggregates (178–182).

Aggregate formation imposes untoward production costs by necessitating additional purification steps and reducing potential yields (183,184). Also, given the increasing demand for antibody therapeutics that can be self-administered, either by subcutaneous injection or via implantable sustained-release devices, and given that aggregation propensity would be exacerbated at the concentrations required for self-administration, the immunogenicity and unpredictable pharmacodynamics of antibody aggregates
pose serious health risks to prospective patients (179,185,186). Particularly upon rapid infusion, aggregated antibodies are known to trigger adverse effects, such as cytokine storms, through clustering of their fragment crystallizable (Fc) domains (187,188), an issue that could be especially problematic in the context of multispecific formats bearing an immune-effector function (e.g., an anti-CD3ε binding domain) (189,190).

It is especially critical that antibody-fragment production and formulation hurdles are overcome insofar as they impede progress towards the development of more ambitious multispecific formats such as heterodimers, particularly those exhibiting specificities for three or more antigens. Heterodimeric multispecific formats, wherein one or both of the unpaired subunits have at least one functional variable domain, would allow efficient screening for cooperative binding-domain specificities and affinities through simple permutation, and such binding-profile optimization in the final format could be critical to eliciting the molecule’s intended effects in vivo (191,192). Comparable screening efforts applied to single-chain or homomultimeric multispecific formats with the same protein-targeting profile would require considerably more cloning steps and thereby drastically increase the time and effort needed to generate them.

Due to their versatility, considerable research has focused on the development of heterodimeric multispecific antibody formats (172–174,176,177). Unfortunately, most conventional heterodimerization techniques cannot fully avoid production obstacles – such as undesired subunit-associations – without introducing amino-acid substitutions into frameworks, and they often require the inclusion of bulky dimerization domains that inevitably reduce tissue penetration (167,172). Collectively, these lingering issues minimize flexibility in the design and engineering of multispecific antibodies, leaving the tremendous potential of this class of therapeutics largely untapped.

Presently, one of the lowest-molecular-weight entities known to dependably retain the full immunoreactivity of a parental antibody is the fragment variable (Fv) (Fig. 3.1) (151). Formed by the assembly of the antibody variable light (VL) and variable heavy (VH) domains, the ~25 kD IgG Fv domain possesses the antibody’s full antigen-binding site. Therefore, designing a multi-subunit, antibody-based multispecif-
The MATCH: a novel multispecific antibody format

ic whose assembly is exclusively driven by association of human $V_L$ and $V_H$ domains would enable the production of modular multispecific antibodies of minimal molecular weight and therefore maximize the potential for tissue penetration. In contrast to formats containing non-human dimerization domains, such a design would benefit from existing immune-tolerance towards its fully human framework sequences. Alas, the frequently poor stability and aggregation propensity of Fvs (193) oftentimes is a limiting factor for the widespread development of heterodimeric multispecific therapeutics whose assembly depends solely on variable domain associations.

Here, we present the development of a novel, broadly-applicable human single-chain Fv (scFv) framework with an exquisite stability profile suitable for the humanization and/or stabilization of antibody

Figure 3.1 | Example of an IgG Fv domain

Cartoon and surface models obtained from the crystal structure of a hu4D5-8 Fv variant (PDB ID: 1FVC) assembled by heterodimerization of the VL (left half) and VH (right half) domains. The murine CDR sets are shown in blue, the human Fv acceptor framework is shown in gray/red and the location of VL FW IV is shown in red.
variable domains. Specifically, the use of a modified human V₅₁-V₅₃ consensus framework in which framework region IV (FW IV) (Fig. 3.1) of the scFv V₅ domain has been replaced with a germ-line sequence from the corresponding position of a human V₅ domain results in scFvs with greatly reduced aggregation propensity and improved storage stability. Critically, these stability enhancements do not come at the expense of antigen affinity.

We were then able to apply our Fv-stabilizing technology to develop a class of novel, heterodimeric multispecifics whose assembly is driven exclusively by variable domain associations. We termed these formats, multispecific antibody-based therapeutics by cognate heterodimerization, or “MATCH” formats. In addition to the limited aggregation propensity of MATCH formats, the rational structure of the MATCH subunits composing the heterodimeric protein largely ensures the absence of undesired intra-subunit associations, allowing greater flexibility in protein design. The results below demonstrate that we have successfully established methods to develop large quantities of stable, non-aggregating tetraspecific antibodies with readily customizable binding profiles and without many of the limitations that plague conventional formats. Moving forward, these formats could support the development of uniquely effective monotherapies for a variety of indications for which there is currently an unmet clinical need and may open up access to several drug targets that are not reliably accessible to biologics under existing technology.
3.1 Results

3.1.1 Full framework domain substitution reduces the aggregation propensity and improves thermal stability of a hu4D5-8 (trastuzumab) scFv variant.

The production and use of human or humanized antibody fragments, particularly scFv fragments, is increasingly common (151,157,183,193,194). Unfortunately, scFv fragments typically do not exhibit the stability profile of full-length IgGs, an issue that researchers have endeavored to remedy via stability-enhancing framework mutations, with some success (157,183,193,195–199). However, these departures from fully human sequences again raise concerns about the potential immunogenicity of these scFvs, and some common mutations, such as those involving the introduction of Cys residues, can render proteins more aggregation-prone (200,201). The goal remains to establish generically-applicable technology to stabilize scFv fragments while ensuring unimpeded functionality.

In the work described by Ewert et al. (202), researchers sought to characterize the stability profiles of several human V\_L and V\_H consensus domains and frameworks, using them to humanize the murine CDR sets from the humanized anti-p185\textsuperscript{HER2} antibody 4D5-8 (hu4D5-8; trastuzumab) (203). Their research uncovered a particularly intriguing finding – that while V\text{\_\lambda}-consensus domains generated the least stable and most aggregation-prone V\_L domains when produced in isolation, upon pairing with a cognate V\text{\_\lambda}-3-consensus domain, they formed the most stable scFvs. Moreover, the resistance of V\_\lambda-V\_H scFvs to denaturant-induced unfolding was in fact greater than that of their respective domains in isolation, suggesting a particularly strong V\_L-V\_H domain interface. However, their analysis of isolated V\_\lambda domains required the replacement of the third CDR loop (V\_L CDR3) of the V\_\kappa hu4D5-8 CDR set with a human-consensus, \lambda-like V\_L CDR3 due to the incompatibility of V\_\lambda-consensus frameworks with the \Omega-loop conformation commonly present at this position in V\_\kappa CDR sets. Furthermore, the high stability of their V\_\lambda-V\_H frameworks was conditional upon the replacement of the hu4D5-8 V\_L CDR3 with the unnatural, \lambda-like
V\_L CDR3. Use of V\_\kappa-V\_H frameworks for CDR grafting would therefore be inadvisable, as one could reasonably anticipate their incompatibility with \sim 70\% of V\_\kappa CDR sets (202).

Figure 3.2 | Putative structural change of V\_L FW IV in the hu4D5-8 Fv variant

The image on the right was obtained by overlaying the crystal structures of the Fab and Fv variants of hu4D5-8, and it reveals an apparently unnatural solvent exposure of I147 and skewing of the C-terminal residues in the Fv variant relative to the constant domain-bearing Fab structure. The image on the left is intended to clarify what is visualized in the image on the right, including the particular Fv framework domains being displayed and where the solvent and protein core exist relative to the amino acids in view.

However, we hypothesized that a V\_\kappa-V\_H Fv framework could profit from the stability-enhancing features of V\_\lambda domains without weakening its compatibility with a V\_\kappa CDR set or enhancing its immunogenicity – the latter being a risk posed by single-point mutations that would render the affected framework
region non-human. Specifically, replacing a full \( V_\kappa \)-consensus framework region with the corresponding region of a \( V_\lambda \) germ-line sequence – i.e., a continuous, fully human stretch flanked by 1-2 CDR loops – could theoretically induce the same strengthening of the \( V_L \)-\( V_H \) domain interface and enhance Fv stability without producing the T-cell epitopes that might arise from single-point mutations. Interestingly, the solubility of the amino acids clustered at the C-terminal end of \( V_L \) FW IV diverge considerably between the respective \( V_L \) gene families, with \( V_\kappa \)-consensus domains containing charged residues at three positions where the corresponding side-chains on \( V_\lambda \) domains are uncharged (202). Furthermore, it was suggested by Ewert et al. that the less-hydrophilic \( \lambda \)-consensus \( V_L \) FW IV was partly responsible for the especially low yields of monomeric \( V_\lambda \) domains. The authors noted that this production issue did not appear to affect the highly stable \( V_\lambda \)-\( V_H \) scFvs, perhaps suggesting improved packing in \( V_\lambda \)-bearing Fv formats. Additionally, \( V_L \) FW IV was considered an attractive location for our proposed substitution as it exhibits considerable structural variability across antibody fragments, and its residues appear to participate strongly in the interface between antibody \( V_L \) and constant domains (constant domains being notably absent from Fvs) while being only minimally involved in antigen-binding (204).

To investigate further, we overlaid crystal structures, retrieved from the RCSB Protein Data Bank (PDB), of fragment antigen-binding (Fab) (PDB ID: 1FVD) and Fv (PDB ID: 1FVC) variants of hu4D5-8 (203) to determine whether this region might assume an unnatural conformation in the absence of Fab constant domains. Indeed, we observed a considerable structural change in \( V_L \) FW IV upon the transition from a Fab to a Fv format (Fig. 3.2). Specifically, the absence of Fab constant domains appears to result in the solvent-exposure of a hydrophobic Ile residue at position 147 in \( V_L \) FW IV as well as a skewing of the downstream hydrophilic residues (NB: for the AHo antibody sequence numbering scheme used in this paper, please refer to the work by Honegger et al (205) as well as the sequence in (Fig. 3.4). In light of these observations, we produced and compared two hu4D5-8 scFv variants, one with (Tras-scFv-\( \lambda \)cap) and one without (Tras-scFv) a \( \lambda \) germ-line sequence (FGGGTKLTVLG) substitution at \( V_L \) FW IV – a substitution referred to, here onward, as the “\( \lambda \)cap.” Each scFv was designed with a N-term–\( V_L \)–peptide linker–\( V_H \)–C-term orientation (peptide linker: (G,S)_4).
3.1 Results

We successfully produced both scFvs (Fig. 3.3A-B), and the protein samples with maximal scFv-monomer content (% of total protein content) possessed 97.4% and 73.3% Tras-scFv-\(\lambda\)cap monomer (0.648 mg/mL) and Tras-scFv monomer (0.780 mg/mL), respectively, providing an early indication that the \(\lambda\)cap renders the scFv less prone to aggregation (Fig. 3.3B). Meanwhile, both antibodies exhibited comparable affinities for recombinant human HER2 (Table 1), again suggesting that the \(\lambda\)capped framework can be applied to humanize even those CDR sets containing the characteristic V\(_k\) \(\Omega\)-loop conformation along V\(_L\) CDR3. Additionally, differential scanning fluorimetry (DSF) assays revealed a higher melting temperature \((n=2)\) of Tras-scFv-\(\lambda\)cap (mean Tm°C ± SD: 71.2 ± 0.1) relative to Tras-scFv (69.9 ± 0.1), indicating a modest increase in thermal stability of \(\lambda\)capped protein (Fig. 3.3C).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>HER2-binding kinetics of scFvs</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(k_{on}(1/\text{M} \cdot \text{s}))</td>
</tr>
<tr>
<td>Tras-scFv</td>
<td>3.81 (\times) 10(^5)</td>
</tr>
<tr>
<td>Tras-scFv-(\lambda)cap</td>
<td>2.70 (\times) 10(^5)</td>
</tr>
</tbody>
</table>

Finally, insofar as oligomerization of scFvs is largely concentration-dependent, we generated scFv aliquots at escalating protein concentrations by centrifuging the protein samples through filters with a 5 kD molecular weight cut-off (MWCO). We then analyzed the samples to determine whether the scFvs exhibited a disparate propensity towards concentration-induced aggregation. The oligomer content in Tras-scFv samples was considerably higher than that of Tras-scFv-\(\lambda\)cap at all concentrations (Fig. 3.3D), plateauing at \(\sim\)10 mg/mL with an oligomer content of \(\sim\)55-60%. The oligomer content of Tras-scFv-\(\lambda\)cap, meanwhile, appears to possess a monomer content of \(<\)25% at \(\sim\)10 mg/mL, and the percentage of oligomer in samples did not exceed \(\sim\)46%, even at a concentration of \(\sim\)30 mg/mL.
3.1.2 λcap substitution enhances the stability of a human $V_{\kappa}1-V_{\lambda}3$ consensus scFv acceptor framework used to humanize an anti-TNFα, rabbit CDR set.

While our initial data supported a considerable stabilizing effect of the λcap in the context of hu4D5-8 scFv variant, it is important to consider that while the framework in the hu4D5-8 Fv variant is highly homologous to the $V_{\kappa}1-V_{\lambda}3$ consensus (204), it differs from a full human consensus by 6 residues.
3.1 Results

Figure 3.4 | Amino-acid sequences of the \( \kappa_1 - \kappa_3 \)-consensus frameworks

These frameworks were used to humanize the rabbit antibodies, aTNFα-scFv and aTNFα-scFv-λcap, respectively. The \( \kappa_3 \) FW IV is highlighted in yellow, and the non-identical (relative to \( \kappa_3 \) FW IV) residues are highlighted in red. The amino acids are numbered according to the AhHo numbering scheme introduced by Honegger & Plückthun (205).

\( \left( \kappa_1: G84R; \kappa_3: S55A, R81A, N83T, L88A, A106S \right) \). Also, while murine CDR sets (such as those displayed by hu4D5-8) are more commonly observed in therapeutic and clinical applications at present, rabbit CDR sets are increasingly considered more desirable as they exhibit greater sequence diversity (206), facilitating the selection of high-affinity antibodies. With this in mind, we sought to evaluate the use of the λcap in a full \( \kappa_1 - \kappa_3 \) consensus framework used to humanize rabbit anti-TNFα CDR sets. To that end, we again designed two scFvs with (aTNFα-scFv-λcap) and without (aTNFα-scFv) the λcap (Fig. 3.4).

Table 2 | TNFα-binding kinetics of scFvs

<table>
<thead>
<tr>
<th></th>
<th>( k_{\text{on}} ) (1/M·s)</th>
<th>( k_{\text{off}} ) (1/s)</th>
<th>( K_d ) (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aTNFα-scFv</td>
<td>3.95 ( \times 10^5 )</td>
<td>1.05 ( \times 10^4 )</td>
<td>2.65 ( \times 10^{10} )</td>
</tr>
<tr>
<td>aTNFα-scFv-λcap</td>
<td>4.19 ( \times 10^5 )</td>
<td>9.41 ( \times 10^5 )</td>
<td>2.25 ( \times 10^{10} )</td>
</tr>
</tbody>
</table>

As confirmed by SDS-PAGE and SE-HPLC, we successfully purified both scFvs (Fig. 3.5A-B) to comparable quality, and SPR analysis (Fig. 3.5C) confirmed the immune-reactivity of the CDR, with each molecule exhibiting comparable affinities for human TNFα (Table 2). We again observed a consistent improvement in the melting temperature \( (n=3) \) of the λcapped scFv relative to its full-consensus counter-
part (mean Tm°C ± SD: aTNFα-scFv-λcap = 63.5 ± 0.1; aTNFα-scFv = 62.5 ± 0.2) in DSF assays (Fig. 3.6A).

![Image of SDS-PAGE gel](image1)

**Figure 3.5 | Successful production and purification of reactive scFvs**

(A) Coomassie-stained SDS-PAGE gel reveals successful purification of both scFvs at the two pre-pared concentrations. (B) SE-HPLC chromatograms of 1 mg/mL samples of each scFv, revealing comparable sample-quality at day 0 of storage-stability studies; peak retention times: 8.13 min (scFv), 7.39 (dimer) (note: the peak at ~10 min corresponds to the buffer matrix). (C) Sensograms generated by SPR analysis by injecting 0.70 – 90 nM of each respective scFv over channels on a sensor chip displaying immobilized, recombinant human TNFα.

Moreover, aggregation propensity was substantially reduced by the λcap substitution, as evidenced by the considerable differences in the monomer content of protein samples we observed after concentration and during storage (Fig. 3.6B-C). Two-week storage at 37°C resulted in monomer losses of 0.03% ± 0.05% and 16.7% ± 0.1% in 1 mg/mL samples (n=3) of aTNFα-scFv-λcap and aTNFα-scFv, starting from monomer contents (at day 0) of 98.0% and 93.5%, respectively. After 2-week storage, 10 mg/mL samples of aTNFα-scFv-λcap were composed of 83.7% ± 0.1% (37°C; n=3) or 93.2% (4°C; n=1) monomeric pro-
tein, whereas comparator aTNFα-scFv samples consisted of 35.8% ± 0.3% (37°C; \( n = 3 \)) or 35.8% (4°C; \( n = 1 \)) scFv-monomer. These results illustrate the significant production advantages conferred by this fully human substitution. Additionally, this enhanced protein stability should translate into a reduced risk of immunogenicity and more dependable reactivity in vivo, pointing to considerable therapeutic advantages (182,196,207).

Figure 3.6 | λcap improves thermal- and storage-stability of rabbit antibodies

(A) Thermal unfolding curves of each scFv variant fitted with the Boltzmann equation to data points (\( n = 3 \); mean ± SD) generated in a DSF assay. (B) Maintenance of scFv monomer content (% of total protein content) during 2-week storage at 37°C; data points (\( n = 3 \)) reflect mean ± SD. (C) SE-HPLC chromatograms of exemplary protein samples at the indicated concentrations and after storage at the indicated durations/temperatures; peak retention times: 8.14 min (scFv), 7.40 min (dimer).
3.1.3 Production of functional and stable MATCH formats

We were able to consistently apply λcap technology to V\textsubscript{κ}1-V\textsubscript{H}3-consensus-derived frameworks to produce functional and stable, humanized rabbit scFvs and scDbs that show limited aggregation propensity (data not shown). The finding that λcapping V\textsubscript{κ}1-V\textsubscript{H}3 scFvs attenuates protein aggregation during sample storage suggested to us that this novel, chimeric framework strengthens the V\textsubscript{L}-V\textsubscript{H} domain interface, while the preservation of antigen-affinities after humanization of V\textsubscript{κ}-bearing IgGs confirms the compatibility of this λ-derived substitution with κ-like CDR sets. This putatively enhanced domain-interface stability inspired us to design a class of heterodimeric, tetraspecific antibody derivatives whose assembly is exclusively driven by interactions between cognate variable domains on each protein subunit. Such a design would obviate the need for non-human (172,174,177) and/or otherwise functionally-irrelevant (176) dimerization domains, which would normally confer distribution-impairing bulk and, possibly, immunogenicity.

Critically, these multispecifics must evince a stability profile that supports generation of homogeneous product that can endure long-term storage and possesses a limited propensity to aggregate – features that only highly-stable V\textsubscript{L}-V\textsubscript{H} interfacing can likely accommodate. Our MATCH concept, as depicted in Fig. 3.7A-B, requires that the dimer subunits consist of a core of two split variable domain pairs, each respective subunit possessing either two V\textsubscript{L} domains or two V\textsubscript{H} domains positioned in tandem, thereby driving heterodimerization of the two protein chains. The dimer-forming tandem variable domains on the respective MATCH chains can be organized in either the same (parallel, Fig. 3.7A) or reverse (antiparallel, Fig. 3.7B) N-term–C-term orientation as their counterpart chain, with traditional co-expression in mammalian cells providing adequate conditions for self-assembly (see Table 3, for more details).
Figure 3.7 | MATCH format concept and MATCH protein designs

(A) Orientation of the MATCH chains in the parallel MATCH format. The two-sided arrows indicate the variable domains whose interaction would represent a cognate association. In the context of MATCH proteins, the term “parallel” indicates that the split, heterodimer-forming variable domains on each chain are organized in the same N-term–C-term order as their cognate variable domains on the complementary MATCH chain. (B) Orientation of the MATCH chains in the antiparallel MATCH format. The two-sided arrows indicate the variable domains whose interaction would represent a cognate association. In the context of MATCH proteins, the term “antiparallel” indicates that the split, heterodimer-forming variable domains on each chain are organized in the opposite N-term–C-term order as their cognate variable domains on the complementary MATCH chain. (C) General designs of the MATCH proteins developed for the research described in this paper.
The MATCH: a novel multispecific antibody format

Table 3 | Design of MATCH protein chains

<table>
<thead>
<tr>
<th>Heterodimer</th>
<th>MATCH chain</th>
<th>N-term to C-term (left-to-right) order of variable domains and peptide linkers in MATCH chains</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMATCH</td>
<td>1</td>
<td>anti-IL-23R Vκ, (G₈S)₉, anti-IL-23R Vλ, (G₈S)₉, anti-TNFα Vκ, (G₈S)₉, anti-CD3ε Vλ, [NA]</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>anti-IL-5R Vκ, (G₈S)₉, anti-IL-5R Vλ, (G₈S)₉, anti-TNFα Vκ, (G₈S)₉, anti-CD3ε Vλ, [NA]</td>
</tr>
<tr>
<td>apMATCH</td>
<td>1</td>
<td>anti-IL-23R Vκ, (G₈S)₉, anti-IL-23R Vλ, (G₈S)₉, anti-TNFα Vκ, (G₈S)₉, anti-CD3ε Vλ, [NA]</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>anti-IL-5R Vκ, (G₈S)₉, anti-IL-5R Vλ, (G₈S)₉, anti-TNFα Vκ, (G₈S)₉, anti-CD3ε Vλ, [NA]</td>
</tr>
<tr>
<td>apMATCH-diS</td>
<td>1</td>
<td>anti-IL-23R Vκ, (G₈S)₉, anti-IL-23R Vλ, (G₈S)₉, anti-TNFα Vκ, (G₈S)₉, anti-CD3ε Vλ, GSC</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>anti-IL-5R Vκ, (G₈S)₉, anti-IL-5R Vλ, (G₈S)₉, anti-TNFα Vκ, (G₈S)₉, anti-CD3ε Vλ, GSC</td>
</tr>
</tbody>
</table>

Combining a selection of four rabbit antibodies humanized with a λ-capped Vₖ1-V₇₃ Vλ scaffold, we applied our MATCH concept to generate both parallel (pMATCH) and antiparallel (apMATCH) tetra-specific heterodimers with therapeutically-nonsensical specificities for human TNFα, CD3ε, IL-5R, and IL-23R (Fig. 3.7C). In pMATCH, we included a lengthier, 15 amino-acid linker (compared to 6-8 amino acids in other MATCH proteins) between the heterodimer-forming, split variable domains on each MATCH chain in order to demonstrate the design flexibility these formats can accommodate. Additionally, we surmised that the antiparallel MATCH formats would be amenable to the introduction of a disulfide bridge by simple addition of a Gly-Ser-Cys sequence at the C-terminal end of each MATCH chain. This was based on our evaluation of juxtaposed scFv models which suggested the close approximation of the MATCH subunits’ C-termini in the heterodimer in our antiparallel (but not parallel) formats. This additionally provided us with a convenient means to visualize and confirm dimerization, simply by staining

Table 4 | Production yields of MATCH protein variants and IgG

<table>
<thead>
<tr>
<th>Protein</th>
<th>Weight (Da)</th>
<th>Yield (mg) per L expression volume</th>
<th>Molar equivalents</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMATCH</td>
<td>106'822</td>
<td>3.4</td>
<td>0.18</td>
</tr>
<tr>
<td>apMATCH</td>
<td>105'219</td>
<td>12.1</td>
<td>0.65</td>
</tr>
<tr>
<td>apMATCH-diS</td>
<td>105'713</td>
<td>17.6</td>
<td>0.94</td>
</tr>
<tr>
<td>anti-TNFα rIgG</td>
<td>147'809</td>
<td>26.2</td>
<td>1.00</td>
</tr>
</tbody>
</table>
3.1 Results

SDS-PAGE gels displaying MATCH proteins that were denatured under non-reducing conditions. Specifically for this purpose, we generated apMATCH-diS (Fig. 3.7C), whose MATCH chains are identical to those of apMATCH but with the added disulfide bridge-forming feature.

The successful expression of pMATCH, apMATCH and apMATCH-diS protein chains was confirmed by SDS-PAGE and SE-HPLC analysis (Fig. 3.8A & D). Using supernatants from transiently-transfected mammalian cells, we were able to produce between 3.4 and 17.6 mg per L expression volume – after protein L-affinity chromatography, size-exclusion chromatography and dialysis – of each MATCH

Figure 3.8 | Expression and purification of stable MATCH proteins

(A) Coomassie-stained SDS-PAGE gel reveals successful co-expression of each ~50 kD MATCH chain. Additionally, non-reduced samples of the MATCH variant with additional C-terminal cysteines, ap-MATCH-diS, contain a protein near the expected gel position (~106 kD) of heterodimeric MATCH protein. (B) Thermal unfolding curves of each MATCH protein fitted with the Boltzmann equation to data points (n=2; mean ± SD) generated in a DSF assay. (C) Each MATCH protein (n=1) largely retains its dimer content (% total protein content) when stored at 1 mg/mL at 37°C, even for a period of 28 days. (D) SE-HPLC chromatograms of 1 mg/mL samples of MATCH proteins at day 0 and day 28 of storage at 37°C; peak retention times: ~7.3 min (MATCH dimer), ~6.6 min (high-molecular-weight impurity), ~8.4 min (low-molecular-weight impurity).
protein, which compared favorably to a parental, full-length rabbit anti-TNFα IgG expressed and purified via similar methods (Table 4). DSF assays (n=2 each) revealed average MATCH protein melting temperatures (mean Tm°C ± SD: pMATCH = 68.5 ± 0.3; apMATCH = 68.0 ± 0.1; apMATCH-diS = 69.4 ± 0.0) that suggested good protein stability (Fig. 3.8B). Additionally, analysis of gels containing non-reduced apMATCH-diS samples revealed a discrete band near the estimated weight of the heterodimer (~106 kDa), supporting inter-MATCH chain associations (Fig. 3.8A). Efficient MATCH chain dimerization was further demonstrated by the remarkable homogeneity of the protein content in protein L-purified samples analyzed by SE-HPLC (Fig. 3.8D). This homogeneity was largely retained even after storage of samples at 37°C for 28 days (Fig. 3.8C-D).

Table 5 | Antigen-binding kinetics of MATCH proteins

The kinetics for scFv (PRO205 and PRO128) and scDb (PRO187) variants of the parental antibodies that provided the CDR set sequences for the MATCH proteins are provided as a comparison.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>TNFα</th>
<th>CD3ε</th>
<th>IL-23R</th>
<th>IL-5R</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{on}$ ($1/M\cdot s$)</td>
<td>$k_{off}$ ($1/s$)</td>
<td>$K_D$ (nM)</td>
<td>$k_{on}$ ($1/M\cdot s$)</td>
</tr>
<tr>
<td>pMATCH</td>
<td>2.23 x 10⁹</td>
<td>6.64 x 10⁻⁹</td>
<td>3.07 x 10⁻⁹</td>
<td>1.19 x 10⁹</td>
</tr>
<tr>
<td>apMATCH</td>
<td>3.59 x 10⁹</td>
<td>7.63 x 10⁻⁹</td>
<td>2.14 x 10⁻⁹</td>
<td>3.18 x 10⁹</td>
</tr>
<tr>
<td>apMATCH-diS</td>
<td>5.13 x 10⁹</td>
<td>1.27 x 10⁻⁹</td>
<td>3.61 x 10⁻⁹</td>
<td>3.18 x 10⁹</td>
</tr>
<tr>
<td>PRO205</td>
<td>5.51 x 10⁹</td>
<td>1.44 x 10⁻⁹</td>
<td>2.81 x 10⁻⁹</td>
<td>5.59 x 10⁹</td>
</tr>
<tr>
<td>PRO187</td>
<td>2.44 x 10⁹</td>
<td>2.24 x 10⁻⁹</td>
<td>9.21 x 10⁻⁹</td>
<td>7.10 x 10⁹</td>
</tr>
</tbody>
</table>

Strikingly, SPR analysis revealed that the affinities of the CDRs within the MATCH formats for each immobilized antigen closely resemble those of their respective donor scFvs and scDb, including the variable domains positioned in the dimer-forming core, whose binding activity we suspected – and later demonstrated (described below) – is dependent upon cognate dimerization (i.e., those displayed by the dimer-forming Fvs targeting TNFα and CD3ε, respectively) (Table 5). Additionally, each of the three tetraspecifics was capable of binding all four target antigens simultaneously, seemingly irrespective of the order of antigen-encounter, as demonstrated by sensograms developed by SPR analysis of immobilized MATCH protein (Fig. 3.9 & Table 6).
Figure 3.9  | MATCH proteins bind all four target antigens simultaneously

The images in this figure depict sensogram data from an SPR analysis in which MATCH proteins were immobilized in individual channels on a sensor chip and each recombinant antigen was sequentially injected over the sensor chip channels at the time, and in the order, indicated on the x-axis of each graph.

Table 6  | Antigen-binding by MATCH proteins does not impair binding of additional targets

<table>
<thead>
<tr>
<th>Sequence 1</th>
<th>1. TNFα</th>
<th>2. IL-5R</th>
<th>3. IL-25R</th>
<th>4. CD3c</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMATCH</td>
<td>NA</td>
<td>81%</td>
<td>78%</td>
<td>57%</td>
</tr>
<tr>
<td>apMATCH</td>
<td>NA</td>
<td>93%</td>
<td>77%</td>
<td>92%</td>
</tr>
<tr>
<td>apMATCH-diS</td>
<td>NA</td>
<td>92%</td>
<td>72%</td>
<td>101%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sequence 2</th>
<th>1. CD3c</th>
<th>2. IL-23R</th>
<th>3. IL-5R</th>
<th>4. TNFα</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMATCH</td>
<td>NA</td>
<td>81%</td>
<td>96%</td>
<td>102%</td>
</tr>
<tr>
<td>apMATCH</td>
<td>NA</td>
<td>94%</td>
<td>95%</td>
<td>100%</td>
</tr>
<tr>
<td>apMATCH-diS</td>
<td>NA</td>
<td>93%</td>
<td>96%</td>
<td>104%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sequence 3</th>
<th>1. IL-23R</th>
<th>2. TNFα</th>
<th>3. IL-5R</th>
<th>4. CD3c</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMATCH</td>
<td>NA</td>
<td>97%</td>
<td>87%</td>
<td>94%</td>
</tr>
<tr>
<td>apMATCH</td>
<td>NA</td>
<td>97%</td>
<td>85%</td>
<td>90%</td>
</tr>
<tr>
<td>apMATCH-diS</td>
<td>NA</td>
<td>95%</td>
<td>90%</td>
<td>95%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sequence 4</th>
<th>1. IL-5R</th>
<th>2. CD3c</th>
<th>3. TNFα</th>
<th>4. IL-23R</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMATCH</td>
<td>NA</td>
<td>100%</td>
<td>98%</td>
<td>73%</td>
</tr>
<tr>
<td>apMATCH</td>
<td>NA</td>
<td>101%</td>
<td>97%</td>
<td>78%</td>
</tr>
<tr>
<td>apMATCH-diS</td>
<td>NA</td>
<td>100%</td>
<td>98%</td>
<td>72%</td>
</tr>
</tbody>
</table>

The RU values obtained when an antigen was injected second, third or fourth in an SPR injection cycle are compared against the values obtained when the antigen was injected first.
3.1.4 Appropriate MATCH chain pairing appears to be considerably favored

It is important to acknowledge that while our initial data suggest proper inter-MATCH chain assembly, they do not necessarily indicate the absence of non-cognate variable domain associations, specifically the inverted heterodimerization of MATCH chains that would produce chimeric CDR sets. It has been suggested that CDR sets influence the efficiency of $V_L$-$V_H$ pairing (198), and our SE-HPLC, SDS-PAGE and SPR data suggest that cognate pairing of MATCH chains is highly favored. However, in an attempt to assess the degree of inverted heterodimerization, we performed a SE-HPLC analysis of antibody and antibody-antigen complexes after incubation of the MATCH proteins with a molar equivalent of trimeric TNFα (i.e., 3-fold excess TNFα epitope). When applying this method of analysis to the parental anti-TNFα scFv used in the dimer-forming core domain of all MATCH formats (Fig. 3.11), SE-HPLC traces revealed convoluted peaks consistent with distinct antibody-antigen complex populations, reflecting the disparate sizes of 1-, 2- and 3-times scFv:TNFα complexes. Additionally, a peak whose retention time was consistent with the presence of residual, non-complexed TNFα in solution was observed, whereas non-complexed scFv was seemingly absent from solution, thus validating the application of this method to identify inactive anti-TNFα antibody.

Separation of MATCH protein and MATCH-antigen complexes was less efficient due to the larger molecular weight range of the different protein populations. However, our results (Fig. 3.10) also clearly revealed the presence of three MATCH-TNFα complex populations and residual, non-complexed TNFα. Additionally, “shouldering” of the 1xMATCH:TNFα complex peaks was observed, which could be explained by the presence of unbound MATCH protein. To estimate the proportion of unbound MATCH protein in solution, a deconvolution of the peaks was performed using PeakFit® v.4.12 software, assuming a tailed distribution for each peak and plotted to optimize goodness-of-fit (Fig. 3.10). This analysis estimated the proportion of unbound MATCH protein to be between 6.6 and 10.7% (apMATCH-diS<apMATCH<pMATCH) of total protein content, possibly indicating the maximal levels of non-
cognate MATCH chain pairings. Meanwhile, to rule out any ability of non-cognate Fvs to complex with TNFα, we also created mispaired scFvs displaying the CDR sets that would theoretically arise from inverted heterodimerization of MATCH chains. These mispaired scFvs demonstrated no capacity to form complexes with TNFα in solution (Fig. 3.11). This confirms that cognate dimerization of MATCH chains is highly favored, particularly in the antiparallel format.

![Figure 3.10 | MATCH protein-TNFα complexing analysis](image)

The overlaid SE-HPLC chromatograms of MATCH protein alone (hashed line, top graphs) and MATCH protein after a 2-hour incubation with excess human TNFα (solid line, top graphs) suggests that a considerable majority of MATCH protein in solution is active insofar as the peak attributable to dimeric MATCH protein is largely absent after reacting with the antigen, and new peaks attributable to higher-molecular-weight complexes appear. After deconvoluting the chromatograms of the MATCH protein-TNFα reaction mixtures with PeakFit® v.4.12 software, our best estimates place the percentage of unbound MATCH protein between 6.6 and 10.7% (bottom graphs).

As an additional proof of the efficiency of MATCH protein assembly, we designed a fourth MATCH protein variant (apMATCH-dis(L/H)) (Fig. 3.12A), wherein we mutated a residue in each variable domain framework (V_L: G141C and V_H: G50C) of the heterodimer-forming anti-CD3ε Fv such that it would include an intra-Fv disulfide bridge. Under this design, the formation of an inter-chain disulfide bridge is entirely conditional on proper assembly of the MATCH protein chains. We managed to produce comparable yields of purified apMATCH-diS(L/H) (14.1 mg per L expression volume) relative to the oth-
er MATCH proteins. SE-HPLC chromatograms of protein samples suggested a highly homogenous product (Fig. 3.12D) with a melting temperature (Tm°C ± SD: 68.5 ± 0.1) consistent with what was observed previously (Fig. 3.12C). Despite the amino-acid substitution in the variable domain frameworks of the anti-CD3ε Fv, the molecule maintained affinity for human CD3ε (K_D (M): 3.39 x 10^8), and it showed

Figure 3.11 | Mispaired CDR sets cannot form complexes with TNFα

(A) Coomassie-stained SDS-PAGE gel indicates the successful production of scFvs displaying the mispaired CDR sets that could arise from inverted heterodimerization of MATCH chains (aTNFα-VL/aCD3ε-VH and aCD3ε-VL/aTNFα-VH) as well as the control, donor anti-TNFα scFv whose CDR is form by proper MATCH chain dimerization (aTNFα Control). (B) Overlays of SE-HPLC chromatograms of complex and control samples reveal that, as expected, the aTNFα Control forms complexes with TNFα. It appears to do so with such efficiency that no inactive scFv evidently remains. (C) Neither of the mispaired CDR sets appear to be able to complex with TNFα, suggesting that any MATCH chains that undergo inverted dimerization would be unable to form such complexes.
good storage-stability, with a 1 mg/mL sample losing only 3.4% of its dimer content after 2-week storage at 37°C (Fig. 3.12E). Crucially, stained SDS-PAGE gels revealed that non-reduced samples display a discrete band at the estimated position of the apMATCH-diS(L/H) heterodimer, suggesting high dimerization efficiency (Fig. 3.12B). This finding was reflected in our TNFα-complexing analysis, which again revealed that production samples contain >90% active MATCH protein (Fig. 3.12F).

Figure 3.12 | Production of stable, functional intra-Fv MATCH protein variant

(A) The design of apMATCH-diS(L/H), which contains a framework mutation to both VH and VL domains of the anti-CD3ε, heterodimer-forming Fv that establishes an intra-Fv disulfide bridge. (B) Coomassie-stained SDS-PAGE gel supports the formation of heterodimeric apMATCH-diS(L/H) in coexpression supernatants. The non-reduced protein sample contains a discrete protein band with the approximate electrophoretic mobility of the ~106 kD MATCH protein heterodimer, whereas reduced samples possess two discrete bands at the approximate weight (~50 kD) of the respective MATCH chains. (C) Thermal unfolding curve of apMATCH-diS(L/H) fitted with the Boltzmann equation to data points (n=3; mean ± SD) generated in a DSF assay. (D) An SE-HPLC chromatogram of purified apMATCH-diS(L/H) indicates that production samples can be prepared at very high purity; peak retention time: 6.78 (MATCH dimer). (E) During 14-day storage at 37°C, the purity of MATCH dimer is largely maintained. (F) apMATCH-diS(L/H)/TNFα complexing experiments are largely consistent with what was observed with classical MATCH proteins – bound MATCH dimer appears to comprise ≥90% of MATCH protein in complex samples.
3.2 Discussion

Considering the remarkable clinical success of bispecifics (163,164), it is difficult to overstate the therapeutic potential of multispecific antibody-based molecules. Unlocking their full potential requires overcoming several obstacles that currently impair multispecifics production and screening as well as their activity *in vivo*. In particular, we believe the considerable potential of heterodimeric multispecifics warrants the widespread, intensive research efforts they have attracted. Generally, with respect to antibody-based therapeutics, a major goal should be to produce technology platforms that optimize therapeutic-design flexibility, such that they can readily accommodate the often-unique pharmacokinetic demands of different therapeutic concepts. Whereas drug stability, selectivity and non-immunogenicity are consistently desirable features, a molecule’s optimal reactivity profile and size often depend on the indication(s) it is intended to treat. Unfortunately, conventional heterodimeric multispecific antibody-based molecules exhibit limited specificities and valences while frequently requiring the inclusion of IgG constant or recombinant domains to drive dimerization (172,174,208). In other words, they are hampered by design rigidity emanating from the structural limitations of their composite variable domains, suggesting a tremendous value to highly stable variable domain frameworks.

While display technologies can now accommodate the discovery and production of fully human antibodies, cloning procedures and/or sub-optimal cell transformations ensure at least some diminishment of a human IgG repertoire that – even when fully intact – possesses a CDR-sequence diversity that does not exceed that found in rabbits (206). For therapeutic applications in human patients, profiting from this considerable rabbit CDR diversity, as well as the various molecular quality control mechanisms exclusively employed *in vivo*, requires a robust discovery platform and a stable scaffold for humanization. Taking it a step further, fulfilling the goal of maximizing flexibility in antibody-based therapeutic design requires that low-molecular-weight antibody derivatives (e.g., fragments) are stable, fully functional, non-immunogenic and amenable to large scale production. To that end, we endeavored to create an entirely
human Fv scaffold for the humanization of rabbit CDRs with a stability profile that would facilitate the production of stable, heterodimeric tetraspecific antibodies consisting only of Fv domains and peptide linkers and assembled solely by variable domain associations.

By replacing V\textsubscript{L} FW IV of a hu4D5-8, V\textsubscript{\kappa}-bearing scFv variant with a V\textsubscript{\lambda} germ-line sequence, we were able to enhance scFv thermal- and storage-stability and reduce the formation of soluble aggregates, suggesting that this substitution alone can induce the strengthened domain-interface previously observed among V\textsubscript{\kappa}-V\textsubscript{H} scFvs by other researchers (202). Additionally, the chimeric scFv maintained its target affinity, suggesting that even a V\textsubscript{\kappa} CDR set with a \Omega-loop conformation at V\textsubscript{L} CDR3 will fully tolerate inclusion of a V\textsubscript{\lambda} sequence at this position. Also, we suspect that \lambda-capping promotes Fv stabilization while mitigating the risk of immunogenicity that could arise from single-point mutations. Meanwhile we have confirmed the broad applicability of this technology to generate stable humanized Fv fragments starting from nearly one hundred independent rabbit antibodies (data not shown).

Equipped with stable human Fv frameworks, we then attempted to implement our vision of a heterodimeric tetraspecific (theoretically up to hexaspecific) format exclusively consisting of fully-human antibody Fv domains and non-immunogenic peptide linkers. With the successful production of these MATCH proteins, we have introduced a new class of heterodimeric antibody-based multispecifics. Under the MATCH format, no non-human or antibody constant domains are required, and the rational design – with tandem V\textsubscript{L} or V\textsubscript{H} domains driving a given chain’s dimerization – prevents intra-subunit domain-associations, theoretically without requiring a geometrically-restricted, rigid peptide linker connecting tandem variable domains. Finally, the MATCH design allows the concomitant, specific engagement of at least four different antigens by a single molecule that is still only ~2/3 the size of a full-length IgG, adding to the versatility of antibody-based therapeutics.

The homogeneity of MATCH production samples, and the stability of dimeric protein contained therein, suggests that these formats will tolerate larger-scale production in a stable mammalian cell-line. Although we acknowledge a risk of non-cognate MATCH chain assembly that would potentially reduce
yields, our results indicate that proper MATCH protein assembly is considerably more efficient than non-cognate pairing, with co-expression of antiparallel MATCH chains in this study evidently producing samples wherein active MATCH protein comprises ~93% of total dimer content. Additionally, various purification techniques could be employed to remove residual, inactive MATCH protein, such as ion-exchange or affinity chromatography. Alternatively, we have explored methods to enhance dimerization efficiency, such as with knobs-into-holes mutations,(174) and found that we can produce MATCH proteins with modified Fvs and achieve similar yields and affinities as the three proteins presented in this paper.

Looking forward, multispecific antibodies present the opportunity to target distinct cell populations with as yet unseen selectivity, with obvious implications for cancer treatment (27). Because cancer cells are frequently distinguished from healthy cells not by a single tumor-specific cell-surface marker, but rather distinctive combinations of membrane antigens, multispecific antibody formats are theoretically perfectly suited to cancer-specific cell recognition with minimal off-target effects (27,199,209). This is because an antibody-based monotherapy exhibiting specificities for multiple cell-surface antigens should bind target cells with exceptionally high avidity, due to its simultaneous engagement of multiple membrane-anchored epitopes. However, the complexity of targeting multiple surface antigens on one cell – and doing so with high cell-type specificity – requires the identification and selection of an optimal combination of binding domains. This speaks to the benefits of modular heterodimeric formats whose design facilitates the production of multiple candidate molecules with unique pharmacokinetics through simple subunit rearrangement. Such modularity should allow the rapid generation and screening of a library of multispecific molecules with diverse binding-profiles and thereby facilitate identification of the optimal cell-binding kinetics for selective tumor-recognition in vivo.

With design flexibility as one of our major goals, the genes that encode MATCH chains possess restriction enzymes flanking the peripheral (i.e., non-dimer-forming) scFvs and the tandem, dimer-forming variable domains, allowing the exchange of Fv-encoding DNA with simple, one-step cloning procedures. This feature, coupled with the ability to co-express various combinations of MATCH chains (as
long as the dimer-forming Fvs remain constant), facilitate rapid dimer recombination and screening. By
virtue of employing a heterodimerization strategy alone, the task of producing MATCH formats with
unique pharmacokinetics can omit cloning steps that would be required of single-chain multispecifics,
saving valuable time, effort and resources.

With MATCH formats, we have overcome limitations in the production, stability and design-
flexibility associated with conventional multispecific antibody fragment-based formats. This should pro-
vide researchers with an unprecedented capacity to proceed from concept to clinic with molecules that
target multiple cell-type-specific antigens, theoretically endowing us with drug candidates possessing ide-
al levels of selectivity.
3.3 Methods

3.3.1 scFv expression and purification

Recombinant amino acid sequences were de novo synthesized and cloned into an expression vector adapted from a pET26b(+) backbone (Novagen), and the resulting construct was used to transform BL12 (DE3) E. coli cells (Novagen), and scFv expression was induced with 1 mM IPTG. Cells were harvested by centrifugation and resuspended in Wash Buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, and 1M Urea) with 2% Triton X-100, and the cell slurry was supplemented with 1 mM DTT, 0.1 mg/mL Lysozyme, 10 mM Leupeptin, 100 μM PMSF and 1 μM Pepstatin. The cells were then lysed by 3 cycles of ultrasonic homogenization, 0.01 mg/mL DNase was added and the homogenate was rotated for 20 min at RT. The sample was then centrifuged at 15,000 g for 15 min, and the pellet was re-suspended in fresh Wash Buffer. This procedure was performed 3x using Wash Buffer with 2% Triton X-100 and 2x using Wash Buffer without Triton X-100. After a final centrifugation step, the inclusion bodies (IBs) in the pellet were then solubilized in IB Solubilization Buffer (100 mM Tris-HCl, pH 8.0, 6M Gdn-HCl, 2 mM EDTA) at a ratio of 1.0 mL per 0.1 g of pellet and rotated for 30 min at RT. Subsequently, 50 mM DTT was added to the solution for an additional 30-min incubation at RT. Insoluble material was removed by centrifugation at 15,000 g for 10 min.

The refolding of the scFvs was performed by rapid dilution to a final protein concentration of 0.5 g/L in Refolding Buffer (100 mM Tris-HCl pH 8.0, 4.5 M Urea, 3 mM Cysteine, 3 mM Cystine, 400 mM Arginine-HCl). The refolding reaction was incubated for 48 h at 4°C with constant stirring. The refolded proteins were purified by affinity chromatography with Capto L (GE Healthcare) on an ÄKTA protein purifier (GE Healthcare) and eluted with 0.1M Glycine-HCl, pH 2.8, followed by the rapid adjustment of sample pH with the addition of 1 mL Tris-HCl, pH 7.5 per 30 mL eluate. Where needed, elution pools

---

1 Unless otherwise indicated, samples were kept on ice or at 4°C during all scFv purification steps.
were polished by size-exclusion chromatography on a Superdex 75 column (GE Healthcare). The isolated monomer fraction was analyzed by SE-HPLC, SDS-PAGE and UV/Vis spectroscopy. The resulting protein solution was concentrated and buffer exchanged by diafiltration with Native Buffer (150 mM NaCl, 50 mM Citrate-Phosphate, pH 6.4).

### 3.3.2 SE-HPLC analysis

Samples were passed through either a Shodex™ (Showa Denko) KW402.5-4F column (for scFv analysis) or a Shodex™ KW403-4F column (MATCH protein analysis) with running buffer (Shodex™ KW402.5-4F: 250 mM NaCl, 50 mM NaOAc, pH 6.0; Shodex™ KW403-4F: 35 mM NaH₂PO₄, 15 mM Na₂HPO₄, 300 mM NaCl, pH 6.0) at a flow rate of 0.35 mL/min. Eluted protein was detected by absorbance at λ=280 nm.

### 3.3.3 Affinity determinations

Antigen affinities of hu4D5-8 scFv variants, MATCH proteins and MATCH parental scFvs/scDbss were determined by surface plasmon resonance (SPR) using a MASS-1 SPR instrument (Sierra Sensors) and the following recombinant proteins (antigens): HER2 (Sino Biological, Inc., Cat. #: 10004-HCCH), CD3ε (Sino Biological, Inc., Cat. #: CT026-H0323H), IL-5R (R&D Systems, Cat. #: 253-5R), TNFα (Peprotech, Cat. #: 300-01A), IL-23R (Trenzyme, Accession #: NP_653302.2 – Gly24-Leu356 (custom synthesis)). Antigens were immobilized at 100-250 RU on a sensor chip (SPR-2 Affinity Sensor High Capacity Amine, Sierra Sensors) using a standard amine coupling procedure. Affinity measurements were performed in HEPES Running Buffer (0.01 M HEPES, 0.15 M NaCl, 0.05% Tween). For TNFα, a standard amine sensor was used. Two-fold serial dilutions of purified scFvs or MATCH proteins ranging from 0 to 90 nM were injected into the flow cells for 3 min (20 µl/min) and dissociation was allowed to proceed for 720 sec. After each injection cycle, surfaces were regenerated with a 45 second injection of 10 mM Glycine-HCl pH 1.5. Affinities were calculated by fitting sensograms of at least six concentrations, and
The MATCH: a novel multispecific antibody format

fits were considered accurate if $\chi^2$ was less than 10% of Rmax. Data were double-subtracted (reference channel and control cycle was subtracted).

Antigen-affinities of aTNFα-scFv-λcap, aTNFα-scFv, and apMATCH-diS(L/H) were determined by SPR analysis using a Biacore T200 System (GE Healthcare). Recombinant human CD3ε was directly coupled to a carboxymethylated dextrane surface on a CM5 sensor chip (GE Healthcare). Biotinylated human TNFα (ACROBiosystems, Cat. #: TNA-H8211) was coupled to a Biotin CAPture Kit, Series S Sensor Chip (GE Healthcare). Production and analysis of sensogram data using the Biacore T200 System was similar to that employed using the MASS-1 SPR instrument described above.

3.3.4 Thermal unfolding assays

The midpoint of transition for thermal unfolding of proteins was determined by Differential Scanning Fluorimetry (DSF), as described by Niesen et al. (210). The DSF assay was performed in a qPCR machine (MX3005p, Agilent Technologies). The samples were diluted in buffer (citrate-phosphate pH 6.4, 0.15M NaCl) containing a final concentration of 5x SYPRO® orange (Sigma-Aldrich) in a total volume of 25 μL. Samples were measured in triplicates and evaluated while subjected to a programmed temperature ramp from 25-96°C.

3.3.5 Stress stability studies

Initial monomer (scFv) or dimer (MATCH protein) content of each sample was determined by SE-HPLC (d0). To calculate the percentage of total protein content that was mono-/multimeric, the area under the curve peaking at the monomer (scFv) or dimer (MATCH) retention time was divided by the total area under curves not attributable to the sample matrix. The samples were stored at 37°C and analyzed repeatedly over a period of 2-4 weeks. Protein degradation was assessed by SDS-PAGE analysis, loading denatured proteins onto Mini-PROTEAN® TGX™ precast gels (Bio-Rad Laboratories) and staining electrophoresed protein with Coomassie Brilliant Blue solution. The protein concentration was monitored at
different time points by UV-Vis spectroscopy with an Infinite® M200 PRO microplate reader (Tecan Group, Ltd.).

### 3.4.6 MATCH expression and purification

The genes for each MATCH chain were *de novo* synthesized and cloned into a modified pcDNA3.1™/V5-His A (Invitrogen) expression vector. The proteins were purified from CHO-S supernatants by protein L-affinity purification, capturing MATCH chains with Capto L resin (GE Healthcare) in a column affixed to an ÄKTA protein purifier (GE Healthcare) and eluted with 0.1 M Citric acid, pH 2.0, followed by the rapid adjustment of sample pH with the addition of 1 mL 1M Tris-HCl, pH 7.5 per 3 mL eluate. Protein solutions were then buffer exchanged with Native Buffer (150 mM NaCl, 50 mM Citrate-Phosphate, pH 6.4) using a PD-10 Desalting Column (GE Healthcare) and finally concentrated using a Vivaspin Protein Concentrator Spin Column (GE Healthcare).

### 3.3.6 MATCH concomitant binding assays

The capacity of MATCH proteins to simultaneously bind all four of their target antigens was assessed by SPR analysis using a MASS-1 SPR instrument (Sierra Sensors). Each MATCH protein was diluted in 10 mM NaOAc, pH 5.0 to a final concentration of 5.0 µg/mL and immobilized on a sensor chip (SPR-2 Affinity Sensor High Capacity Amine, Sierra Sensors). Saturating concentrations of each antigen (CD3ε: 1’200 nM; IL-5R: 90 nM; IL-23R: 180 nM; TNFα: 90 nM) were sequentially injected, each for 3 min (20 µL/min), into sensor chip flow cells presenting immobilized MATCH protein or mock. As an additional control, sensor chip flow cells presenting immobilized MATCH protein were treated with buffer only for 3 min (20 µL/min) four times prior to initiating antigen injections (pre-antigen buffer injections), and the sensor chip responses were measured. Data were double-subtracted.
3.3.7 MATCH/scFv-TNFα complexing analysis

The requisite amount of MATCH protein or scFv was added to a solution containing 4.0 μg trimeric TNFα (Peprotech) to reach a molar equivalent between antibody and antigen (i.e., 3-fold excess of TNFα epitope). Solution volumes were adjusted to 20 μL by adding ddH₂O. Samples were incubated for 2 h at RT, agitating gently throughout. Reaction mixtures were then analyzed by SE-HPLC, passing each sample through either a Shodex™ KW402.5-4F column (for scFv-TNFα complexing analysis) or a Shodex™ KW403-4F column (for MATCH-TNFα complexing analysis) under the conditions described above. The MATCH-TNFα complexing reactions produced poorly separated elution curves in SE-HPLC chromatograms, complicating efforts to determine the proportions of protein and protein-complex populations in the samples. In order produce estimates of these proportions, raw data were analyzed in PeakFit v4.12, a deconvolution of the protein peaks was performed assuming a tailed distribution and optimizing goodness-of-fit (all R² values were ≥ 0.99).
Chapter 4

Effects of N-Glycosylation of the human cation channel TRPA1 on agonist-sensitivity

This chapter has been accepted for publication (citation: Egan TJ, Acuña MA, Zenobi-Wong M, Zeilhofer HU, Urech D. Effects of N-Glycosylation of the human cation channel TRPA1 on agonist-sensitivity. Biosci Rep. 2016 Aug 31. pii: BSR20160149. [Epub ahead of print]). The final version of record is available from: http://www.bioscirep.org/. Timothy James Egan is first author on the manuscript, having written it and having performed all experiments.
4 Characterizing N-glycosylation of TRPA1

Transient receptor potential (TRP) channels (reviewed in 1–7) are a family of nonselective, cation-permeable integral membrane proteins that commonly act as receptors in various sensory processes. Functional TRPA1 channels are composed of four subunits, each containing six transmembrane regions (S1−S6), two extracellular domains (E1 and E2) and an ion-permeable pore formed by the S5-S6 regions. TRP channels are modulated by diverse chemical and physical stimuli and simultaneous exposure to combinations of these stimuli often trigger unique channel responses, revealing cooperative effects. In addition, a single TRP channel gene can give rise to many structurally and functionally diverse channels due to the ability of subunits to heteromultimerize as well as display post-/co-translationally-added modifying groups such as N-glycans (218).

TRP channels are highly conserved, and studies have described the N-linked glycosylation of many of them (218–226). This common protein modification entails the co-translational addition, and subsequent processing, of an oligosaccharide to side-chains of lumenal Asn residues that are displayed within a specific N-X-S/T consensus sequence (where X is any amino acid other than Pro) (227). In general, a protein-bound N-glycan can exhibit one of three structures: high-mannose, hybrid or complex (227). The role of N-glycans identified in studies has ranged from involvement in protein folding and cellular localization to protein function (218,228). In the case of TRP channels, N-glycans have been shown to influence cell-surface (CS) expression (219,221,222,224,225), sensitivity to agonists (223), activity regulation (219,226) and temperature sensitivity (222).

The TRP channel “ankyrin” 1 (TRPA1) is most prominently expressed on nociceptive afferent fibers of dorsal root ganglia (DRG) and trigeminal ganglia (TRG) neurons and is activated by various exogenous and endogenous chemical stimuli in vivo. In addition, channel responses to cold (<17°C) and mechanical stimulation have been reported (41,42,47,48,50–53,229–238). TRPA1 is structurally distinguished from other members of the TRP family by the 14 to 18 ankyrin repeat motifs along its cytosolic...
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N-terminal domain (236,237,239). Its N-terminus is further characterized by reactive Cys and Lys residues, which act as sites for reversible covalent binding by membrane-permeable electrophiles exhibiting sulfhydryl-/amine-reactive groups (54,232). Reversible covalent binding of these residues is the mechanism by which several pungent chemicals, such as cinnamaldehyde (CA) and allyl isothiocyanate (AITC), activate TRPA1 (54,232).

Figure 4.1 | Structure of TRPA1 and orientation of its N-glycosylation site.

(A) Graphical representation of the structure of an hTRPA1 subunit, including the N-glycosylation sites along the E1 domain, the N-terminal ankyrin repeat domain, the locations of reactive Cys (five) and Lys (one) residues, and the location of the two residues on TM5 critical to menthol activation (S873 & T874). (B) Sequence alignment of the E1 domain of TRPA1 (retrieved from the NCBI database) across various mammalian species, revealing the conservation of both glycosylation sites. Residues highlighted in yellow indicate non-identity with human TRPA1

Mammalian TRPA1 contains two highly conserved (Fig. 4.1) lumen-exposed, N-glycosylation consensus sequences (at N747 and N753) along its E1 domain. Meanwhile, Western Blot (WB) analysis of denatured lysates from cells expressing human TRPA1 (hTRPA1) display a characteristic doublet band at the approximate position of the channel’s subunit, presumably due to the disparate weights of “mature”
and “immature” glycoprotein. This study sought to confirm the presence – and characterize the role – of hTRPA1-linked glycans with the aim of offering valuable insights into protein function and regulation.

Our present data confirm the di-glycosylation of TRPA1 and indicate a functional role for the glycan at position N747. These findings present further evidence of the functional significance of N-glycosylation, especially as it relates to ion channels.
4.1 Methods

4.1.1 Cloning Wild-type and Mutant hTRPA1-FLAG

For subsequent cloning and transfection, hTRPA1/pENTR223.1 cDNA (NCB Accession #: NP_015628) was supplied by Fisher Scientific AG. hTRPA1-FLAG cDNA was amplified and cloned into a pcDNA™5/FRT (Life Technologies) transfection vector between Kpn I and Xho I restriction sites, generating the open reading frame (ORF) using the following primers: forward, 5’-AAAGGTACCATGAAGCAGCAGCCTGAGG-3’; reverse, 5’-ATTCTCGAGCTATTTGTGCATCGTCTTTTGATGTCAGGTCTC AAGATGGGTGTGTTTTGCG-3’. To produce cDNA with Asn-Gln mutations encoded, site-directed mutagenesis was performed by overlapping PCR, using the mutating primers (N747Q: forward 5’-CCAGGAATGGCTTTCCAGTAAGTCATC-3’; reverse 5’-GATGCCAGTTGACTGGAAAGCCATTCCTGG-3’; N753Q: forward 5’-CAACTGGCATCATCCAGGAccaACTAGTGATC-3’; reverse, 5’-GATCAA ACTA GTTTCCCTGGATGATGCCAGGT-3’) in combination with primers for cloning between HpaI and BamHI restriction sites located within the hTRPA1 ORF (forward, 5’-ATTGTTAACACAACCGATGGA TGTCATGAGACC-3’; reverse, 5’-ATTGGATCTCTGTAAAATTCAGGACTGATGATGAAAGGC-3’).

4.1.2 Transient Transfection of HEK293 cells and Western Blot Experiments

HEK293 cells were seeded on poly-L-Lysine (0.01% Solution) (Sigma-Aldrich) treated growth surfaces at ~60,000 cells/cm² and grown for 16-24 h in Dulbecco’s Modified Eagle’s Medium (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal calf serum (v/v), 1% Pen-Strep (v/v), and 2 mM L-Glutamine (complete DMEM). The cells were transfected using the jetPRIME™ system (Polyplus Transfection), according to manufacturer’s instructions, and the culture medium was exchanged ~12-16 h after transfection. Cells were always harvested 36-48 h after transfection.
To harvest, intact cells were rinsed in ice-cold, 1X Phosphate Buffered Saline, pH 7.4, without Ca\textsuperscript{2+} or Mg\textsuperscript{2+} (PBS) and treated with Lysis Buffer (1% Triton X-100 in PBS w/ protease inhibitors) for 30 min on ice, agitating intermittently. The samples were then centrifuged at 10,500 g for 15 min at 4°C, and the pellets were discarded. The protein concentration of samples was then determined by the DC™ Protein Assay (Bio-Rad) according to manufacturer’s instructions, and sample concentrations and volumes were equalized. To denature the lysate samples, SDS-PAGE Loading Buffer (0.4M Tris-HCl (pH 8.5), 5% SDS (w/v), 40% Glycerol (v/v), 0.5 mM EDTA, 0.01% Bromophenol Blue (w/v), 50 mM DTT) was added (1:2) and the samples were incubated for 30 min at RT and then boiled at 95°C for 5 min. For WB detection, M2 mouse anti-FLAG® mAb (Sigma-Aldrich, Catalogue #: F3165) served as the primary antibody and a peroxidase-conjugated, rabbit anti-mouse IgG (Jackson ImmunoResearch, Catalogue #: 315-035-046) was used as a secondary antibody. Lectin-mediated hTRPA1 detection was also performed, applying a Concavalin A (ConA)-peroxidase conjugate (Sigma-Aldrich, Catalogue #: L6397) to transfer membranes displaying immobilized, purified hTRPA1-FLAG (purification method described in next section). WB data analysis was performed using signal intensities (a.u.) normalized to total protein transferred, which was revealed by staining transfer membranes with 1X Amido Black Staining Solution (Sigma-Aldrich) and destaining overnight in 15% methanol and 10% acetic acid in ddH\textsubscript{2}O. To compare protein expression levels, the combined, normalized signal intensities of all TRPA1 subunit glycoforms detected in the TRPA1 mutant samples – mature and immature glycoprotein (in N747Q and N753Q samples) or non-glycosylated protein (in N747/753Q samples) – were compared to the combined signal intensities of all TRPA1 subunit glycoforms detected in WT samples.

4.1.3 Endo H and PNGase F Digestion of Denatured Protein

HEK293 cell-transfections were carried out in flasks with a 75 cm\textsuperscript{2} growth-surface area. After incubation, the cells were rinsed three times in ice-cold PBS and lysed in 1 mL Lysis Buffer. Wild-type (WT) or mutant hTRPA1-FLAG was immuno-precipitated out of whole-cell lysates by incubation with 50
µL M2 anti-FLAG® magnetic beads (Sigma-Aldrich), rotating the samples overnight at 4°C. After rinsing the beads 3X with ice-cold PBS, the proteins were eluted from the beads with 100 µL, 100 mM Glycine-HCl, pH 2.8 (rocking the samples for 15 min at RT), and the pH of the eluate was promptly adjusted by addition (1:30) of 1M Tris-HCl, pH 9.5.

From each eluate, three 9 µL samples were aliquoted and the protein samples were denatured for subsequent glycosidase (or mock) digestion, according to manufacturer’s (New England Biolabs) instructions, with either 75’000 units/mL Endo H, 50’000 units/mL PNGase F or ddH₂O (mock). The samples were incubated with their respective glycosidase (or ddH₂O) for 2 hours at 37°C. The samples were then analyzed by Western Blot.

4.1.4 Cell-surface Biotinylation

HEK293 cell transfections were carried out in flasks with a 25 cm² growth-surface area. After incubation, the intact cells were rinsed three times in ice-cold PBS and incubated in 1.0 mg/mL EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific) in PBS for 30 min at 4°C. The biotin was quenched by washing the cells 3X in ice-cold, 100 mM glycine–PBS, and then three additional washes with ice-cold PBS were performed. The cells were lysed in 500 µL Lysis Buffer. After equalizing the protein contents, aliquots from each lysate sample were set aside (CL samples) and the remaining lysate was incubated with 50 µL Pierce™ Streptavidin Agarose (Thermo Fisher Scientific), rotating overnight at 4°C.

The agarose was pelleted by centrifugation at 1,000 g and 4°C, supernatants were discarded and the agarose was washed 6X total: 3X in Wash Solution A (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA), 2X in Wash Solution B (50 mM Tris-HCl, pH 7.4, 500 mM NaCl) and 1X in Wash Solution C (50 mM Tris-HCl, pH 7.4). The streptavidin-affinity precipitate was eluted from the beads with 80 µL SDS-PAGE Sample Loading Buffer, incubating for 30 min at RT (while gently agitating) and then boiling for 5 min at 95°C. All samples were then analyzed by WB using signal intensities normalized to total precipitate transferred, as determined by detection with streptavidin-peroxidase. To confirm the absence of
substantial intracellular contamination in the cell-surface fraction, an anti-Glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) IgG (Sigma-Aldrich, Catalogue #: G9545) was applied as a primary antibody to be detected by an HRP-conjugated goat, anti-rabbit IgG (Jackson ImmunoResearch, Catalogue #: 111-035-046).

4.2.5 Membrane Depolarization Assays on the FlexStation 3

HEK293 cells were seeded in poly-L-lysine-treated, 96-well plates (~65,000 cells/cm²) and incubated for 16-24 h. Transfection samples (100 µL jetPRIME™ Transfection Buffer and 2 µL jetPRIME™ Transfection Reagent per 1 µg plasmid) were diluted in pre-warmed complete DMEM at a concentration of ~1.6 µg plasmid/mL. The old medium was discarded, and ~220 ng/cm² of the appropriate plasmid were added to each well. The cells were then incubated for 18-26 h.

The medium was discarded and 100 µL 1X Membrane Potential Assay Kit RED Dye in Component B Buffer (Molecular Devices, Inc.), a voltage-sensing dye, were added to each well and the plates were incubated for 30 min at either 37°C or 23°C. The plates were analyzed in a FlexStation® 3 scanning fluorometer (Molecular Devices, Inc.) at 525 nm excitation and 565 nm emission (550 nm cut-off) wavelengths. Initiation of the scanning program resulted in the transfer of 0.02-1,000 µM of the TRPA1 agonist – cinnamaldehyde (CA), allyl isothiocyanate (AITC), or menthol – to each well. Fluorescence intensity data were collected using SoftMax® Pro v5.4 (Molecular Devices, Inc.) for 2.5 min after application of the agonist. These data were then baseline-subtracted, yielding the relative fluorescence units (RFU) presented here. Normalized fluorometric data are presented as % maximum RFU. All dose-response curves were fitted to data up to the maximal channel response using GraphPad Prism® 6 software.

4.1.5 Electrophysiological Recordings

HEK293 cells were maintained in complete DMEM and plated on 35 mm dishes 16-24 h before transfection. Cells were co-transfected with 1 µg of plasmid encoding hTRPA1-FLAG (WT or mutant)
and 1 μg of plasmid encoding enhanced green fluorescent protein (eGFP, used as a marker of successful transfection) and then incubated for 16-24 h. On the day of experiment, the medium was discarded and replaced with pre-warmed extracellular solution (37°C) containing (in mM): 150 NaCl, 10 KCl, 2.0 CaCl₂, 1.0 MgCl₂, 10 HEPES (pH 7.4), and 10 glucose. Whole-cell patch-clamp recordings were performed at a holding potential of -60 mV using an EPC7 amplifier and Patch Master v2.11 software (HEKA Elektronik, Lambrecht-Pfalz, Germany). Recording electrodes (3.5-4.5 MΩ) were pulled from borosilicate glass capillaries and filled with internal solution containing (in mM): 120 CsCl, 10 EGTA, 10 HEPES (pH 7.35), 4 MgCl₂, 0.5 GTP, and 2 ATP. Only eGFP-positive cells were used for recordings. Whole-cell TRPA1-mediated currents were activated by 0.5-1.5 min application of CA fed through an outlet tube (200 μm ID) of a custom-designed, gravity-fed, microperfusion system positioned 50–120 μm from the recorded cell. The temperature of solution in the cell’s microenvironment was maintained at 37°C using a Heat/Cooled Temperature Control System (AutoMate Scientific®).
4.2 Results

4.2.1 hTRPA1 is Glycosylated in vitro

To investigate N-glycosylation at both N747 and N753, we applied three strategies: 1) site-directed mutagenesis of cDNA to replace the critical Asn residues with structurally similar Gln (220,222–226); 2) glycosidase digestion of hTRPA1-FLAG purified from whole-cell lysates (CLs) (220,221,224); and 3) lectin-mediated detection of glycosylated hTRPA1-FLAG (225).

![Figure 4.2](image-url)
WB analysis of HEK293 cells transiently expressing hTRPA1-FLAG WT and N747Q and N753Q mutants revealed a doublet band at the estimated size of the TRPA1 subunit (~128 kD) (Fig. 4.2A). This doublet was conspicuously absent in lysates containing the N747/753Q double-mutant (Fig. 4.2A). Further, the TRPA1 glycoforms exhibiting the lowest electrophoretic mobility lost considerable signal intensity when subjected to PNGase F, but not Endo H, digestion (Fig. 4.2C), suggesting that the mature glycoprotein is modified with either a hybrid or complex glycan (i.e., one bearing terminal sialic acid) at both Asn residues. It also seems likely that the faster-migrating TRPA1 subunits in the respective doublets have been modified with a core glycan because, unlike the N747/753Q double-mutant, they were detected by ConA (Fig. 4.2D), which possesses an affinity for terminal α-D-mannose or α-D-glucose (240). Moreover, ConA failed to detect any of the slower-migrating subunits in the doublets, suggesting that both N-glycosylation sites ultimately bear a complex glycan possessing three or more antennae (240).

4.2.2 Expression of hTRPA1 is Affected by N-Q mutation

Over the course of 5 independent HEK293 transfections, the presence of either single N-Q mutation reduced total protein expression as measured by normalized (to total protein content) WB signal intensities relative to WT (mean ± SEM) (N747Q: 0.72 ± 0.05; N753Q: 0.55 ± 0.08), while the N747/753Q mutant consistently displayed the weakest signal intensity (0.42 ± 0.06) (Fig. 4.2F). Unexpectedly, WB analysis also indicated that the absence of the N747 glycosylation site enhances the glycoprocessing of the N-glycan at N753, as determined by differences in the ratio of complex-/core-glycosylated protein signal intensities (N747Q: 0.93 ± 0.07; WT: 0.49 ± 0.04) (Fig. 4.2F).

In addition to the differences observed in overall protein expression, we detected disparate CS expression between mutant and WT hTRPA1 after 5 independent HEK293 transfections (Fig. 4.2E-F). The lower cell-surface expression of N753Q and N747/753Q mutants relative to WT (N753Q: 0.49 ± 0.09; N747/753Q: 0.49 ± 0.16) was apparently consistent with the reduction in total protein expression, determined by analysis of mean ratios of WT-relative cell-surface expression to WT-relative total hTRPA1 ex-
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Expression (N753Q: 0.88 ± 0.07; N747/753Q: 1.08 ± 0.27). However, expression of hTRPA1 N747Q at the cell surface was evidently greater than WT (1.24 ± 0.10), indicating that removal of the glycan at position N747 results in a greater proportion of total protein localizing to the cell membrane (1.76 ± 0.19).

Figure 4.3 | Effects of N-glycosylation on TRPA1 agonist-sensitivity.

Dose-response curves depicting membrane depolarization of HEK293 cells expressing hTRPA1-FLAG glycoforms as measured on a FlexStation® 3 (data are expressed as mean ± SEM). (A) Response of hTRPA1-FLAG to indicated concentrations of CA. (B) Normalized (% maximum RFU) response of hTRPA1-FLAG to indicated concentrations of CA. (C) Normalized response of hTRPA1-FLAG to indicated concentrations of menthol. (D) Normalized response of hTRPA1-FLAG to indicated concentrations of AITC.

Despite the WB results we obtained, we did not observe significant differences between glycoforms in maximal changes in relative fluorescence and peak amplitudes in our fluorometric voltage measurements and electrophysiological current recordings, respectively (Fig. 4.3A, 4.4, 4.5B). However, fluorometric assays did reveal mean maximal fluorescence changes that were consistent with reductions in cell-surface expression of N753Q mutants (in RFU) (WT: 1155; N747Q: 947; N753Q: 902; N747/753Q: 866).
Meanwhile, the absence of a negatively-charged, cation-attracting glycan on hTRPA1 N747Q may provide a reason why maximal cell responses could be diminished despite slightly higher cell-surface expression.

### 4.2.3 TRPA1 Gating Mechanics are Altered by N747Q Mutation

Fluorometric activity assays \( (n=4 \text{ independent experiments})^2 \) revealed that TRPA1 mutants lacking a glycosylation site at N747 exhibit decreased sensitivity to CA (EC\(_{50}\) (in μM), 95% confidence interval (CI)) (WT: 9.5, 8.7-10.4; **N747Q**: 32.5, 30.6-34.6; **N753Q**: 10.5, 9.3-11.9; **N747/753Q**: 20.9, 19.8-22.0) (Fig. 4.3B, 4.4). While the EC\(_{50}\) of CA differed considerably between glycoforms, differences in saturating concentrations were not obvious. Indeed, the steeper slope factors of the CA dose-response curves plotted for N747Q mutants relative to WT, combined with reduced RFU values at lower concentrations, might suggest that the absence of an N-glycan at N747 results in altered activation kinetics rather than reduced agonist affinity.

In theory, neither mutation should alter the affinity of lipophilic, sulphydryl-/amine-reactive TRPA1 agonists, as they are known to interact with N-terminal Cys and Lys side chains (54,232). This gave rise to the idea that the functional differences between glycoforms were the result of glycan participation in gating rather than agonist binding. To test this hypothesis, and to determine whether the altered sensitivity we observed was specific to N-terminal-domain-binding TRPA1 agonists, we performed additional fluorometric measurements applying a similarly-reactive, TRPA1-agonizing electrophile, AITC (\( n=2 \) independent experiments), as well as menthol (\( n=3 \) independent experiments), which activates TRPA1 via interaction sites along the S5 domain (19) (Fig. 4.3C-D). The significant disparities between AITC and menthol dose-response curves were consistent with the glycoform-specific changes to CA sensitivity we observed (EC\(_{50}\) (in μM), 95% CI) (AITC: **WT**: 1.8, 1.2-2.5; **N747Q**: 4.2, 3.3-5.2; **N753Q**: 1.6, 1.1-2.3;

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\(^2\) Each fluorometric experiment (i.e., 1 independent experiment) described consisted of triplicate readings at each agonist concentration.
Characterizing N-glycosylation of TRPA1

N747/753Q: 3.0, 2.3-3.8; Menthol: WT: 29.2, 24.8-34.3; N747Q: 68.9, 64.6-73.6; N753Q: 34.4, 30.1-39.2; N747/753Q: 58.0, 51.3-65.5 (Fig. 4.3C-D).

Finally, results from electrophysiological experiments also support the apparent functional significance of the N-glycan at N747. In whole-cell patch-clamp experiments, (n≥4 cells each) we observed similarly reduced sensitivity to lower concentrations of CA among hTRPA1 glycoforms bearing the N747Q mutation (EC$_{50}$ (in µM), 95% CI) (WT: 35.0, 22.2-55.0; N747Q: 141.0, 116.1-171.2; N753Q: 42.0, 28.5-62.0; N747/753Q: 142.3, 95.9-211.2) (Fig. 4.5A). Once again, these data support a two- to four-fold increase in agonist concentration required for half-maximal activation of hTRPA1 that is non-glycosylated at position N747.

Figure 4.4 | Robust activity of both N-glycosylated and non-N-glycosylated TRPA1.
Fluorescence traces collected on a FlexStation® 3 (set to 37°C); representative of 4 independent experiments.
4.2 Results

Figure 4.5 | Electrophysiological assays confirm reduced sensitivity of N747Q mutants.

Data collected in whole-cell patch-clamp set-up (data are expressed as mean ± SEM). (A) Normalized peak amplitudes (% maximum amplitude for the respective cell) after application of indicated concentrations of CA (n≥4 cells each); CA was applied until current stabilized or began to desensitize during application. (B) Average maximum amplitudes of cells expressing each respective glycoform (n≥4 cells each); the amplitudes were compared by one-way ANOVA (p>0.05). (C) Examples of patch-clamp current traces for each hTRPA1 glycoform during CA application/removal.

4.2.4 Glycoform-specific Cooperative Effects of Temperature and CA

An apparent cooperativity between cold temperature and chemical agonists in the activation of TRP channels has been reported previously (229,241,242). Bandell et. al observed a roughly 9-fold lower EC₅₀
of menthol on TRPA1 when experiments were conducted at an ambient temperature (23°C) rather than physiological temperature (37°C) (229). Additionally, Pertusa et al. identified an N-glycan at position N934 of TRPM8 that influenced channel temperature sensitivity (222). Accordingly, we ran fluorometric activity experiments at 23°C in parallel to those conducted at 37°C to determine whether such cooperativity between temperature and CA was evident and whether it could be influenced by N-glycosylation.

Figure 4.6 | Cooperative effects of temperature and CA on TRPA1 activity.

Dose-response curves depicting normalized (% maximum RFU) membrane depolarization of HEK293 cells, at 23°C and 37°C, expressing hTRPA1-FLAG glycoforms as measured on a FlexStation® 3 (n=4 independent experiments)(data are expressed as mean ± SEM).

In our experiments (n=4 independent experiments), we observed a slight, albeit significant, decrease in the sensitivity of hTRPA1-FLAG WT and the N753Q mutant at 23°C relative to recordings at 37°C (EC$_{50}$ (in μM), 95% CI) (WT: 13.5, 12.8-14.2; N753Q: 18.0, 17.3-18.7) (Fig. 4.6A&C). In contrast, the
absence of the N747 glycosylation site led to enhanced sensitivity of TRPA1 at ambient temperature (EC$_{50}$ (in μM), 95% CI) (N747Q: 18.2, 17.7-18.8; N747/753Q: 14.8, 13.7-16.9) (Fig. 4.6B&D). Despite these findings, we did not, however, observe any direct agonistic effect of noxious cold (as low as 4°C) on hTRPA1-FLAG expressed in HEK293 cells (data not shown). The cold-sensitivity of TRPA1 remains controversial, with our data supporting some findings (48,243,244) but contradicting others (45,47,245).
4.3 Discussion

Our study into the effects of N-glycosylation of hTRPA1 produced several new insights. In addition to confirming that both consensus sites can be modified with an N-glycan \textit{in vitro}, we determined that the removal of these sites does not prevent functional assembly or cell-surface trafficking of the protein. On the contrary, it appears that the absence of the glycan at position N747 results in higher levels of protein localized to the cell surface. Additionally, we identified what appear to be important functional roles of N-glycosylation at the N747 consensus site. Specifically, we first observed that the N747Q mutation influences the efficiency of glycoprocessing of the N-glycan at N753. Later, we observed that the absence of the N747 glycan reduces hTRPA1 sensitivity to a variety of agonists, and those effects were more dramatic at physiological temperatures.

The effects of protein glycosylation are highly variable (228). Because of this diversity, the task of characterizing N-glycans must – at least currently – be performed on a glycoprotein-by-glycoprotein basis. This appears particularly true in the case of TRP channels. For example – in evident contrast to hTRPA1 – removal of the N-glycan on rat TRPV1 appears to enhance agonist sensitivity, with researchers observing a ~5-fold reduction in the EC\textsubscript{50} of capsaicin in non-glycosylated mutants (223). As such, determining the specific function of N-glycosylation for each TRP channel plays a crucial role in expanding our understanding of both this protein family and, more broadly, the structural and functional protein traits that can be conferred by glycans. Several researchers have already contributed heavily in this regard (218–226), but many TRP channel-bound N-glycans remain unstudied.

Given the potential clinical significance of TRPA1 (38,39,46,246–250), insights into its structure and function could be of considerable value. Our data suggest a critical role of an N-glycan in TRPA1 gating mechanics, with its absence resulting in diminished channel sensitivity to lower concentrations of a broad array of agonists. While that finding may be of greatest interest within the context of our research, it bears mentioning that confirming TRPA1 glycosylation and eliminating the possibility that it is required for either a) protein assembly, or b) trafficking to the cell surface, is in itself highly relevant. This is espe-
cially true of the latter, as the N-glycosylation of TRP channels has been occasionally shown to have a profound impact on the levels of cell-surface expression (218,219,225).

While the expression data we collected revealed that a greater proportion of hTRPA1 N747Q was localized to the cell surface than that of hTRPA1 WT, it is important to also consider our finding that the removal of the smaller glycan at position N747 resulted in more robust expression of mature (relative to immature) glycoprotein. This might suggest that the enhanced surface expression of this TRPA1 mutant arises due to its more efficient glycoprocessing rather than by the absence of the N-glycan at N747 per se. This would point to a critical role for the complex N-glycan at N753, namely that its presence promotes optimization of cell-surface expression. The expression patterns of hTRPA1 proteins bearing the N753Q mutation appear to support this idea. Both proteins possessing this mutation exhibited reduced total expression relative to WT and, consequently, reduced cell-surface expression. It is unclear whether these reductions signify less efficient protein processing or increased susceptibility to degradation. Regardless, it appears that optimal hTRPA1 expression patterns may rely on di-glycosylation.

The heightened level of mature hTRPA1 elicited by the N747Q mutation could arise from several possible causes. For example, it is becoming increasingly clear that sequences adjacent to the N-X-S/T consensus site can influence glycosylation and/or glycoprocessing (251–253). Additional evidence highlights the influence that the distance between N-glycosylation acceptor sites can have on the efficiency of glycoprocessing (254). While one could hypothesize that an upstream N747Q mutation might result in unnatural glycoprocessing at position N753 (potentially resulting in the altered protein functionality), that contradicts our finding that the N747/753Q double-mutant produces similar agonist-induced behavior to that of hTRPA1 N747Q. We find it more likely that close approximation of the glycosylation sites and/or the amino acids nearby the respective sequons are the cause of the apparent interplay between these two sites.

In light of the disparate levels of cell-surface expression we observed, it was surprising that the maximal TRPA1-specific responses observed in whole-cell patch-clamp and fluorometric assays did not differ significantly between TRPA1 variants. However, maximal functional responses may not directly
correlate with TRPA1 cell-surface expression. Activation of TRPA1 in intracellular organelles, whose expression is not reflected in our membrane fraction WB analysis, may influence current and voltage-sensitive dye readouts. In addition, in the patch-clamp experiments, we may have had a bias towards selecting cells with a low level of TRPA1 expression, as these cells are likely to appear “healthier” than cells with a higher expression level. Ultimately, the production of stable cell lines would be useful in resolving these discrepancies.

Given the consistency of our results from the functional analysis, both across different assays and between agonists with distinct reactivity profiles, we are confident in the functional significance of the glycan at position N747. As further support, the EC$_{50}$ values we generated from fluorometric measurements of hTRPA1-FLAG WT-mediated membrane depolarization closely resemble those observed by other researchers using HEK293 cells in similar assays, for all three agonists (231,233,255), and we likewise observed IC$_{50}$ values of the TRPA1-selective antagonist, HC-030031, that were consistent with the literature (249) (data not shown). These findings, to our thinking, validate the use of transiently-transfected HEK293 cells in fluorometric assays.

In our review of the literature, we did not uncover many full dose-response curves of TRPA1 generated in a whole-cell patch-clamp format in heterologous expression systems. However, Chung et al. used precisely such a format to evaluate the response of TRPA1 to its agonist, eugenol (238). We found that TRPA1-expressing HEK293 cells were highly amenable to electrophysiological analysis, with serial dilutions of CA applied to single cells producing large, slowly-inactivating inward currents that waned only slowly upon removal of the agonist (Figure 4C). This enabled us to confirm the effects of the N747Q mutation, which once again demonstrated a notable loss of agonist-sensitivity. Although the EC$_{50}$ values determined by whole-cell patch-clamp and fluorometric assays differed considerably, we considered this a consequence of the differences inherent to the respective assays. In addition to discrepancies in assay sensitivities, other factors, such as voltage control and/or the treatment of CA-naïve cells (in the FlexStation) vs. single-cells exposed to escalating CA concentrations (in patch-clamp experiments) likely contributed to the observed differences.
Recently, Deering-Rice et al. (256) published results from a functional analysis of non-glycosylated TRPA1 variants. Their work entailed analyzing calcium influx after a single application of a saturating concentration of TRPA1 agonists to HEK293 cells transiently expressing either WT or N747A and/or N753A TRPA1 variants. To rule out disparate sub-cellular protein distribution as a cause of disparate channel responses, the researchers endeavored to activate intracellular TRPA1 in isolation by applying TRPA1 agonists in the presence of the membrane-impermeable TRPA1 inhibitor, ruthenium red. While they found diminished responses among TRPA1 variants bearing the N753A mutation, they did not – as we did – identify significant differences in protein distribution. We believe this is likely a consequence of the different analytical approaches, namely the use of immunoblotting of isolated cell surface proteins vs. an intracellular activity assay, to determine differences in TRPA1 cell-surface expression.

The influence of N-glycosylation on the apparent cooperative effects of temperature and CA was a surprising finding in this study. In particular, it is interesting that the presence of both glycosylation sites appears to optimize TRPA1 agonist-sensitivity at physiological and ambient temperatures. Perhaps in the case of TRPA1, di-glycosylation served as a kind of evolutionary calibration, especially in light of the fact that the N753 glycosylation site is more evolutionarily recent, with cold-blooded amphibians and reptiles lacking it. It would be interesting to see whether similar patterns are present among other thermosensitive TRP channels.

There are some limitations inherent to this analysis that bear acknowledgement. Specifically, it remains unclear whether TRPA1 N-glycosylation occurs in native cell lines in vivo. However, results published by other research groups consistently indicate that TRP channels are N-glycosylated in vivo (222,224), supporting extrapolation of findings obtained in vitro. In addition, we cannot exclude that the Asn-Gln mutations induce confounding changes to protein structure that may obscure other effects of glycosylation. Also, there were multiple TRPA1 subunit glycoforms present in single cells, as evidenced by the doublet bands observed in single samples in WB analysis. Individual TRPA1 variant glycoforms may contribute disparately to functional readouts, and the experiments we performed effectively aggregate these contributions.
Finally, TRPA1 is potentiated and inactivated by Ca\(^{2+}\) (257). It is possible that WT and mutated TRPA1 channels analyzed in our experiments differ in their permeability to Ca\(^{2+}\), which in turn could affect current amplitudes through Ca\(^{2+}\)-dependent TRPA1 potentiation (257). One might consider such disparities as an alternative explanation for the observed discrepancies between our expression and functional data. However, it is unclear what effects such disparities would have, if any, on the dose-response relationships between agonists and the TRPA1 variants under the experimental design we applied. In our experiments, the absence of significant differences in maximal TRPA1-specific response supports the statistical comparison of normalized dose-response curves. Furthermore, we did not observe a discernible pattern in TRPA1 channel function that was suggestive of the presence of confounding structural changes or disparate Ca\(^{2+}\)-dependent potentiation brought on by the mutation to either site. On the contrary, the two variants bearing the N747Q mutation exhibit very different expression patterns but produce similar maximal signals and dose-response curves when stimulated by diverse agonists and under different experimental approaches.

One of the common features of TRP channels is their functional and structural versatility. They are typically polymodally activated, ubiquitously expressed, and can both hetero- and homo-multimerize (211–217). The revelation that TRPA1 behavior can be modified by altered glycosylation patterns potentially unlocks even more functional diversity. Glycosyltransferases are differentially expressed during development and across human cell-types (258–260). Therefore, there is some potential that cell-type-specific transferase expression could ensure the targeted presence of functionally unique TRP channels, allowing proteins with identical primary structures to accommodate different functional roles. In the context of thermosensitive TRP channels, processes such as glycosylation may also be a means to optimize function over the range of temperatures to which an expression locus is typically exposed. For example, sensory receptors innervating the skin are exposed to lower temperatures than that of the core, and the functional demands of these respective environments could likewise differ.

This study provides new insights into the complex channel behavior and its tightly controlled regulation. Our confirmation of TRPA1 di-glycosylation, and the indications that this modification carries
considerable functional importance for the protein, enhances our understanding of not only TRPA1 behavior, but also of the broader TRP channel family as well as of the versatility of N-glycans.
The work performed for this thesis has contributed to the knowledge base regarding cation channel structure and function, and it has helped to add a novel, multispecific antibody-based molecule to the existing multispecific classes already documented in the public domain. Despite these advances, application of this information and technology to the production and development of cation channel-modulating mAbs remains elusive. Numab is in the ideal position to develop such mAbs because they employ the use of a proprietary, high-throughput antibody discovery platform, enabling them to find the “needle in the haystack” – selectively identifying and isolating antigen-reactive B-cells among the many B-cells isolated from the spleens of immunized animals. By contrast, the cation channel-inhibiting mAbs described in previous chapters utilize conventional selection techniques, relying on random B-cell isolation and hybridization in the hope that some may be antigen-reactive. Indeed, Numab’s immunization campaigns relying on standard peptide-KLH immunizations have produced several ion channel binders (data not shown) but ultimately produced no cation channel-inhibiting mAbs. Therefore, it seems clear that the most considerable work still to be done is either on the side of antibody recombination or immunogen design.

To that end, it is critical that the MATCH format technology is further developed and characterized to determine its amenability to various combinations of CDR sets, and, of course, the long-term goal will be the development of a MATCH format with potential therapeutic applications. One could envision circumstances under which a MATCH format, in particular, could provide highly selective binding and inhibition of a cation channel, engaging multiple epitopes across multiple domains/subunits in an effort to “suture” the ion channel pore shut. Additionally, Numab has relied primarily on peptide, genetic and/or cellular immunizations for the generation of antibodies and have selected B-cells on the basis of binding to channel-derived peptides. Designing new immunogens, particularly small vesicles that can be fully internalized by B-cells carrying full-length or recombinant cation channels, possibly in conjunction with immune-stimulating molecules and with modifications for breaking immune-tolerance, could allow discov-
ery and development of IgGs with CDR sets that engage conformation-dependent epitopes. Meanwhile, the selection strategy should be designed to facilitate identification of such conformation-dependent immunobinders by utilizing, for example, pore mimetic structures such as the fusion peptide produced by Gómez-Varela et al. (72). In the next two subsections, proposals for further experiments to address these lingering issues are described.
5.1 Developing the MATCH format

The assembly of MATCH subunits into functional MATCH proteins was surprisingly efficient. In light of the fact that such assembly is exclusively driven by variable domain associations, the only explanation for this would seem to be that the rabbit CDR sets heavily contribute to the strength of the variable domain interface. This has already been described in the literature (198). However, an important caveat to consider is that the dimer-forming Fvs in all MATCH proteins that were produced were identical. It is not clear whether the CDR sets that were displayed by dimer-forming Fvs were particularly unamenable to mispairing. In fact, the unusual behavior of only one of the mispaired scFvs developed (Fig. 3.11) possibly suggests a particular incompatibility between CDR sets in the proof-of-concept MATCH proteins that would not be commonly observed in other MATCH proteins, which could produce lower yields of functional heterodimer. This could complicate the production of MATCH proteins developed for therapeutic applications.

In a new experiment, it would be highly instructive to shuffle the CDR sets in the proof-of-concept MATCH protein, so as to determine whether different CDR sets in the dimer-forming positions would compromise the yield of functional MATCH protein due to a reduced efficiency of cognate pairing. To perform such analysis, variants of these proteins could be modified with disulfide bridges and various framework mutations to enhance cognate dimerization efficiency, allowing a direct comparison with the unmodified MATCH proteins to analyze whether any marked improvement in yields occurs by virtue of such modifications.

Additionally, it is crucial to the long-term development of MATCH formats that experiments are carried out to optimize their purification. With ~10% of inactive MATCH protein remaining in solution after protein L-affinity and SEC purification steps, current production methods are not sufficient for clinical development. Removal of inactive MATCH protein from solution could potentially be accomplished by, for example, ion exchange or affinity chromatography. Experiments should be conducted that im-
prove the purity to the maximum extent possible via these methods. Not only would this validate the potential therapeutic application of this technology, it would also provide a sense of what kind of production yields, bottlenecks and costs can be anticipated.

Finally, Numab is in possession of hNa\textsubscript{V}1.7-binding rabbit mAbs targeting both the E3 and PG regions of DI and DIII, respectively, which were raised in the course of standard KLH-peptide immunization campaigns. It would be interesting to determine whether the inclusion of CDR sets from these mAbs into a bispecific, heterodimeric format could elicit the channel-inhibition that the mAbs failed to in isolation. This would be a considerable undertaking, as it would require recombination of the full-length rabbit IgGs into humanized scFvs, followed by pharmacokinetic and stability analysis, and finally the cloning, production and screening of several multispecific molecules of variable lengths. However, results from such an analysis, positive or negative, could be highly informative for future cation channel-modulation strategies, and it would be at the very least interesting to determine whether antibody hNa\textsubscript{V}1.7-selectivity can be improved by using a molecule that binds multivalently.
5.2 Designing new immunogens to raise cation channel-targeting mAbs

To properly complement Numab’s differentiating antibody discovery technology, experiments should be performed to determine optimal immunization strategies for the development of mAbs that inhibit complex multispanskers generally, and cation channels specifically. So far, only the peptide immunization campaigns performed in the course of work for this dissertation successfully raised anti-cation channel antibodies, but none of the resulting mAbs have displayed any capacity to modulate protein function. Perhaps this is because the mAbs that have been selected bind only to single regions of single subunits/domains. A protein immunization strategy that displays conformation-dependent ion channel epitopes in a lipid bilayer, therefore, should be attempted.

Particularly, modifying the exosome-display approach that was described by Delcayre et al. (141) by using a parental cell-line of rabbit origin could produce small vesicles heterologously expressing multispanskers in functional form and in the proper orientation while the background protein content is likely to be non-immunogenic in rabbits. These vesicles would then be small enough for complete uptake by B-cells, subjecting even intracellular and transmembrane regions of the channel to protease digestion and presentation as antigenic peptides on MHC II molecules, making it more likely to produce Th-cell epitopes for B-cell activation and differentiation. Another advantage of such vesicles is that if DCs are used to develop them, they will likely present immune-stimulatory proteins which could specifically activate B-cells displaying exosome-reactive BCRs (261).

Further, the heterologously expressed multispanskers could be modified via fusion with CD40L (146,147) and deglycosylated by mutation or glycosidase digestion so as to break immune-tolerance and promote the development of antigen-specific antibodies. Alternatively, one could potentially use rabbit T-cell derived exosomes, which natively display many B-cell stimulating molecules, including CD40L. Implementation of additional protein recombination approaches to specifically target the pore-forming region
could be explored, as well, including the development of pore-only constructs (149,150) in the hope that such constructs may be passively taken up as protein cargo in exosomes.

Employing many variations of these immunization strategies in parallel, followed by a careful evaluation of anti-cation channel antibody titers could establish a robust method for the generation of multi-spanner-inhibiting mAbs. Numab’s remarkable discovery platform could then be tuned to ensure that B-cells are selected on the basis of binding to multiple subunits/domains simultaneously. The use of antigen-expressing exosomes derived from rabbits could potentially accommodate such selection, because – as mentioned – off-target IgG interactions should be minimal. Alternatively, one could use a biological construct that mimics the target domain, such as a variation of the tetrameric fusion peptide used to generate the anti-Kv10.1 mAb (72). Part of work during this doctoral campaign (not included in this dissertation) was devoted to the production of a tetrameric display of the hTRPA1 PG region, scaffolding the peptides with the macrocyclic chemical compound, calixarene. Such a display could be used to select pore-occluding anti-hTRPA1 mAbs, for example.
5.3 Final statement

The work performed for this thesis was largely focused on developing strategies for the discovery and development of mAbs that inhibit cation channels. The challenges that this presented led to a considerable expansion of the focus, including: 1) efforts to develop a novel immunogen in collaboration with the Organic Chemistry Department at ETHZ; 2) building up Numab’s Fv-stabilizing technology as well as their multispecific antibody platform; and 3) performing research into the structure and function of an ion channel pain target. Ultimately, the results emanating from these efforts have yielded new insights into cation channel function and added to the already-considerable versatility and potential of antibodies as therapeutic instruments. Hopefully, the information presented herein will soon yield cation channel-inhibiting antibodies with sufficient selectivity and potency for therapeutic development.
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WORK EXPERIENCE
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The Medicines for Malaria Venture, Kampala, Uganda (Field Office)
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 Researched the effects of supply-side subsidies of a first-line antimalarial on purchasing trends and pricing in rural Uganda
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