Therapeutic Antibodies Targeting Potassium Ion Channels

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Abstract

Monoclonal antibodies combine specificity and high affinity binding with excellent pharmacokinetic properties and are rapidly being developed for a wide range of drug targets including clinically important potassium ion channels. Nonetheless, while therapeutic antibodies come with great promise, K^+ channels represent particularly difficult targets for biologics development for a variety of reasons that include their dynamic structures and relatively small extracellular loops, their high degree of sequence conservation (leading to immune tolerance), and their generally low-level expression in vivo. The process is made all the more difficult when large numbers of antibody candidates must be screened for a given target, or when lead candidates fail to cross-react with orthologous channels in animal disease models due to their highly selective binding properties. While the number of antibodies targeting potassium channels in preclinical or clinical development is still modest, significant advances in the areas of protein expression and antibody screening are converging to open the field to an avalanche of new drugs. Here, the opportunities and constraints associated with the discovery of antibodies against K^+ channels are discussed, with an emphasis on novel technologies that are opening the field to exciting new possibilities for biologics development.

Keywords

Biologic · Ion channel · Kv1.3 · Potassium channel · Therapeutic antibody

1 K^+ Channels as Targets for Therapeutic Antibody Development

As detailed throughout this volume, potassium-selective ion channels are involved in a wide range of physiological processes important in human health and disease. While not all K^+ channels are validated drug targets, many are, and the past decade has seen notable successes in the discovery and development of both small and large molecule drugs that can modulate the activity of a number of important disease-related K^+ channel targets. Antibodies that can recognize and modulate the activities of human Kv1.3, Kv10.1, Kv11.1, and TASK3 have now been described and their potential use in the context of disease is discussed in detail at the end of the chapter (Sects. 4.1–4.4).

First, we consider the advantages of antibodies as therapeutic compounds, the mechanisms by which they achieve their effect, the strategies underlying their discovery and development (Fig. 1; Box 1) along with the practical aspects of antigen design, protein production, immunization, and screening to identify potential lead compounds that can move forward through clinical trials.

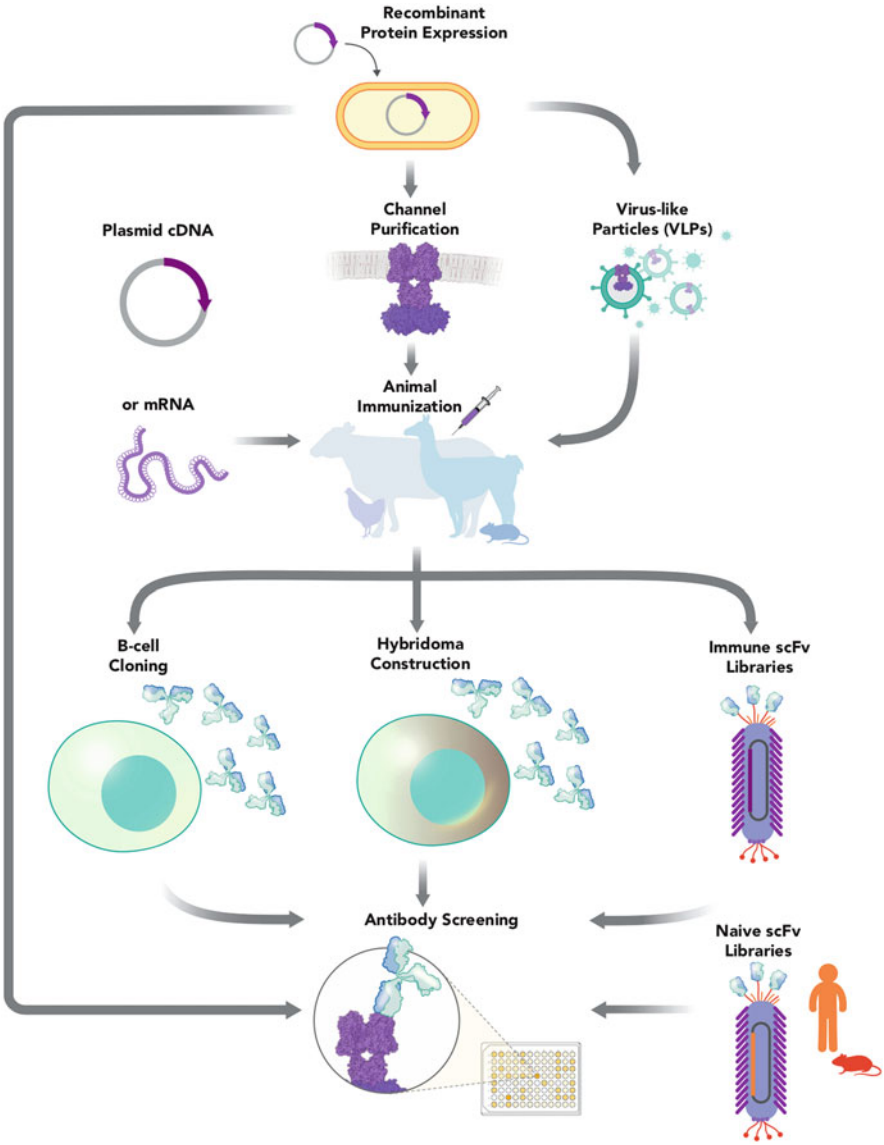

Fig. 1 Alternative routes toward antibody discovery

Box 1 Antibody Discovery

Obtaining therapeutic antibodies that can modulate the activity of K^+ channels can proceed via different routes depending, to some extent, on the source

(continued)

Box 1 (continued)
material for antibody generation and screening. As shown in Fig. 1, traditional approaches involve immunizing animals with purified channel proteins (or, in some cases, virus-like particles containing channel proteins) that are expressed recombinantly in heterologous systems. Alternatively, genetic immunization with nucleic acids encoding relevant proteins (either in the form of cDNA or mRNA) offers yet another way to induce channel-specific antibody production in animal hosts. Following immunization, culture supernatants from cloned B-cells or immortalized hybridoma cell lines that secrete antibodies are then screened for binding to channels of interest. As an alternative to screening culture supernatants, B-cells from immunized animals can be harvested and used to construct phage or yeast libraries that display single-chain antibody fragments (scFvs) on their surface as a way to indirectly screen for antibodies that bind and modulate channel activity. In fact, animal immunization can be precluded altogether using single-domain antibody display libraries constructed from B-cells of naïve animals or human patients (bottom right). These libraries are often of sufficient depth that they can be mined for rare antibodies that might never be produced in response to immunization but are valuable therapeutic candidates, nonetheless. Finally, regardless of the source of antibodies used for discovery, recombinant channel proteins that are either purified or expressed in mammalian cell lines for functional (patch-clamp) studies, provide necessary material for antibody screening.

2 Antibody Discovery and Development: Challenges and Opportunities

Based on their long half-lives and exquisite target selectivity, antibodies have distinct advantages relative to both small molecule drugs, which often lack selectivity, and peptide toxins that can be highly selective but are rapidly cleared from the bloodstream (Hutchings et al. 2019; Posner et al. 2019; Wulff et al. 2019). With regard to the latter, approaches toward increasing the half-lives of peptide toxins (including the use of antibody scaffolds (see Sect. 2.1)) are being actively pursued, however, maintaining the native binding activity of re-engineered toxins remains a significant issue (Lau and Dunn 2018; Murray et al. 2019).

In the case of small molecules, the drug-binding pockets where these entities typically exert their effect are often conserved among members of a given ion channel family, have relatively small surface area (~300 $Å^2$ (Coleman and Sharp 2010)), and suffer from general promiscuity at the level of both the pocket and ligand. Conversely, the large solvent accessible surface area of the paratope/epitope interaction site formed between the complementarity-determining regions of immunoglobulins and their cognate antigens (~1,500–2,000 $Å^2$ (Ramaraj et al.

2012)) drives high specificity binding for a given target and is one of the principal advantages of antibodies compared to small molecule drugs.

High-affinity binding is clearly a hallmark of therapeutic antibodies, however, in order to achieve clinical relevance, antibodies targeting K^+ channels must not only bind the target, they must also alter a specific physiological process in vivo either by blocking or potentiating channel activity, and/or drive the elimination of disease-causing cells by engaging immune effector mechanisms. This has been a difficult problem to solve for reasons that are both theoretical and practical.

2.1 Theoretical Considerations

Although much is known about the gating properties of K^+ channels, there are relatively few examples of antibodies that can modulate channel activity and there is little-to-no structural information that can inform us as to how such antibodies either inhibit or potentiate channel activity. Thus, in the absence of a solid framework for understanding how antibodies alter the functional activity of a channel, a rational approach toward generating such antibodies remains elusive.

That being said, among the handful of antibodies that can modulate K^+ channel activity, a majority are directed against the small extracellular loop structures of their cognate channel proteins. While this may seem obvious given that these loop structures are the sole regions on K^+ channels available for antibody binding, an antibody directed against TASK3 appears to inhibit the channel indirectly by driving receptor-mediated endocytosis of the channel itself. Inhibitory antibodies to Kv1.3 and Kv10.1, on the other hand, appear to block their respective channels more directly.

In the absence of detailed structural studies, one can only speculate that antibodies in this latter group act through either steric hindrance (that is, blockage of the pore) or allosteric interactions that interfere with the normal cycling between conformationally distinct (resting, open, and inactivated) states of the channel in each case (Kuang et al. 2015). In the second instance, antibodies that recognize unique epitopes associated with a particular state could potentially stabilize or lock a channel into a given conformation leaving it irreversibly opened or closed. Alternatively, it is possible that binding to an epitope (s) shared by all conformational states could prevent cycling or force a channel into a particular state. Either way, such antibodies could be useful from a therapeutic standpoint and, in the case of true "state-dependency," could offer an additional layer of selectivity on target binding. Indeed, recent evidence has suggested that a lead antibody targeting Kv1.3 is capable of blocking channel activity in a use-dependent manner by recognizing an epitope associated with the open-state of the channel accessible in activated human T_{EM} cells but not in "resting" Kv1.3 expressing CHO cells (Colussi, personal communication).

The idea that K^+ channels may expose state-dependent epitopes is clearly consistent with voluminous studies showing that these ion channels are structurally dynamic and that conductance changes are generally accompanied by changes in the 3-dimensional conformation of the channel itself (Tombola et al. 2006; Gupta

et al. 2010; Vargas et al. 2012; Kuang et al. 2015; Islas 2016). Although it is unclear whether such conformational shifts generate novel B-cell epitopes, studies using stabilized GPCR structures (Hutchings et al. 2017; Soave et al. 2018) suggest possible strategies for screening antibodies that recognize unique determinants associated with particular conformational states of a given K^+ channel. Additionally, active consideration is being given to the use of biparatopic and bispecific antibodies that can bind different epitopes on the same or different proteins (in the case of homomeric or heteromeric channels, respectively) as a way to prevent dynamic structural changes thereby blocking channel activity. Finally, it should be noted that antibodies that can modulate K^+ channel activity have been identified using purified full-length proteins in an undetermined conformational state as the starting immunogen (Colussi, unpublished).

As noted above, inhibition of channel activity by antibodies may result from steric blockage of the pore. In this case, single-domain antibodies (nanobodies) from camelids or sharks may be particularly useful due to their small size and apparent ability to bind recessed epitopes (Henry and Mackenzie 2018). While a precise mechanism (s) is lacking, several camelid single-domain constructs have been shown to inhibit K1.3 (see Sect. 4.1). Along the same lines, antibodies produced in some species have ultra-long hypervariable regions in their heavy chains. This is perhaps best exemplified in cows where the complementarity-determining region 3 (CDRH3) of the heavy chain folds into an extended "stalk" and disulfide-bonded "knob" structure. Wang and colleagues took advantage of this structure to build chimeric humanized antibodies containing the extended bovine CDRH3 stalk domain but substituting the knob region with the peptide toxins Moka-1 or Vm24 that bind to the outer vestibule of the Kv1.3 channel pore (Wang et al. 2016). In each case, the chimeric antibodies blocked Kv1.3 channel activity, presumably by interfering with conductance through the pore. While such chimera are highly engineered constructs, the fact that the ultra-long bovine CDRH3 can gain access to the outer regions of the pore suggests that cows and other species that generate antibodies with novel properties could be ideal platforms for producing pore-blocking antibodies.

2.2 Practical Considerations

From a practical standpoint, immunization and screening campaigns to identify modulating antibodies typically require large amounts of high-quality starting material to achieve success. Nevertheless, K^+ channels tend to be expressed at low levels in their native contexts and, as multi-span transmembrane proteins that can be toxic when overexpressed, are often difficult to produce as recombinant proteins. This problem is compounded when considering heteromeric K^+ channels where more than a single polypeptide must be expressed and assembled at the proper stoichiometric ratios before antibody generation can begin. Despite this, a number of successful approaches are being used to generate sufficient levels of antigen for animal immunization including the use of novel expression systems (Sect. 3.2).

A more basic problem relative to antibody generation is the high level of sequence conservation between K^+ channels of different species. Tolerance mechanisms during B- and T-cell development dampen the immune response to autoantigens and can be difficult to overcome when challenging a given species with antigens that are evolutionarily conserved and therefore similar or identical to self. The most relevant regions of the K^+ channels in this case are the small extracellular loops where antibodies are expected to bind. As illustrated for the Kv1.3 channel (Table 1), these regions show varying degrees of sequence conservation in orthologs from humans and mice and, as expected, greater divergence in more distantly related species (specifically, chickens). Interestingly, when comparing homologous K^+ channel family members of a given species, extracellular loop structures tend to be less conserved than the transmembrane domains separating them (Table 1) and, theoretically, provide sufficient sequence diversity to elicit production of highly selective antibodies in a given animal host.

It is also worth noting that while the number of potential B-cell epitopes associated with the extracellular loops is somewhat limited due to their small size (roughly 10–20% of total protein for the annotated human K^+ channels (Table 1)), the proteins themselves exist as multimers of the same or different subunits. As a result, surface epitopes are displayed in a repetitive fashion on native proteins. Such repetitive features may be conducive to antibody production via crosslinking of the Ig receptor on B-cells and may provide some advantage for antibody screening based on binding avidity. Certainly, the ability to generate native loop structures on recombinant proteins together with the use of strong adjuvants and evolutionarily diverse species for immunization offer logical approaches to antibody generation (Sects. 3.2 and 3.4). And, while peptide constructs corresponding to loop structures tend to be weakly immunogenic on their own, methods are being developed to enhance their potency including multimerization of antigen constructs. A notable example in this case is mAb56, which blocks the Kv10.1 channel (Sect. 4.2) and was generated by linking a peptide from the third extracellular loop of the channel to a tetramerization domain from the C-terminal region of the same protein (Hartung et al. 2011).

Beyond the need to generate antibodies, screening to identify not only binders, but also antibodies that can modulate channel activity in vivo has its own unique challenges. This is a complex task that involves surveying vast repertoires of antibody molecules for antigen binding, recovering genetic sequences for antibodies that bind, and screening potentially hundreds of candidates for their ability to modulate channel activity. While these steps are often fraught, a variety of novel technologies are being married with more traditional approaches to screen and identify candidates of interest. A more detailed description of these technologies is provided in Sect. 3.4.

The preceding discussion has focused on the discovery of antibodies that can modify channel function by blocking or potentiating current flow. While this is arguably the most difficult goal to achieve in the development of therapeutic antibodies against K^+ channels, it is worth noting that modulating channel activity is not the sole mechanism for achieving both specificity and biological effect. As

Table 1 Average percent identities calculated from percent identity matrices generated by Clustal Omega alignments

Channel family	Members (n)	Whole protein	Average % identity														ECL (% total protein)
			TMD						Re-entrant loop (RL)		Extracellular						
			S1	S2	S3	S4	S5	S6	RL	RL2	ECL1	ECL2	ECL-RL	RL-ECL	ECL-RL2	RL2-ECL	
KCa2.x	3	68.1	96.7	85.0	74.6	87.3	96.8	100.0	100	NA	56.0	80.7	89.5	91.7	NA	NA	8.2
KNav1.x	2	76.7	85.7	60.0	61.1	100	76.2	81.3	81.0	NA	35.3	100.0	55.6	50.0	NA	NA	3.9
Kv1.x	8	67.0	79.1	73.5	78.0	96.8	98.9	97.4	89.5	NA	35.9	49.3	64.5	78.6	NA	NA	15.1
Kv2.x	2	67.0	90.9	100.0	94.7	100.0	100.0	100.0	100.0	NA	75.0	100.0	84.6	100.0	NA	NA	6.2
Kv3.x	4	73.5	90.4	76.7	93.9	100.0	100.0	100.0	100.0	NA	49.1	95.5	69.1	90.5	NA	NA	13.5
Kv4.x	3	70.0	96.8	79.4	82.5	100.0	100.0	100.0	96.7	NA	65.7	81.5	74.4	90.5	NA	NA	8.5
Kv6.x	4	49.5	53.8	50.8	53.2	76.2	65.9	62.9	82.5	NA	43.3	34.9	36.7	66.7	NA	NA	11.9
Kv7.x	5	47.8	63.8	46.2	52.9	84.6	80.5	73.2	85.2	NA	42.0	77.5	35.8	63.3	NA	NA	4.9
Kv8.x	2	39.4	40.9	38.1	52.4	100.0	52.4	42.9	61.9	NA	16.7	33.3	38.5	33.3	NA	NA	8.6
Kv9.x	3	51.6	48.5	46.4	59.8	84.1	71.2	78.2	83.3	NA	41.5	10.0	38.5	76.2	NA	NA	10.5
Kv10.x	2	76.8	90.5	90.5	94.7	100.0	91.3	81.0	81.8	NA	71.4	100.0	68.8	71.4	NA	NA	7.8
Kv11.x	3	63.4	96.8	85.7	93.7	100.0	93.7	96.8	100.0	NA	33.3	77.8	67.6	100.0	NA	NA	6.4
Kv12.x	3	56.5	81.0	69.8	76.2	90.5	81.0	96.8	87.3	NA	22.2	41.7	58.5	86.7	NA	NA	7.4
Kir2.x	4	64.4	64.0	67.4	NA	NA	NA	NA	79.0	NA	45.3	77.8	NA	NA	NA	NA	8.3
Kir3.x	4	63.2	62.0	76.5	NA	NA	NA	NA	85.1	NA	54.9	83.3	NA	NA	NA	NA	7.7
Kir4.x	2	61.0	68.0	77.3	NA	NA	NA	NA	94.7	NA	48.0	66.7	NA	NA	NA	NA	9.0
Kir6.x	2	69.7	80.0	77.3	NA	NA	NA	NA	94.7	NA	43.5	88.9	NA	NA	NA	NA	8.9
K2P	15	28.9	41.5	46.0	24.5	37.8	NA	NA	57.2	56.9	NA	NA	22.9	45.5	40.7	16.9	22.7

Approximate boundaries of transmembrane domains (TMD: S1-S6), Re-entrant loops (RL), Extracellular loops (ECL) and ECLs either N-terminal (ECL-RL) or C-terminal (RL-ECL) of re-entrant loops were based on annotation in UniProtKB, Clustal alignments and where necessary manual alignment. Kv5.1 is the only member of the Kv5 family and therefore it was not analyzed. *NA* not applicable

noted above, a monoclonal antibody targeting the TASK3 channel inhibits human lung cancer xenografts and murine breast cancer metastasis in mice through internalization of the channel/antibody complex (Sun et al. 2016). Furthermore, Fc-mediated effector functions such as antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC) offer additional mechanisms by which depletion of diseased cells could achieve therapeutic benefit (de Taeye et al. 2019; Graziano and Engelhardt 2019). In the same vein, engineering approaches involving optimization of Fc domain structure (Lobner et al. 2016; Saunders 2019), the use of monovalent, bi- and multi-valent formats (Brinkmann and Kontermann 2017; Husain and Ellerman 2018), antibody drug conjugates (Joubert et al. 2020), as well as cellular approaches that engage chimeric antigen receptors on engineered T cells (Golubovskaya and Wu 2016) represent additional opportunities for K^+ channel antibody development.

3 Workflows in Antibody Production and Screening

As outlined in Fig. 1, having chosen a particular target, a wide range of options is available in the path toward therapeutic antibody development. This begins with decisions around whether to immunize animals or rely on surface display technologies to select for antibodies (typically in single-chain scFv or Fab formats in the case of phage, yeast, or ribosome display) that can bind the target of interest (Fig. 1; Box 1). Since therapeutic antibodies must contain humanized or fully human heavy and light chain scaffolds, one advantage of surface display is the potential to screen commercially available libraries prepared from human DNA. Indeed, such libraries have been constructed from both naïve and autoimmune patient samples and are of sufficient depth that one can mine the entire B-cell receptor repertoire of a given individual, albeit, without native heavy and light chain pairings (Lee et al. 2018; Rouet et al. 2018). Alternatively, one can choose to immunize animals and create de novo surface display libraries, which, theoretically, could increase the likelihood of selecting for antibodies of interest, particularly in cases where a given channel protein is highly conserved across species boundaries and pre-existing libraries might lack strong binders.

Regardless of the direction one chooses, success in antibody discovery ultimately depends on having an antigen to screen with (Fig. 1). If the initial choice is to immunize animals with protein antigens, the immunogen itself can be used for screening purposes in combination with any of a variety of binding assays including conventional ELISA and bead-based assays that allow colorimetric or fluorescence read-outs. Similarly, if one opts for nucleic acid immunization, or relies solely on surface display to select for antibodies of interest, the screening entity can be a peptide, a protein fragment, or a full-length protein purified directly from native tissue or produced recombinantly and bound to a solid support (see Sect. 3.1). Cell-based flow cytometry offers an additional method of screening but suffers a number of pitfalls including low resolution (if the channel is not abundantly expressed) and

the potential to miss antibodies that bind a particular conformational state. Despite these problems, flow cytometry has the major advantage that it can potentially select for only those antibodies that bind surface exposed loops on channel proteins and is especially useful at later stages of screening when binders have already been identified.

Among the most powerful technologies now being applied to antibody discovery are manual and automated high-throughput B-cell cloning methods that allow one to screen the secreted products of thousands to hundreds-of-thousands of individual plasma cells from naive or immunized animals. These include the optofluidic BEACON platform created by Berkeley Lights, Inc. (Emeryville, CA, USA), the Genovac Nano platform (Aldevron LLC, Fargo, ND, USA), and Abcellera's microfluidic platform (Vancouver, Canada) among others. A somewhat different approach involves the capture of individual B-cells in porous microspheres that can be directly screened under a microscope using diffusible fluorescent probes (Izquierdo et al. 2016). Of course, the power of these single-cell technologies is that cDNA sequences encoding the heavy and light chains of potentially every B-cell clone in their native pairings can be readily recovered by standard RT-PCR and sequencing to regenerate the antibody of interest either as a native immunoglobulin or a chimeric antibody containing the variable regions of the original clone within a human immunoglobulin scaffold. Although not routinely available to academic laboratories, the platforms themselves can be accessed through commercial sources (Aldevron, LLC, Fargo, NC; Abcellera, Vancouver, Canada; Ligand Pharmaceuticals Inc., San Diego, CA).

Lastly, antibodies identified in binding assays must be further screened for their ability to modulate K^+ channel activity and/or induce a biological effect in vitro and in vivo. Depending on the channel and target cell, these assays will differ but assuming the desired effect is achieved, the antibody will then be evaluated for binding affinity, selectivity, cross-reactivity with non-human orthologs, potency, manufacturability (expression yield, solubility, thermal and long-term stability), and immunogenicity and, if necessary, subjected to additional engineering prior to selection of a final lead and initiation of preclinical development. While these latter criteria are beyond the scope of this chapter, the sections that follow provide additional detail on key aspects of antibody generation and screening beginning with the selection of immunogens.

3.1 Choice of Immunogen

For programs involving animal immunization, antibody development begins with the choice of immunogen, which are grouped here into four categories, namely, (1) peptides and soluble protein fragments; (2) native full-length proteins; (3) recombinant full-length proteins; and, (4) nucleic acids encoding antigens of interest. The use of peptides and protein fragments enables production of antibodies against targeted regions of the channel that are accessible at or near the cell surface and likely to play a role in ion flux. When larger fragments or full-length proteins are

used as immunogens, screening methods designed to preferentially select those antibodies that can bind extracellular regions of the channel (either on a solid support or on target cells) are preferred (Bednenko et al. 2018).

Although peptides and small protein fragments have been used to generate antibodies against a number of K^+ channels (Hemmerlein et al. 2006; Gómez-Varela et al. 2007; Sun et al. 2016; Hartung et al. 2020; Fan et al. 2020), they are weakly immunogenic and lack the full spectrum of multi-domain, conformational epitopes present in the full-length, correctly folded and functional channel proteins (Dodd et al. 2018). Efforts to improve their immunogenicity and solubility include linking such fragments to sequences that promote oligomerization (Hemmerlein et al. 2006; Gómez-Varela et al. 2007; Hartung et al. 2020).

By comparison, full-length proteins provide all possible B- and T-cell epitopes associated with a given target and, when available, are the antigens of choice for generating inhibitory antibodies against channel proteins; the principal caveat being that K^+ channels are not abundantly expressed in their native cell types and their overexpression in heterologous cells can be toxic resulting in limited yields. Modulating antibodies against a small number of ion channels (the calcium channel, Orai1 (Lin et al. 2013); the ligand-gated ion channel P2X7 (Buell et al. 1998); and the cation channel TRPA1 (Lee et al. 2014)) have been generated using proteins derived from mammalian expression systems. While this has not been demonstrated in the case of the K^+ channels, alternative approaches using DNA immunization (Stortelers et al. 2018), peptide/carrier protein conjugates (Fan et al. 2020), virus-like particles (Adam et al. 2014; Doms et al. 2014) and proteins produced in protozoan expression systems (Bednenko et al. 2018) have had success targeting the important potassium channel, Kv1.3.

Lastly, vector-encoded antigens (in the case of DNA immunization) and, more recently, mRNA immunization (Pardi et al. 2018; Zhang et al. 2019) obviate the need to generate and purify proteins altogether and are discussed in more detail below (Sect. 3.2).

3.2 Expression Platforms for Antigen Generation

While expression in *Escherichia coli* and other bacteria are among the most robust platforms in terms of protein yield, they are generally not recommended for mammalian membrane proteins due to their reducing cytosolic environments along with significant differences between prokaryotic and eukaryotic protein processing and membrane insertion mechanisms (Pandey et al. 2016). However, several eukaryotic systems have demonstrated consistently strong performance in expression of membrane protein drug targets. We took advantage of the Protein Data Bank (PDB; rcsb.org) to obtain information on production of eukaryotic full-length K^+ channels for structure analysis (Table 2). Of the 21 potassium channels in this archive, 9 were expressed in human embryonic kidney HEK293 cells using BacMam technology, 6 in methylotrophic yeast *Pichia pastoris*, 5 in insect cells (*Spodoptera frugiperda* Sf9 and *Trichoplusia ni* High Five cells), and 1 in rat insulinoma INS-1832/13 cells.

Table 2 Potassium ion channels that were expressed as full-length proteins for structural analysis (source: Protein Data Bank, rcsb.org)

Potassium channel	Species	Expression system	PDB accession number	References
Kv1.2	*Rattus norvegicus*	*Pichia pastoris*	2A79	Long et al. (2005)
Kv7.1	*Homo sapiens*	Human HEK293S GnTI$^-$ cells (BacMam technology)	6UZZ	Sun and MacKinnon (2020)
Kv7.1	*Xenopus laevis*	Human HEK293S GnTI$^-$ cells (BacMam technology)	5VMS	Sun and MacKinnon (2017)
Kv10.1	*Rattus norvegicus*	Human HEK293S GnTI$^-$ cells (BacMam technology)	6PBY 6PBX 5K7L	Whicher and MacKinnon (2016), (2019)
Kv11.1	*Homo sapiens*	Human HEK293S GnTI$^-$ cells (BacMam technology)	5VA1 5VA2 5VA3	Wang and MacKinnon (2017)
KCa1.1	*Homo sapiens*	Human HEK293S GnTI$^-$ cells (BacMam technology)	6V3G 6V38 6V35 6V22	Tao and MacKinnon (2019)
KCa1.1	*Aplysia californica*	*Trichoplusia ni* (High Five cells)	5TJI 5TJ6	Hite et al. (2017), Tao et al. (2017)
KCa3.1	*Homo sapiens*	Human HEK293S GnTI$^-$ cells (BacMam technology)	6CMN 6CNN 6CNO	Lee and MacKinnon (2018)
KCa4.1	*Gallus gallus*	*Spodoptera frugiperda* Sf9 cells	5A6E 5U70 5U76	Hite et al. (2015), Hite and MacKinnon (2017)
Kir2.2	*Gallus gallus*	*Pichia pastoris*	3JYC 3SPC 3SPG 3SPH 3SPI 3SPJ 5KUK 5KUM 6M84 6M85 6M86	Tao et al. (2009), Hansen et al. (2011), Lee et al. (2016b), Zangerl-Plessl et al. (2020)
Kir3.2	*Mus musculus*	*Pichia pastoris*	3SYA 3SYC 3SYO 3SYP	Whorton and MacKinnon (2011), (2013)

(continued)

Table 2 (continued)

Potassium channel	Species	Expression system	PDB accession number	References
			3SYQ 4KFM	
Kir6.2	*Homo sapiens*	Human HEK293S GnTI^{-} cells (BacMam technology)	6C3O 6C3P	Lee et al. (2017)
Kir6.2	*Mus musculus*	Human Free-style HEK293-F cells (BacMam technology)	5YKE 5YKF 5YKG 5YW8 5YW9 5YWA 5YWB 5YWC 6JB1	Wu et al. (2018), Ding et al. (2019)
Kir6.2	*Rat norvegicus*	Rat insulinoma INS-1 832/13 cells transduced with adenovirus	5TWV 6BAA	Martin et al. (2017a, b, 2019)
Kir6.2	*Mus musculus*	HEK293S GnTI^{-} cells (BacMam technology)	5WUA	Li et al. (2017)
TWIK1	*Homo sapiens*	*Pichia pastoris*	3UKM	Miller and Long (2012)
TREK1	*Homo sapiens*	*Spodoptera frugiperda*	4TWK	To be published
TREK2	*Homo sapiens*	*Spodoptera frugiperda* Sf9 cells	4BW5 4XDJ 4XDK 4XDL	Dong et al. (2015)
TRAAK	*Homo sapiens*	*Pichia pastoris*	3UM7 4I9W 4RUE 4RUF 4WFE 4WFF 4WFG 4WFH	Brohawn et al. (2012), (2013), (2014), Lolicato et al. (2014)
TRAAK	*Mus musculus*	*Pichia pastoris*	6PIS	Brohawn et al. (2019)
TASK1	*Homo sapiens*	*Spodoptera frugiperda* Sf9 cells	6RV2 6RV3 6RV4	Rödström et al. (2020)

Both BacMam and insect cell platforms utilize modified double-stranded DNA baculoviruses as vehicles for gene delivery. Baculoviruses do not replicate in mammalian cells, and high-level transient expression in BacMam system is achieved by the presence of a mammalian expression cassette containing a strong CMV promoter (Goehring et al. 2014).

While insect cells are capable of generating high protein yields, mammalian cells offer additional advantages, most importantly, native N-glycosylation and membrane lipid composition (e.g., higher cholesterol content), which increases the likelihood of correct protein folding (Goehring et al. 2014). However, overexpression of recombinant ion channels can be toxic for mammalian cells, presumably due to unregulated activity of the channel in a heterologous environment (Claire 2006). To avoid cytotoxicity, expression can be carried out in eukaryotic or prokaryotic cell-free systems that may include detergents and/or lipids to enhance membrane protein solubility. Both Kv1.1 and Kv1.3 have been successfully expressed using cell-free technologies (Renauld et al. 2017; Cortes et al. 2018), although it is important to note that cell-free systems typically produce only moderate amounts of protein, are relatively expensive and often difficult to scale up.

As an alternative to mammalian and insect cells, fast-growing and efficient microbial eukaryotes such as yeast (especially, *Pichia pastoris* and *Saccharomyces cerevisiae*) or the ciliated protist, *Tetrahymena thermophila*, have also been explored for the expression of recombinant potassium channels (see Table 3 for comparison of *P. pastoris* and *T. thermophila*). *P. pastoris* owes its success in large part to a very powerful and tightly regulated methanol-inducible AOX1 promoter as well as to its ability to reach very high cell densities in culture (Vogl and Glieder 2013; Guyot et al. 2020). Growth of *P. pastoris* either in shake flasks or bioreactors at the liter scale can be sufficient to produce milligram quantities of purified membrane protein to support animal immunization and screening trials (Guyot et al. 2020).

Tetrahymena, on the other hand, is a free-living protist with biological properties that make it ideal for the production of eukaryotic membrane proteins. These properties include the absence of a cell wall, the ability to introduce foreign genes at very high copy number (~18,000 copies per cell), near uniform N-glycosylation (primarily 3Man2GlcNAc) and a metabolism that is geared toward the production of membrane and secreted proteins (Nusblat et al. 2012; Guerrier et al. 2017). Additionally, the *T. thermophila* genome is characterized by expanded gene families encoding proteins involved in membrane dynamics and transport, including more than 300 potassium voltage-gated ion channels (Eisen et al. 2006). Typically, it takes 1–2 weeks to obtain *Tetrahymena* transformants with confirmed recombinant protein expression, and 2–3 more weeks to accumulate sufficient biomass for production of 2–10 mg of purified membrane protein. TetraGenetics Inc. (Arlington, MA, USA) has employed this system for production of a number of human K^+ channels being targeted for therapeutic antibody development in the treatment of cancer, autoimmune and fibroproliferative disorders (Bednenko et al. 2018, Shim et al. 2020; Bednenko and Colussi, unpublished).

Some antibody development technologies do not require immunogen purification. For example, Integral Molecular (Philadelphia, PA, USA) produced

Table 3 *Pichia pastoris* and *Tetrahymena thermophila*

	Pichia pastoris	*Tetrahymena thermophila*
Cell size	4–6 μm	30–60 × 50–100 μm
Cell wall	Yes	No
Typical cell density at the time of induction of protein expression	10^8–10^9 cells/ml	10^6 cells/ml
Examples of high membrane protein expression yield	90 mg purified human aquaporin 1 from 1 l of culture (Nyblom et al. 2007) 13 mg purified mouse P-glycoprotein from 100 g of cells (Bai et al. 2011)	3 mg purified human Kv1.3 from 1 l of culture (Bednenko et al. 2018)
Inducible promoter	AOX1, methanol-inducible	MTT, cadmium-inducible
Typical growth temperature	27–30°C	30–37°C
Typical induction temperature	20–27°C	24–37°C
Doubling time	2–3.5 h (1–3 h)	2–3 h
Induction time	16–60 h	2–24 h
N-glycosylation	8–14 mannose residues per side chain GlycoSwitch technology: engineer your own strains to obtain controlled, human-like N-glycosylation (Jacobs et al. 2009)	Primarily $Man_{2-5}GlcNAc_2$ (Calow et al. 2016)
O-glycosylation	Very little	None
Membrane lipid composition	Ergosterol is the major sterol. Strains producing cholesterol have been engineered (Hirz et al. 2013)	No sterol synthesis, tetrahymanol as a sterol surrogate. Membrane lipid composition can be modified by addition of cholesterol and other lipids into a growth media (Nusblat et al. 2012)

Kv1.3-containing murine leukemia virus-like particles (VLPs) in HEK293T cells, with the yield of 400 pmol Kv1.3/mg total VLP protein, or ~ 2.6% w/w (Adam et al. 2014; Doms et al. 2014). Due to their repetitive surface structures, VLPs are known to elicit robust humoral and cellular immune responses (Mohsen et al. 2017).

As noted above, nucleic acid immunization technologies short-circuit the need for protein antigens altogether (Liu et al. 2016). Typically, DNA expression vectors encoding the antigen of choice are delivered into animal tissues by intradermal microparticle bombardment or by intramuscular injection, while liposome formulations and/or electroporation are used to increase transfection efficiency. Ablynx (Ghent, Belgium) immunized llamas with Kv1.3-encoding plasmid DNA to develop functional anti-Kv1.3 single-domain nanobodies (Stortelers et al. 2018). The caveat with DNA immunization strategies is that they often yield low antibody

titers and may need to be supplemented with a purified recombinant protein for animal boosting and/or antibody screening (Bednenko et al. 2018). Along with DNA, in vitro transcribed messenger RNA (mRNA) can serve as a template for antigen synthesis in vivo following cell entry. Chemically formulated mRNA vaccines have demonstrated encouraging results in the field of vaccine development and may become an indispensable tool in the fight against emerging infectious diseases and cancer (Pardi et al. 2018; Corey et al. 2020; Espeseth et al. 2020).

3.3 Purification and Formulation of Target Immunogens

An important consideration when designing purification strategies for K^+ channels is maintenance of physiological activity, which in the end can be validated using one or more analytical methods depending on the formulation (e.g., ligand binding, ion-flux, electrophysiology). This is particularly important when modifying native sequences with various tags to aid in purification (6-10xHis, GFP, antibody epitopes such as FLAG, Rho1D4, and others), or when truncations are introduced to the N- and C-termini to limit aggregation and impart stability (Wang and MacKinnon 2017; Whicher and MacKinnon 2019; Sun and MacKinnon 2020). While intracellular domains are often irrelevant for therapeutic antibody discovery, care should be taken to retain regions of the protein that are responsible for oligomerization and membrane trafficking (Jenke et al. 2003). An additional consideration for immunogen design is whether auxiliary proteins such as beta or gamma subunits (Tao and MacKinnon 2019), or calmodulin for some Kv and KCa channels (Wang and MacKinnon 2017; Lee and MacKinnon 2018; Whicher and MacKinnon 2019; Sun and MacKinnon 2020) need to be co-expressed and purified along with the primary alpha channel subunit.

Extraction of K^+ channels from membranes typically requires solubilization of the lipid bilayer with detergents, preferably those deemed more gentle and likely to stabilize the channel such as the maltoside series of non-ionic detergents (e.g., DM, DDM, Cymal-series). Additionally, incorporation of cholesterol analogs (e.g., cholesteryl hemisuccinate), phospholipids (alone or in mixtures, e.g., POPC, POPG, POPE, POPA, POPS) can also help preserve activity of detergent-stabilized K^+ channels (Hansen et al. 2011; Whorton and MacKinnon 2011; Sun and MacKinnon 2020).

While some antibody discovery programs have utilized ion channels in detergent solution (Brohawn et al. 2013; Shcherbatko et al. 2016), reconstituting the target in a membrane-like environment is the preferred option to stabilize the channel in preparation for immunization, screening, and binding assays. Liposomes, which are composed of synthetic or natural lipids that are sonicated or extruded to obtain spherical unilamellar membranes, are the most commonly used formulations for this purpose. Following reconstitution into liposomes (Seddon et al. 2004), the resulting proteoliposomes can be used directly for immunization, as well for screening antibody libraries and testing channel activity in flux assays (Wang and Sigworth 2009; Bednenko et al. 2018; Lee and MacKinnon 2018). Nevertheless, a drawback

of liposome formulations is the difficulty in controlling the orientation of a reconstituted membrane protein which is dependent on various empirical factors, including the reconstitution method (Yanagisawa et al. 2011), lipid composition (Hickey and Buhr 2011), and protein structure. This issue can be addressed via engineered tags located on intracellular loops or termini that can tether a protein to a solid support prior to reconstitution into lipids, thereby forcing the channels to orient with the extracellular loops facing out (Sumino et al. 2017). Such approaches can be effective for screening phage or yeast display libraries to preferentially recover antibody fragments that recognize surface epitopes.

In recent years, numerous surfactants have been developed for keeping membrane proteins stable in detergent-free solutions. Such surfactants offer additional options for presenting K^+ channels during immunization and screening and fall into two general categories: (1) polymer-based surfactants such as amphipols (Tribet et al. 1996; Popot et al. 2003) along with styrene-maleic acid (SMA) copolymers (Lee et al. 2016a); and (2) peptide-based surfactants that are variations on membrane-interacting proteins such as saposins or apolipoproteins (Frauenfeld et al. 2016; Denisov et al. 2004; Denisov and Sligar 2016). Each of these platforms results in disc-like membrane protein-surfactant complexes that theoretically expose both the intracellular and extracellular domains of the protein in the same complex. Currently, the most established surfactant technology is the protein-based nanodisc (Denisov et al. 2004; Denisov and Sligar 2016) in which a dimer of an engineered apoA1 construct (Membrane Scaffold Protein, or MSP) encircles a lipid membrane containing an embedded membrane protein. This approach has been used successfully in the reconstitution of a number of K^+ channels (Xu et al. 2015; Matthies et al. 2018; Shenkarev et al. 2018; Sun and MacKinnon 2020). Supporting their utility in antibody discovery, membrane protein nanodiscs have been used to identify Fab fragments from phage display libraries (Dominik et al. 2016). Amphipols, such as the polyacrylate derivative A8–35 and PMAL-C8, have been used successfully to stabilize K^+ channels (Spear et al. 2015; Lee et al. 2017) and amphipol formulations have been employed to generate monoclonal antibodies against other membrane protein antigens in rodents (Agosto et al. 2014; Vij et al. 2018; Storek et al. 2019). SMA lipoparticles (SMALPs) have also been demonstrated to solubilize K^+ channels (Dörr et al. 2014), including human channels expressed in mammalian cells (Karlova et al. 2019) but, to date, there have been no reports of their use in antibody discovery.

3.4 Antibody Platforms and Initial Phases of Screening

As noted above, the decision tree leading to antibody discovery (Fig. 1) requires a fundamental choice between the use of animal immunization, surface display libraries, or some combination of the two. Each of these approaches has advantages and disadvantages and, with sufficient resources, an argument can be made to pursue parallel paths.

In the case of animal immunization, one has the opportunity to generate antibodies with high affinity that arise naturally through a process of reiterative B-cell selection following somatic hypermutation of heavy and light chain genes in the germinal centers of the spleen and secondary lymph nodes of vaccinated animals. The principal downsides of animal immunization are the potential for tolerance mechanisms to interfere with antibody production and the possibility that the immune system will focus the B-cell response on so-called immunodominant epitopes (to the exclusion of others) that are irrelevant with respect to therapeutic potential of resulting antibodies. Additionally, while antibodies produced against a given target may, in fact, be excellent drug candidates, downstream manufacturing of those antibodies requires linking phenotype (that is, antibody binding and/or modulating activity) to genotype (or more precisely, the sequences of the hypervariable regions of the heavy and light chain genes that produce the antibody). Accomplishing this requires identification and isolation of individual B-cell clones responsible for antibody production, which, until recently, relied on standard mouse hybridoma technology that has since been expanded to other species (Parray et al. 2020). The ability to immortalize human B-cells (Kwakkenbos et al. 2016), along with single-cell methods that permit high-throughput screening of secreted antibodies from large numbers of B-cell clones is proving to be extremely powerful as well and may eventually supplant hybridoma technology altogether (see below).

In choosing to immunize, a variety of factors including animal species/strain, antigen dose, choice of adjuvant, route of injection, vaccination schedule must all be considered before initiating the process. With regard to the animal host, specialized mouse strains, as well as evolutionary distant species provide opportunities to overcome tolerance mechanisms limiting the response to highly conserved channel proteins (Sect. 2.2; Table 1). At the same time, antibodies produced in llamas, cows, and sharks have unique structures that may be well suited to blocking channel activity (see below).

Although standard mouse lines (especially BALB/c) have long been used for monoclonal antibody production, the development of hyperimmune strains that produce autoantibodies such as DiversimAb (Abveris, abveris.com, Canton, MA, USA) and others (Perry et al. 2011; Lee et al. 2012), as well as mice that overexpress the neonatal Fc receptor (Cervenak et al. 2015; Schneider et al. 2015) has proven to be useful in generating immune responses to antigens with high homology to endogenous mouse proteins. Additionally, tolerance issues should be precluded in knock-out mice that do not express channels of interest (Hrabovska et al. 2010) and strains lacking *KCNA3*, *KCNA5,* and *KCNN4* genes all appear to be viable (London et al. 2001; Koni et al. 2003; Begenisich et al. 2004). Finally, in addition to engineered mouse strains, evolutionary distant species provide another option to overcome tolerance mechanisms. Chickens, in particular, are capable of generating high-affinity antibodies against broad sets of epitopes on mammalian proteins (Abdiche et al. 2016; Bednenko et al. 2018). Indeed, Bednenko and co-workers identified 9 functional anti-Kv1.3 mAbs following immunization of a single animal with full-length Kv1.3 expressed recombinantly in *T. thermophila* (Bednenko et al. 2018) (Sect. 4.1).

Along with chickens and mice, other species have garnered attention from ion channel researchers based on their ability to generate antibodies with unusual structures. In cows, for example, roughly 10% of antibodies contain ultra-long CDRH3 heavy chain loops with protruding "stalk-and-knob" structures that extend away from the main antibody scaffold and play a predominant role in antigen binding (Wang et al. 2013; Dong et al. 2019; Stanfield et al. 2018, 2020). Researchers have explored the use of these unique structures in the development of broadly neutralizing therapeutic antibodies against HIV (Sok et al. 2017). Furthermore, the "knob" domains of cow antibodies have been modified to incorporate various polypeptides including protease inhibitors and cytokines, as well as peptide toxins that have been shown to block the activity of Kv1.3 (Zhang et al. 2013a, b; Liu et al. 2015; Wang et al. 2016).

In camelids (i.e., llamas, alpacas, and camels) and cartilaginous fish (sharks), a subset of immunoglobulins (or immunoglobulin-like molecules in the case of sharks) is entirely devoid of light chains (Könning et al. 2017). The variable domains of these heavy chain-only isotypes (referred to as VHHs, or nanobodies, in camelids and V-NARs, for New Antigen Receptors, in sharks) represent autonomous antigen-binding units. While having different evolutionary origins (Flajnik et al. 2011; English et al. 2020), camelid nanobodies and V-NARs share an immunoglobulin-like fold along with a number of other features including small size (12–15 kDa), protruding CDR3 loops (up to 40 amino acids in length), enormous structural diversity, modular design, and high stability and solubility (Könning et al. 2017; Mitchell and Colwell 2018; Feng et al. 2019). Camelid VHH genes are highly homologous to human VH genes while shark V-NARs arose from a non-immunoglobulin lineage. Generally, camelid nanobodies exhibit low immunogenicity in humans but nevertheless require humanization (Vincke et al. 2009), a process which is far more challenging for V-NAR sequences (Steven et al. 2017).

Over the past decade, camelid nanobodies have dominated the single-domain antibody discovery field and are viewed as next generation tools for use in medical and biotechnological applications (Hoey et al. 2019). Nanobody-derived products are at various stages of clinical development and at least one (caplacizumab; Cablivi) received FDA approval in 2019 for treatment of acquired Thrombotic Thrombocytopenia Purpura (aTTP) (Hanlon and Metjian 2020; Jovčevska and Muyldermans 2020; Kaplon et al. 2020). A number of promising shark single-domain antibodies, including a V-NAR against the ion channel, P2X3, are currently in preclinical development (English et al. 2020; ww.ossianix.co.uk).

As with bovine antibodies, single-domain entities can access buried epitopes, such as enzyme active sites and, potentially, the transmembrane pores of ion channels. Small size, high tissue/tumor penetration, and fast renal clearance make single-domain antibodies particularly useful for in vivo diagnostics. To enable half-life extension (HLE) for therapeutic purposes, nanobodies have either been PEGylated, engineered in a bivalent or multivalent format with an albumin-binding domain, or fused to human antibody scaffolds containing the Fc domain. For example, trivalent anti-P2X7 and anti-Kv1.3 nanobodies with extended half-lives have been developed that contain two identical channel blocking domains linked to

an anti-albumin binding domain (Danquah et al. 2016; Stortelers et al. 2018). Other examples of engineered multivalent nanobodies include a biparatopic dimer targeting distinct epitopes of CXCR2 (Bradley et al. 2015) and an engineered fusion of the anti-mGluR1 IgG with the blood brain barrier-penetrating nanobody FC5 (Webster et al. 2016).

As indicated above, regardless of the species, antibodies developed in animals must be humanized in order to limit immunogenicity in therapeutic applications. This is a complex process and often needs to be coupled to affinity maturation. An alternative strategy relies on the immunization of transgenic animals carrying human immunoglobulin loci (Green 2014). The first rodent strains that utilized this approach, the HuMAbMouse® and XenoMouse® (Lonberg et al. 1994; Mendez et al. 1997), produced fully human antibodies, while subsequent versions (the OmniRat and VelocImmune mouse) focused on substituting human variable domains for the rodent versions without modifying rodent constant regions allowing for a more efficient immune response in immunized animals (Osborn et al. 2013; Murphy et al. 2014). Following hit identification, human constant regions are appended to fully human variable regions using standard molecular biology tools. To date, more than 20 monoclonal antibodies discovered in transgenic mice have been approved by the FDA (Lu et al. 2020) and many different species – rats, chickens, cows – have been transformed to create human antibody-producing factories. Some humanized commercial platforms not listed above include IntelliSelect (kymab.com), OmniMouse, OmniChicken, OmniFlic, OmniClic (omniab.com), H2L2 Mouse and HCAB (harbourantibodies.com), Trianni mouse (Trianni) and Tc-bovines (sabbiotherapeutics.com).

As a way around animal immunization, investigators have the option to screen combinatorial libraries that display variable regions of antibodies on the surfaces of either phage particles or microbial cells (bacteria or yeast) to screen for potential binders to K^+ channels of interest. Surface display technologies have been in widespread use for more than two decades and have seen enormous advancement in both the underlying display platforms and types of molecules that are displayed, and in the case of antibodies, allow the user to mine vast numbers (on the order of 10^{12} or more) of heavy and light chain pairings from naïve or immune animals, while at the same time, have access to their sequence information.

Variations on the types of surface display platforms and their relative advantages and disadvantages are too great to adequately cover in this chapter, although several excellent reviews are available in the literature (Almagro et al. 2019; Kunamneni et al. 2020; Ministro et al. 2020). Briefly, the libraries themselves are classified as immune, naïve, synthetic and semi-synthetic depending on the source material and the way in which the libraries are constructed. Naïve libraries are antigen independent and are constructed from non-rearranged V gene segments from humans or animals, as well as synthetic and shuffled V genes. In some respects, they are considered "universal" libraries since they are used to screen for binders to essentially any target. Immune libraries, on the other hand, are constructed from humans or animals that have been immunized or are expected to contain antibodies against specific proteins as a result of a medically relevant condition. While these are

typically more circumscribed in their overall sequence coverage, V_H and V_L regions have already undergone gene rearrangement and somatic hypermutation in vivo, thereby increasing the likelihood of identifying high affinity binders to the antigen of interest. By contrast, identification of antibody fragments with high binding activity displayed on naïve, synthetic, or semi-synthetic libraries often depends on the size of the library being screened. As suggested by their names, much of the diversity of V_H and V_L domains in synthetic and semi-synthetic libraries is generated artificially through oligonucleotide synthesis and/or random in vitro assembly of V and DJ gene segments. In either case, such libraries are commercially available and can be mined in-house or on a fee-for-service basis using commercial suppliers.

One of the inherent issues with combinatorial antibody display libraries has been the non-native pairing of heavy and light chains generated during library construction that can give rise to antibodies having lower affinity and selectivity than bona fide immunoglobulins produced in response to immunization. Additionally, non-natively paired immunoglobulin can be prone to less than desirable biophysical properties (e.g., aggregation, etc.) although more recent engineering approaches to library design and construction have mitigated these drawbacks. Using a novel single-cell emulsion and oligo-dT mRNA capture approach, Wang and co-workers have recently succeeded in retaining the native V_H and V_L pairings in scFv expressing yeast display libraries prepared from human patient plasmablasts (Wang et al. 2018). This approach yielded broadly neutralizing antibodies against HIV, as well as high-affinity antibodies to the Ebola virus glycoprotein and influenza HA (Wang et al. 2018) and will likely have numerous applications in antibody discovery in the future.

3.5 Functional Screening Assays

Following generation of anti-K^+ channel antibodies in immunized animals, or, alternatively, interrogation of surface display antibody libraries, screening to identify mAbs with desired characteristics typically requires a multi-tiered approach. Generally, initial antibody screening establishes specificity and identifies binders that recognize the target K^+ channel. Protein-based binding assays such as ELISA and HTRF-FRET are highly sensitive, amenable to high throughput and can be used to screen unpurified samples such as hybridoma supernatants and bacterial periplasmic extracts. However, cell-based assays (e.g., flow cytometry) using either endogenous or recombinant cell lines expressing the K^+ channel of interest in a more physiologically relevant conformation can offer a significantly more robust method to identify surface binders that are more likely to modulate channel function. Nevertheless, while the latter is typically viewed as superior to the former, this is not always the case where, for example, antibodies bind an exposed epitope on a purified protein preparation in an ELISA format that is not necessarily exposed on cells owing to the particular state of the channel.

Identification of antibody binders is typically followed by functional screening to isolate clones capable of modulating K^+ channel function. Analytical methods used

for functional screens can be broadly divided into ion flux-based assays and electrophysiology that directly measures electrical currents produced by potassium ion movement through the channel. Ion flux assays include methods that use radioactive tracers such as ^{86}Rb that can pass through K^+ channels and can be detected by atomic absorption spectrometry (Karczewski et al. 2009). However, hazards associated with radioactivity can make these assays inconvenient to perform (Gill et al. 2003; Yu et al. 2016) and alternative methods using ion sensitive dyes have been developed and are preferable for some laboratories. For example, K^+ channels are also permeable to thallium (Tl^+) ions, and Tl^+-sensitive dyes have been developed and applied to K^+ channel screens (Weaver et al. 2004). In Tl^+-based ion flux assays, cells expressing the K^+ channel of interest are initially loaded with a non-fluorescent, Tl^+-specific, and membrane permeant reporter dye such as the BTC-AM or FLUXOR. Tl^+ is added to the medium and, following stimulation, flows through the open channel, activating the dye, thereby generating a fluorescent signal that can be measured by imaging plate-readers (Beacham et al. 2010). Tl^+ flux assays have been used to identify modulators of multiple types of K+ channels including voltage-gated (Cheung et al. 2012; Yu et al. 2016) inward rectifier (Wang et al. 2011; Kaufmann et al. 2013; Wydeven et al. 2014; Swale et al. 2016), two-pore domain (Sun et al. 2016; Wright et al. 2017), and K_{Ca} channels (Jørgensen et al. 2013).

An alternative method of detecting ion flux is based on monitoring the change of membrane potential induced by movement of ions across the membrane using a voltage-sensitive dye. Multiple voltage-sensitive dyes utilizing different mechanisms have been developed (Miller 2016) and include: (1) electrochromic probes such as amino-naphthyl ethenyl-pyridinium (ANEP) dyes whose fluorescence spectra change in response to changes in the surrounding electric field. ANEP dyes can detect sub-millisecond membrane potential changes, but typically suffer from low sensitivity; (2) lipophilic redistribution dyes (e.g., oxonol dye DiBAC4) that have superior sensitivity, but slower response times; and, (3) fluorescence resonance energy transfer (FRET) based probes which typically combine high sensitivity with fast response times (Yuan et al. 2013). Additionally, a new type of voltage-sensing dye has been developed in recent years that utilizes a photo-induced electron transfer (PET) mechanism (Kulkarni et al. 2016).

The direct measurement of electrical currents by patch-clamp electrophysiology is still generally considered the gold standard for ion channel research, with the whole-cell patch-clamp configuration being widely used for investigating ion channels and membrane potential. The whole-cell patch-clamp technique uses a glass micropipette electrode to form a high resistance seal with the cell membrane allowing it to record and manipulate ionic current across the whole-cell membrane (Liu et al. 2019). The inherent flexibility of manual patch recording makes it ideal for characterizing ion channel functional properties as well as investigating mechanisms of action of modulating compounds. Indeed, several modulating antibodies targeting K^+ channels have been successfully characterized using manual patch clamp techniques including Kv1.3 (Bednenko et al. 2018) and Eag1 (Gómez-Varela et al. 2007). For screening purposes, manual patch clamp is hampered by several factors

including the fact that it is labor-intensive, can only be performed by highly trained experimentalists, and has relatively low throughput.

To meet the demand for large-scale screening of drug candidates, several automated patch clamp systems have been developed that can be divided into two types, based on their patching system (Dunlop et al. 2008; Annecchino and Schultz 2018). The first type inherited the glass micropipette of conventional patch clamp and includes Flyscreen, AutoPatch, and roboPatch for mammalian cells, and the robocyte17 and OpusXpress 6000A for Xenopus oocytes. More popular automated platforms use micro-fabricated planar electrode-based patch clamp that replaces the glass micropipette in conventional patch clamp and include IonWorks Barracuda and PatchXpress from Molecular Devices (Xu et al. 2003; Kuryshev et al. 2014; Cerne et al. 2016), Patchliner and Syncropatch 384/786i from Nanion (Farre et al. 2009; Obergrussberger et al. 2018), and QPatch and Qube 384 from Sophion (Asmild et al. 2003; Chambers et al. 2016).

Compared to manual patch clamp, the appeal of automated platforms is their semi-high throughput capabilities that enable compound library screening or acquisition of multiple data points in a single experiment. Nevertheless, automated systems suffer from some limitations including compatible cell types (typically recombinant as opposed to primary), inability to establish long recording times and generally lower quality recordings compared to manual patch (Annecchino and Schultz 2018), though the latter continues to improve in association with platform advancements (Bell and Dallas 2018). Based on their respective advantages and disadvantages, automated platforms are typically involved in early drug discovery while confirmation and in-depth characterization of compounds of interest rely on manual patch clamp techniques.

It is important to note that whichever approach is used, screening antibodies has at least two unique challenges. First, any functional effect bestowed by an antibody can reasonably be expected to be directly related to the kinetics of association and dissociation from the target on a timescale that will likely be represented in minutes not seconds as is typically observed with small molecule modulators. Second, a number of antibodies targeting K+ channels demonstrate some level of state-dependency (e.g., Kv1.3 [Colussi, personal communication], Eag1 [Gómez-Varela et al. 2007]) that should be considered when designing screening experiments in order to mitigate the potential of false-negative results. Such state-dependency may depend on expression of the channel in its native context and be missed completely in recombinant cell lines.

4 Current Status of the Field

Despite the challenges associated with antibody generation and screening, successful attempts to produce antibodies that can modulate the activities of K^+ channels have been made. While these antibodies are at various stages of clinical development, we fully anticipate that therapeutic antibodies targeting K^+ channels will find their way into clinical use for a wide range of human diseases in the not too distant future.

4.1 Kv1.3

Based on its essential role in the activation and proliferation of chronically stimulated effector memory T-cells (T_{EM} cells), the voltage-gated Kv1.3 channel is a well-established drug development target for autoimmune and inflammatory diseases such as type 1 diabetes, psoriasis, and rheumatoid arthritis (reviewed in Feske et al. 2015; Chandy and Norton 2017). Additionally, Kv1.3 is expressed in a variety of other blood cells including platelets (McCloskey et al. 2010; Feske et al. 2015; Fan et al. 2020) where it is thought to play a role in platelet-dependent thrombosis (see below). It is also considered a key therapeutic target for neuroinflammatory diseases (Wang et al. 2020) and there is accumulating evidence that inhibition of the Kv1.3 channel may be beneficial in cancer therapy (Teisseyre et al. 2019). While a variety of small molecules, peptides, and peptide/antibody fusions have been reported to downregulate Kv1.3 activity (Schmitz et al. 2005; Wang et al. 2016; Chandy and Norton 2017), there remains a substantial need for selective anti-Kv1.3 monoclonal antibodies that can be used therapeutically in humans.

To develop antibodies against the Kv1.3 channel, Bednenko et al. utilized a powerful *Tetrahymena thermophila* protein expression system to express and purify multi-milligram quantities of Kv1.3 formulated as particulate antigens on polysterene beads, magnetic beads, or proteoliposomes (Bednenko et al. 2018). Two phylogenetically diverse species, chickens and llamas, were used to generate antibodies. Chickens were immunized with Kv1.3-containing proteoliposomes, and individual B-cell clones were screened for the production of antibodies against Kv1.3 displayed on magnetic or polystyrene beads using gel-encapsulated microenvironment (GEM) assays (Izquierdo et al. 2016). Llamas were immunized with a DNA vaccine encoding Kv1.3 and boosted with Kv1.3 proteoliposomes, followed by construction of phage libraries displaying single-chain variable-region antibody fragments (scFv) with non-native heavy and light chain pairings. Libraries were then screened using recombinant Kv1.3 linked to beads to identify scFv clones with binding activity. Finally, chicken antibodies or llama scFv fragments were used to construct chimeric scFv-Fc clones that were analyzed by whole-cell patch clamp electrophysiology with L929 mouse fibroblasts transiently expressing human Kv1.3. Nine chicken and one llama antibody were found to inhibit Kv1.3 activity by 44–82% within 10 min of antibody addition. Select antagonist mAbs demonstrated high potency (IC50 $<$ 10 nM), cross-reactivity to rat and cynomolgus monkey Kv1.3, and significant selectivity over related Kv1.x family members, hERG and Nav1.5.

In independent studies with llamas, genetic immunization enabled the production of nanobodies that successfully blocked Kv1.3 function as demonstrated by electrophysiology (Steeland et al. 2016; Stortelers et al. 2018). These nanobodies recognized the first extracellular loop (ECL1) of Kv1.3 and cross-reacted with rat and cynomolgus monkey Kv1.3 channels. In addition, they displayed functionality in primary T cell assays. Formatting monovalent nanobodies into bivalent and trivalent constructs resulted in increased potency. Additionally, a construct

comprised of two monovalent nanobody components and one albumin-binding nanobody component (Nb12–12-alb11) was created, and it demonstrated efficacy in vivo in a rat delayed-type hypersensitivity model.

Recently, Fan and co-workers utilized mouse hybridoma technology to develop a monoclonal antibody (6E12#15) against a 14 amino acid peptide EADDPTSGFSSIPD corresponding to a portion of the extracellular loop 3 (ECL3) of human Kv1.3 (Fan et al. 2020). This antibody recognized both the human and mouse Kv1.3 channels and inhibited their activity in transfected HEK293 cells. Consistent with the important role Kv1.3 plays in the regulation of membrane potential and calcium signaling in platelets (McCloskey et al. 2010), mAb 6E12#15 inhibited aggregation, adhesion, and activation of platelets isolated from humans and wild-type mice but not mice lacking the *KCNA3* gene. Moreover, 6E12#15 impaired platelet-driven thrombus formation when injected into wild-type, but not *KCNA3* knock-out mice, opening up the possibility for developing therapeutics for the treatment of platelet-dependent thrombosis.

4.2 Kv10.1

Kv10.1, or Eag1, belongs to the ether-á-go-go (EAG) subfamily of voltage-gated K^+ channels and it is recognized for its pathological overexpression in solid tumors (Cázares-Ordoñez and Pardo 2017; Barros et al. 2020). In healthy human tissues, Kv10.1 is primarily expressed in the brain. Kv10.1 is implicated in a variety of cellular processes, including proliferation, migration, and adhesion, as well as cell cycle progression. In addition, Kv10.1 has been genetically linked to two developmental disorders, Temple-Baraitser syndrome and Zimmermann-Laband syndrome (Kortüm et al. 2015; Simons et al. 2015).

In the search for specific inhibitors of Kv10.1 that did not interfere with the function of other EAG family members such as Kv10.2 and Kv11.1 (hERG), Gómez-Varela et al. developed a monoclonal antibody, mAb56, using standard mouse hybridoma technology (Gómez-Varela et al. 2007). A 79 residue ECL3 sequence fused to the C-terminal Kv10.1 tetramerization domain was used to immunize mice (Hemmerlein et al. 2006). Following hybridoma construction, mAb56 was selected based on its ability to bind Kv10.1 and to inhibit Kv10.1 activity, but not hERG currents in whole-cell patch clamp experiments with Kv10.1-expressing HEK293 cells (~40% inhibition of current amplitude with an IC50 of ~73 nM). mAb56 displayed cross-reactivity with rat and mouse Kv10.1, and its cognate epitope has been mapped to a linear peptide, GSGSGKWEG, near the middle of ECL3. Consistent with a role for Kv10.1 in cancer, mAb56 has been shown to decrease proliferation of cancer cells in vitro and to reduce tumor growth in mouse xenograft models (e.g., MDA-MB-435S human breast cancer cell xenografts; PAXF1657 pancreatic cancer patient-derived xenografts). The mAb56 antibody is commercially available through Sigma-Aldrich (catalog # MABN378) and has been used as a highly selective Kv10.1 inhibitor for research purposes (Chen et al. 2011; Hernández-Reséndiz et al. 2020).

Ideally, therapeutics against cancer should not only reduce tumor growth, but also mediate cancer cell death. Toward that end, Hartung et al. designed an anti-Kv10.1 antibody fusion with TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) with the goal of inducing apoptosis in Kv10.1-positive cancer cells (Hartung et al. 2011, 2020; Hartung and Pardo 2016). In the first set of studies, mAb62 antibody, which was selected in the same immunization campaign as mAb56 (Hemmerlein et al. 2006; Gómez-Varela et al. 2007), was used as a template to generate the single-chain variable fragment, scFv62, and then a trimeric scFv62-TRAIL fusion. mAb62 has been shown to interact with Kv10.1, but not with its closest relative Kv10.2. Unlike mAb56, however, mAb62 did not inhibit Kv10.1 K^+ currents, and its linear epitope, DYEIFDEDT, was located within the N-terminal portion of ECL3, proximal to the mAb56 epitope. The scFv62-TRAIL fusion induced apoptosis in cancer cells when used in combination with cytotoxic drugs, such as doxorubicin (Hartung et al. 2011; Hartung and Pardo 2016).

A third antibody with high affinity for ECL3 of Kv10.1, VHH-D9, was developed by immunization of llamas with a Kv10.1 ECL3-containing construct similar to the one used in the mAb56 and mAb62 development campaigns. This single-domain antibody (nanobody) was then fused to three TRAIL-encoding sequences in tandem to produce the potent apoptosis-inducing construct VHH-D9-scTRAIL (Hartung et al. 2020). Together, these studies suggest a promising approach to cancer therapy that utilizes antibody fusion constructs to target pro-apoptotic factors (in this case TRAIL) to the surface of tumor cells.

4.3 Kv11.1 (hERG)

Another member of the EAG subfamily, Kv11.1 (in humans, referred to as hERG), plays a fundamental role in cardiac repolarization. While hERG is also ectopically expressed in many types of cancer cells and may play a role in cancer progression (He et al. 2020), suppression of Kv11.1 function in vivo, either by genetic mutation or, in some cases, by administration of small molecule drugs, can lead to the potentially fatal long QT syndrome (Barros et al. 2020; Garrido et al. 2020). Since the vast majority of drugs associated with QT prolongation have been shown to interact with hERG, lead compounds are counter-screened against human Kv11.1 early in drug discovery to rule out potential interactors.

Despite this, antibodies against hERG that specifically recognize intracellular epitopes within the N-terminal portion of the protein have been developed as biochemical, immunofluorescence, and electrophysiological tools (Hausammann et al. 2013; Harley et al. 2016). In the first instance (Hausammann et al. 2013), mice were immunized with the purified full-length hERG expressed in *Spodoptera frugiperda* Sf9 cells. Twelve hERG-specific monoclonal antibodies were identified based on their ability to bind hERG in detergent-permeabilized hERG-expressing CHO cells. Most antibodies in this series recognized linear epitopes within the intracellular region spanning amino acid residues 130 through 320 corresponding to the disordered linker region between the N-terminal Per-Arnt-Sim (PAS) domain

and the voltage-sensor domain. Antibodies developed in this study were utilized to develop sensitive ELISA-based protocols for quantification of hERG in cell lysates and membrane preparations.

Harley and colleagues developed and characterized single-chain variable fragments recognizing the PAS domain of Kv11.1 (Harley et al. 2016). In this case, a phage display library containing scFv fragments derived from chickens immunized with the N-terminal region of the protein (amino acid resides 1–135) was screened with the purified PAS domain. Following biochemical and functional characterization of various scFv fragments, two clones, designated scFv 2.10 and scFv 2.12, were selected. Both were able to modulate hERG activity in whole-cell patch electrophysiology assays when delivered into the cytoplasm of either hERG-expressing HEK293 cells or stem cell-derived human cardiomyocytes via the patch pipette. Interestingly, both antibodies appeared to augment hERG channel activity although by different mechanisms, consistent with them interacting with two different regions in the PAS domain.

4.4 TASK3

The TWIK-related Acid-Sensitive K^+ channel 3 (TASK3) is a member of the K2P family of potassium channels expressed primarily in the central nervous system (Bittner et al. 2010; Feliciangeli et al. 2015). TASK3 is also overexpressed in cancer cells. Expression of the TASK3-encoding *KCNK9* gene was found to be amplified threefold to tenfold in ~10% of breast tumor samples (24 out of 247) and *KCNK9* mRNA expression was elevated between 2 and >100-fold in 36% of lung tumors and in 44% of breast tumors (Mu et al. 2003). Furthermore, wild-type TASK3, but not the G95E mutant with minimal-to-no channel activity, promotes tumorigenesis in mice, presumably by enhancing cancer cell survival under hypoxic or serum-deprived conditions (Pei et al. 2003). Based on these findings, TASK3 has generated interest as a potassium channel target for cancer therapy development.

To develop a monoclonal antibody against TASK3, Sun and colleagues (Sun et al. 2016) took advantage of a specific structural feature of the K2P channels, a large extracellular cap that extends above the selectivity filter (Brohawn et al. 2012; Miller and Long 2012; Dong et al. 2015; Rödström et al. 2020). A 59 amino acid region of the human TASK3 extracellular loop 1 corresponding to the cap-forming domain was expressed in mammalian cells as a fusion protein with the Fc domain of mouse IgG2a and then used to immunize mice (Sun et al. 2016). The most potent monoclonal antibody, Y4 (an IgG1), demonstrated binding to human TASK3 with sub-nanomolar affinity, cross-reactivity with mouse TASK3, and selectivity when tested against other human K2P family members. Although no inhibitory effect on channel activity was observed in electrophysiology experiments after relatively short periods of incubation and recording (20 min), Y4 functionality was demonstrated in ion flux assays using human TASK3-expressing HEK293 cells after preincubation for >6 h. The delayed effect of Y4 is presumably due to antibody-induced internalization of the channel which has been shown to occur in both recombinant TASK3-

expressing HEK293 cells as well as in selected human and murine cancer cell lines (Sun et al. 2016). Importantly, following prolonged incubation (24–72 h), the Y4 antibody resulted in decreased viability of TASK3-expressing cancer cells, an effect exacerbated when the cells were grown in low serum. Furthermore, Y4 suppressed tumor growth and metastasis in vivo when administered to mice (Sun et al. 2016).

5 Concluding Remarks

Targeting potassium channels with antibodies remains a challenging endeavor. However, the ability to achieve high selectivity, potency, and favorable pharmacokinetics using biologics that can modulate the activity of a wide range of K^+ channels implicated human disease make this approach well worth the effort. Indeed, the growing list of functionally active K^+ channel antibodies identified to date, along with concomitant advances in target expression, formulation and methods for antibody discovery, suggests that this burgeoning field will continue to expand leading ultimately to novel and effective antibody therapeutics.

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