mAbs 6:3, 577–618; May/June 2014; © 2014 Landes Bioscience

Antibody Engineering & Therapeutics, The Annual Meeting of The Antibody Society

December 8-12, 2013, Huntington Beach, CA

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Keywords: antibody-drug conjugate, bispecific antibodies, protein scaffolds, antibody therapeutics,

Abbreviations: AID, activation-induced cytidine deaminase; aa, amino acid; ADC, antibody-drug conjugate; ADCC, antibody-dependent cell-mediated cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; bNAbs, broadly neutralizing antibodies; CDR, complementarity-determining region; CDC, complement-dependent cytotoxicity; DAR, drug:antibody ratio; EGFR, epidermal growth factor receptor; FACS, fluorescence-activated cell sorting; GPCR, G-protein-coupled receptor; HER, human epidermal growth factor receptor; HIV, human immunodeficiency virus; HVL, hypervariable loop; IL, interleukin; MS, mass spectrometry; NK, natural killer; NGS, next-generation sequencing; PK, pharmacokinetics; TNF, tumor necrosis factor

The 24th Antibody Engineering & Therapeutics meeting and The Annual Meeting of The Antibody Society brought together a broad range of participants who updated attendees on the latest advances in antibody research and development. Sessions were organized by distinguished members of The Antibody Society and the meeting was seamlessly implemented by IBC Life Sciences. Preconference workshops on 3D modeling and delineation of clonal lineages were featured, and the conference included sessions on a wide range of topics at the forefront of our field that are very important for researchers: a Keynote Session that discussed the role of systems biology in immunobiology, antibody and vaccine engineering; antibody deep sequencing and repertoires; the effects of antibody gene variation and usage on antibody response; directed evolution; knowledge-based antibody and protein design; antibodies in a complex environment; polyreactive antibodies and polyspecificity; the interface between antibody therapy and cellular immunity in cancer; antibodies in cardiometabolic medicine; antibody pharmacokinetics, distribution and off-target toxicity; optimizing antibody formats for immunotherapy; polyclonals, oligoclonals and bispecifics; antibody discovery platforms; and antibody-drug conjugates.

Note: The views expressed herein are those of the authors and do not necessarily reflect the views of their respective employers. Speakers who wrote a summary of their own presentation are listed in the header for the relevant session. The majority of the other summaries in this report were based on PDFs of the presentations provided by speakers or on post-meeting input from the speakers. The authors thank all speakers who contributed to the report. When speakers could not contribute either the PDF or input, detailed summaries of their presentations were not prepared, although the names, affiliations and topics of all speakers were included in the report.

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Submitted: 02/20/14; Accepted: 02/20/14; Published Online: 03/03/2014
http://dx.doi.org/10.4161/mabs.28421

Pre-conference Workshop
Three-dimensional Structure Antibody Modeling

Juan Carlos Almagro, Gary L Gilliland

The success of antibody-based drugs has fueled the refinement of three-dimensional (3D) structure antibody modeling methods as a critical component of the antibody engineering process to generate more efficacious, safer and developable therapeutic antibodies. In this workshop organized as part of the 24th annual Antibody Engineering & Therapeutics meeting, the state of the art in 3D modeling of the variable region (Fv) of antibodies was discussed. Eleven unpublished, high-resolution x-ray Fab crystal structures (provided by investigators at Janssen R&D, LLC and The Scripps Research Institute) from diverse species and covering a wide range of antigen-binding site conformations were used as a benchmark to compare Fv models generated by seven structure prediction methodologies, including Accerlys, Inc, Chemical Computer Group, Schrödinger, Gray's laboratory at John Hopkins University, Macromoltek, Astellas/Osaka University and PIGS (Prediction of ImmunoGlobulin Structure). The modelers presented their results and discussed opportunities to improve current methodologies. The assessment group presented on the overall quality of the models, and the relative strengths and weaknesses of the methods and compared of the results with the first antibody modeling assessment conducted two years ago.[1]

Juan Carlos Almagro (Pfizer, Inc.) and **Gary Gilliland** (Janssen R&D, LLC), organizers of the workshop, introduced the agenda, speakers and goals of the workshop. In discovery of potential antibody therapeutics, the initial focus is on the desired binding profile and mechanism of action. Nevertheless, Dr. Almagro highlighted the equal importance of engineering antibodies with the appropriate developability profile, i.e., high solubility, low aggregation, long high-life, high human content, and thus, low immunogenicity in therapeutic settings, as well as cross-reactivity with relevant orthologs to be able to conduct proof of concept and toxicology studies in animal models, among other desirable characteristics of antibody-based drugs. It has become apparent in the last decade that the earlier in the drug discovery process developable antibodies are engineered, the better the chances of success in developing the manufacturing and formulation processes required for the production and use of therapeutic antibodies. Central to the antibody engineering process is the availability of antibody structures to guide optimization and further development. X-ray crystallography is the gold standard method for fulfilling this need, but homology modeling also can provide guidance in a much shorter timeframe and may be the only option when crystallization is not fruitful. The question is "How reliable are the antibody models?"

The workshop aimed to address this question by presenting and discussing the results of the second 3D Antibody Modeling Assessment (AMA-II). This exercise, inspired by CASP (Critical Assessment of Protein Structure Prediction) explored the current state of the art in antibody 3D modeling and compared the results with a first assessment (AMA-I) conducted two years ago.[1] For AMA-II, modelers tackled eleven antibody Fv regions whose structures were solved, but were not yet deposited in the Protein Data Bank (PDB; www.rcsb.org/).

Dr. Gilliland (Janssen R&D, LLC) expanded on the antibody discovery and optimization processes and discussed the antibody structures used as benchmark. Nine structures were contributed by Janssen R&D, LLC and two structures were provided by Robyn Stanfield of The Scripps Research Institute (TSRI). Dr. Gilliland provided a detailed description of the x-ray statistics and refinement parameters of the benchmark structures, as well as the canonical structure classification of their hypervariable loops (HVLs).[2,3] He explained that the set of benchmark Fabs used in AMA-II was larger and more diverse than the one used in the first assessment. For AMA-II, the organizers were able to include the Fab structures of antibodies from three different sources: four human, including a lambda type antibody (the first study only included kappa type antibodies), six mouse and one rabbit.

Six modeling groups participated in AMA-II: Accelrys Software, Inc., Chemical Computing Group, Inc., Johns Hopkins University (Gray's lab), Astellas Pharma, Macromoltek, and Schrödinger, Inc. Also, the sequences of the benchmark structures were submitted to the PIGS[4] web server to generate models for comparison. The models were evaluated by the organizers relative to the crystal structures which also were provided to the participants for self-evaluation. The assessment was conducted in two stages. Similar to AMA-I, the first stage (Stage (1) of AMA-II was aimed at evaluating the quality of the Fv models generated by the participants. The second stage (Stage (2) was designed to evaluate HCDR3 modeling methods and explore how the context of the HCDR3 loop affects its structural prediction.

For Stage 1, each of the groups generated three models for every Fv sequence, each of which was produced using variations of the primary homology modeling protocols. For Stage 2, the modelers were provided with the structural coordinates of the Fv regions, excluding HCDR-3, to determine whether this structural context enabled more accurate prediction of this loop. Dr. Gilliland mentioned that there are practical instances where the Fv structure is available, but it lacks the HCDR3 loop structure, e.g., antibodies isolated from HCDR3-focused libraries designed for affinity maturation or antibodies where the loop can't be seen in the electron density map. For Stage 2, five models of HCDR3 for each of the Fv structures were generated using variations of the primary modeling protocols. The rabbit antibody was not included in the Stage 2 due to failure to obtain a reasonable model in Stage 1.

After the introductory remarks, five participants presented their results and discussed their views on the strengths and weaknesses of their corresponding modeling methodologies. The presenters were **Marc Fasnacht** (Accelrys Software, Inc.), **Brian D. Weitzner** (Jeffrey Gray's laboratory, Johns Hopkins University), **Hiroki Shirai** (Astellas Pharma/Osaka University), **Monica Berrondo** (Macromoltek) and **Kai Zhu** (Schrödinger, Inc.). In the final presentation, **Jinquan Luo** and **Alexey Teplyakov,** both at Janssen R&D, LLC, described the evaluation strategy, summarized the results, compared the quality of

the models obtained by all the participants, provided an overview of progress with respect to AMA-I and discussed challenges for the future of antibody 3D modeling. A summary of these talks follows.

Accelrys Software, Inc.

Marc Fasnacht described the methodology and results obtained at Accelrys (http://accelrys.com/). Accelrys' Antibody modeling module, distributed as part of Discovery Studio, is centered on template-based modeling. The templates are selected from an antibody database using sequence similarity to the target in the framework regions (FRs). Depending on the quality of the template hits, models of the FRs are built either using a single, chimeric or multiple template approach. The HVLs in the initial models are rebuilt by grafting the corresponding regions from suitable templates onto the model. For the HCDR3, further refined models using *ab initio* methods are obtained. Finally, the models are subjected to constrained energy minimization to resolve severe local structural problems. The analysis of the models obtained using this procedure showed that Accelrys tools allow the construction of quite accurate models for the FRs and the canonical HVLs, with backbone root mean square deviations (RMSDs) to the X-ray structure on average below 1.0 Å for most of these regions. The results also showed that accurate prediction of the HCDR3 remains a challenge.

Gray's lab at Johns Hopkins University

Brian D. Weitzner presented the results from Jeffrey Gray's laboratory at Johns Hopkins University (http://graylab.jhu.edu/). Brian explained that Gray's laboratory applies protein structure prediction methods based on Rosetta modeling tools. The application developed for antibody modeling, RosettaAntibody, can be found through the web server ROSIE (http://rosie.rosettacommons.org/). By using this application Gray's group produced accurate, physically realistic models, with all FRs from the benchmark structures and 42 of the 55 non-HCDR3 loops predicted to under 1.0 Å RMSD. The performance was notable, he explained, when modeling HCDR3 on a homology FR, where RosettaAntibody produced the best model among all participants for four of the eleven targets, two of which were predicted with sub-Å accuracy. The most common limitation was template unavailability, underscoring the need for more antibody structures or better *de novo* loop methods. In some cases, better templates could have been found by considering residues outside of the HVLs. *De novo* HCDR3 modeling remained challenging at long loop lengths, but constraining the C-terminal end of HCDR3 to a kinked conformation allowed near-native conformations to be sampled more frequently. They also found and discussed that incorrect VL:VH orientations caused models with low HCDR3 RMSDs to score poorly, suggesting that correct VL:VH orientations will improve discrimination between near native and incorrect conformations. He concluded that these observations will guide their future development of RosettaAntibody.

Astellas Pharma/Osaka University

Hiroki Shirai represented the group of Astellas Pharma/Osaka University. This group used a semi-automated template-based structure modeling approach involving several steps, including template selection for FR and canonical structures of the HVLs, homology modeling, energy minimization and expert inspection. The models they submitted in Stage 1 had RMSDs of 1.1 Å and represented the most accurate models for 4 of 11 benchmark structures. They found that the successful modeling in Stage 1 was primarily due to expert-guided template selection for HVLs, especially for HCDR3. In choosing the right template for this HVL, they based the selection on their pioneering work in developing knowledge-based rules to model HCDR3,[5] as well as the use of a scoring function called position specific scoring matrix (PSSM). Dr. Shirai mentioned that loop refinement using fragment assembly and multicanonical molecular dynamics (McMD) was applied to the HCDR3 in Stage 2. Fragment assembly and McMD produced putative structural ensembles with low free energy values that were scored based on the OSCAR (optimized side chain atomic energy) all-atom force field and conformation density in PCA (Principal Component Analysis) space, respectively, as well as the degree of consensus between the two sampling methods. The quality of 8 out of 10 targets improved as compared with Stage 1. For 4 out of 10 Stage-2 targets, the method developed by this group generated top-scoring models with RMSD values of less than 1.0 Å. In the most successful targets, the side-chain conformations were highly similar to those found in the crystal structures. Dr. Shirai also acknowledged that AMA II helped to develop the server Kotai Antibody Builder (http://kotaiab.org/) for automated antibody modeling.

Macromoltek

Monica Berrondo, CEO and founder of Macromoltek (www.macromoltek.com/), explained that Macromoltek develops tools for antibody modeling and analysis, which includes algorithms such as SmrtMolAntibody. Dr. Berrondo acknowledged that AMA-II served as their first blind test of the algorithms and that the results are guiding the further development of the software. Macromoltek's main focus has been on developing algorithms that can quickly and efficiently produce antibody structures for use in subsequent modeling and analysis, such as epitope mapping and antibody humanization. Dr. Berrondo explained that for the models to be useful for such experiments, there must be a balance between accuracy and speed, and the results should be consistent. In fact, in 10 of 11 benchmark structures, the results generated by SmrtMolAntibody showed good agreement between models and x-ray crystal structures. In Stage 1, the average RMSD was 1.4 Å. Average RMSD values for the FR was 1.2 Å and for the HCDR3 was 3.0 Å. In Stage 2, there was a slight improvement with an RMSD for the H3 loop of 2.9 Å, she concluded.

Schrödinger, Inc.

Kai Zhu presented the results of the antibody modeling group at Schrödinger, Inc. (www.schrodinger.com/). They used the antibody structure prediction methods as implemented in BioLuminate and Prime. Dr. Zhu explained that Schrödinger developed a novel knowledge-based approach for modeling the HVLs using a combination of sequence similarity, geometry matching, and the clustering of database structures. The homology models are further optimized with a physical-chemistry based

energy function VSGB2.0, which improves the model quality significantly. He pointed out that although HCDR3 modeling remained the most challenging task, their *ab initio* loop prediction performed very well in predicting the HCDR3 structures in the crystal structure context (Stage 2), and allowed improved results when refining the HCDR3 loops in the context of homology models. Excluding the rabbit antibody target—a challenge for all the modeling groups involved in AMA II—the average RMSDs for the Fv models and the FRs for the 10 targets were 1.2 Å and 0.7 Å, respectively. The non-HCDRH loop RMSDs ranged from 0.6 Å to 1.1 Å, and the HDCR3 loop RMSD was 2.9 Å. The *ab initio* HCDR3 loop predictions yielded an average RMSD of 1.3 Å when performed in the context of the crystal structure scaffold (Stage 2), and 2.7 Å in the context of the homology modeled structure (Stage 1).

Comparison of the modeling methodologies and concluding remarks

After the presentations by the five modeling groups, **Jinquan Luo** and **Alexey Teplyakov** provided an overview of the criteria used to assess strengths and weaknesses of the methods and compared the models obtained by all the modeling groups. They started by noting the basic steps to obtain an antibody model, e.g., (1) the choice of a template from the PDB for VL, VH and Fv when VL and VH template structures are from different antibodies; (2) grafting of HVLs with canonical structures from the template of choice onto each template and assemble into an Fv; and (3) *ab initio* modeling of HLVs without canonical structures, including HCDR3 with option to refinement by energy minimization. Then, they discussed bottlenecks and pitfalls in this process for the participant groups.

They emphasized the crucial importance of the step 1, i.e., the template selected for homology modeling. The templates provide grafting points for HVLs but also define the packing of the VL and VH domains which can be characterized by a relative tilt angle. Based on the analysis of the models and precedents in the field, they concluded that since the tilt angle is extremely important for the relative positioning of HVLs, the choice of the Fv template should include specific filters beyond overall sequence similarity. Moreover, an analysis of the templates used by the participant groups revealed the somewhat unexpected observation that many of the errors in the models were derived from the errors in the structures available from the PDB. They inspected the electron density maps (from ED server) of a small sample of templates used in AMA-II and six high resolution structures used in the elbow angle survey by Stanfield et al.[6] Of the 33 structures, 10 showed various structure refinement issues, including peptide bond flips, missing residues, solvent molecules, incorrect rotamer conformations and poor backbone geometry. These errors are propagated in the current models and possibly in the crystal structures determined by molecular replacement. Therefore, they strongly recommended a careful curation of the antibody structures available at the PBD to generate a set of templates devoid of errors and suitable for modeling.

For step 2, i.e., grafting of HVLs with canonical structures, they mentioned the results obtained by fully automated PIGS modeling, which is based on the canonical structure model. PIGS generated the lowest-RMSD backbone structures for at least five of the sequences in AMA-II. The other PIGS models were also within the common range of agreement, with the only outlier resulting from the unfortunate use of a template that contains serious errors in the PDB coordinates.

Regarding loops without canonical structures, they emphasized that modeling HCDR3 remains the biggest challenge in antibody modeling. The HCDR3 is so diverse in sequence, length and conformation that it appears impractical at this time to use a template-based approach. Building HCDR3 *ab initio* is perhaps the most promising path as shown by a number of successful cases in AMA-II. Although the current software is able to generate correct conformations, it is not yet possible to identify them within the pool of options, thus speaking to the need for a more sophisticated weighting scheme to select a correct model from the ensemble of structures.

Drs. Luo and Teplyakov also mentioned that all the methodologies assessed in AMA-II produced similar and reliable models of the FR. Overall, the models deviated by < 1.0 Å from the experimental structures, and with a few exceptions, several due to the quality of the template, the RMSD values were close to 1.0 Å in the HVLs with canonical structures. Compared with CASP these results are very encouraging. The models obtained by current modeling methods are reliable for some antibody engineering applications, such as humanization, library design for affinity maturation and developability enhancement. A trend was also shown towards improvement in the modeling of HCDR3 with respect to AMA-I, in particular for short loops. Nevertheless, further improvement is needed if the models are to be used in applications demanding higher accuracy, for instance docking with the corresponding ligand.

The results of AMA-II will be published in a Special Issue of *PROTEINS: Structure, Function, Bioinformatics*, which will be composed of twelve papers describing in detail all the modeling methodologies and results of AMA-II. Participants concluded that, without a doubt, one of the critical outcomes of AMA-II has been the creation of a discussion forum for antibody engineers and antibody modeling method developers, which has helped with the identification of bottlenecks in antibody modeling and have pushed the accuracy of antibody prediction methods to the sub-Å level in the FR and close to one Å in the antigen-binding site, with the exception of HDCR3. Therefore, the participants agreed that it would be valuable to continue this initiative through additional rounds of modeling assessments with an increasing level of challenging structures and more refined modeling methods, and perhaps extend the assessment to prediction of antigen-antibody complexes in the near future.

Availability of Models and Supplementary Material

The coordinates of benchmark structures and models, as well as Supplementary Material of AMA-II and the first assessment, can be downloaded from: http://www.3DAbMod.com. The coordinates of the benchmark structures are also available from the PDB. Alternatively, the models and the material presented in the workshop will be made available upon request to Juan C. Almagro at: jcalmagro@hotmail.com.

Pre-conference Workshop
Identifying Clonal Lineages From Next-Generation Data Sets of Expressed V_h Gene Sequences

Felix Breden, Jamie K Scott

This workshop was organized by **Jamie K. Scott** and **Felix Breden** of Simon Fraser University, and was held Sunday afternoon before the main meeting. It was motivated by the rapid expansion in the number of labs that are "deep sequencing" the antibody (Ab) gene repertoires expressed by B-cell and plasma-cell populations. This so-called "next-generation sequencing" (NGS) technology, or "NextGen" sequencing technology, produces hundreds of thousands to millions of Ab gene sequences from the cDNA of B cells or plasma cells from a patient, control individual (or phage library). Ab-gene sequence repertoires can also be obtained for various B-cell subsets, such as antigen-binding B cells or B cells bearing particular cell-surface markers.

In introducing the purpose of the workshop, Dr. Scott reviewed the origins of the Ab repertoire's huge genetic diversity. Expressed Ab genes are produced by joining, through genetic rearrangement, of germline V, D and J gene segments to form a VH gene region encoding the variable domain of the immunoglobulin (Ig) heavy chain. The V, D and J gene segments are ligated together imprecisely and their ends can be modified by several mechanisms beforehand, including the addition of non-templated (N) nucleotides by thymidine deoxynucleotide transferase (TdT). The region of the VH gene encompassing the 3'-end of the V gene segment through to the 5'-end of the J gene segment encodes the most variable region of the Ab, the third complementarity-determining region of the heavy chain (CDR-H3). During B-cell development, V, D and J gene segments rearrange first in the heavy chain locus (IgH); on successful production of the heavy chain protein, the cell divides to produce ~100 daughters. Next, at the light-chain loci (kappa and lambda) the germline V and J gene segments rearrange to form the VL gene regions encoding the variable domain of the kappa and lambda light chains (VL). With the successful production of a light chain, the B cell bears a full Ab on its surface, and will mature, undergoing negative and positive selection to become a naïve B cell bearing surface IgM and IgD containing identical VH and VL domains. After encounter with antigen, naïve B cells, whose surface Ab binds antigen, will divide and develop into plasma cells that secrete Ab, and into memory B cells that recirculate through the secondary lymphoid tissues. Depending on a number of factors, and especially T-cell help, antigen-stimulated B cells will upregulate activation-induced cytidine deaminase (AID) to produce somatic mutations in the VH and VL gene regions, and to mediate Ig class switching of the heavy chain isotype (to Ig G, A or E). Thus, Ab diversity is concentrated in the VDJ joint encoding an Ab's CDR-H3 region (contributed by imprecise joining and N-nucleotide additions), with diversity also being contributed by combinatorial diversity of the multiple germline V, D and J gene segments, and of heavy and light chain pairing.

The full sequence of large numbers of VH and VL gene regions can be obtained through NextGen sequencing of B-cell or plasma-cell cDNA, allowing in-depth characterization of expressed Ab-gene repertoires for parameters, such as somatic mutations, V-gene usage and CDR-H3 length, than was possible a few years ago. Since a large portion of Ab sequence diversity is located in the gene region encoding the CDR-H3, several researchers have developed bioinformatic tools that use VH-gene sequence data to deduce B-cell clonal lineages, as defined by an original naïve B cell clone and its somatically mutated descendants. However, recall that the same VH sequence can reside in different B-cell clones (i.e., B-cells sharing the same VH gene but having different VL genes). How does one decide that highly related VH-gene sequences should be grouped into a single clonal lineage, as opposed to different clonal lineages?

The purpose of the workshop was to review and discuss the various approaches that have been developed to delineate clonal lineages. It was suggested that the definition of B-cell clonal lineage should refer to each distinct combination of VH and VL heavy and light chains, and all descendant B cells. In contrast, "clonotype" was used variably, as an operational definition for VH genes that share V and J germline genes, similar CDR-H3 lengths, as well as a given level of homology (e.g., 70%) within the DNA encoding, or amino-acid sequence of, the CDR-H3.

The first talk in the workshop was presented by **Marie-Paule Lefranc** (CNRS; University of Montpellier), founder and director of the IMmunoGeneTics (IMGT) web portal. Entitled "IMGT/HighV-QUEST Paradigm for Immunoglobulin IMGT Clonotype Diversity and NGS Immunoprofiling," Professor Lefranc reviewed the IMGT program V-Quest for the analysis of Abs, which identifies the most likely germline V, D and J genes contributing to an Ab, the mutational pattern leading to the expressed Ab, and parameters such as CDR-H3 length. Prof. Lefranc also described the development of HighV-Quest, which now allows the analysis of up to 500,000 sequences in one submission, and which is essential to the analysis of NextGen Ab data bases. Professor Lefranc also described the clonotype analysis function of IMGT, which defines an IMGT clonotype (AA) for the Ab heavy chain as a unique V-D-J rearrangement with a unique CDR3-IMGT AA (in-frame) junction sequence. Tools for clonal analysis in IMGT allow for description of clonal diversity and expansion.

The talk, "Using Phylogenetic Techniques to Identify B-cell Lineages," was then delivered by **Felix Breden**, who reviewed approaches to defining species as independent lineages in evolutionary biology. He applied these concepts to defining B-cell clonal lineages in Ab repertoires. He first separates large Ab datasets into "clusters," defined as sequences using the same V and J gene, and with the same CDRH3 length. This is a common clustering technique, and few Ab sequences from the same B-cell clone should fall into different clusters defined in this way. Within such clusters, inter-sequence divergences fall into two major categories: (1) those representing small divergences of sequences from the same presumed clonal lineage, and (2) those representing inter-clonal differences. Dr. Breden then applied this categorization technique to Ab repertoires from the individual from whom the m66 anti-HIV Ab was discovered, and to Ab repertoires of HIV+ and control individuals.

Thomas Kepler (Boston University) delivered a talk entitled, "Statistical Methods for Immunoglobulin Gene Repertoires, Clones, and Sequences." Prof. Kepler presented a statistical maximum-likelihood model that describes the development of Ab sequences from the original V-D-J recombinant, including N and P addition/deletion, followed by expected patterns of somatic hypermutation. He then showed how this model can be used to identify clonally-related Ig sequences from large Ab repertoires, and finally to infer the unmutated ancestor of each set of clonally-related sequences. This full model is implemented in an Ab analysis pipeline available from his lab.

Paul Kellam (Wellcome Trust Sanger Institute; University College London), discussed different BCR sequencing methods and network analysis of B-cell populations. He first compared various high-throughput NGS techniques and found that they produced similar patterns of clonality and gene usage when applied to Ab samples from the same patient. His group has developed a method of analysis of clonality in large Ab repertoires based on networks, defining a clone as any group of sequences connected in the same network. Based on this definition, Ab repertoires from individuals with leukemia were dominated by one or two large expanded networks, while those from healthy individuals showed a more even distribution of network sizes. This network analysis pipeline is also available from the Kellam lab.

Duane Wesemann (Brigham and Women's Hospital; Harvard Medical School) reported on primary B cell receptor diversification in the gut mucosa. RAG-mediated "receptor editing," which can replace the first VκJκ exon with one that generates a new specificity, allows the local environment to influence the primary B cell repertoire. Using lineage comparisons, Dr. Wesemann showed that RAG-mediated V(D)J recombination occurs in gut mucosa-resident developing B lineage cells, leading to a distinct pre-immune repertoire. Microbial colonization of germ-free mice led to an increase in the expression of lambda versus kappa light chains, producing a markedly altered Ab repertoire.

Jacob Glanville (Distributed Bio) discussed benchmarking Ab lineage grouping and paratope convergence. Dr. Glanville reported on studies of Ab repertoires from monozygotic twins that examined the effect of genetics, environment and disease treatment on the development of the Ab repertoire, especially V-gene usage and CDR-H3 length. These studies enabled clear parameter generation for human Ab genetic lineage classification. Dr. Glanville reviewed these methods and used comparisons of related sequences within individuals (possibly representing clonal lineages), and between individuals (which cannot be offspring of the same B-cell clone), to evaluate the performance of these measures. Such comparisons suggest that clones consist of Ab sequences that have no more than two amino-acid changes in the CDR-H3 junction region.

The final talk in the workshop, "Identifying Ab Clonal Lineages from Next-generation Sequencing Data," was given by **Bryan Briney** (The Scripps Research Institute). He reported on a suite of computational tools for advanced Ab sequence analysis, which include error-correction and clonal-lineage assignment algorithms. This analysis package, which is available from the Burton lab at Scripps, integrates with major cloud computing platforms, ensuring that the analysis tools can scale with future increases in sequencing throughput. Dr. Briney used these tools to show that Abs with long CDR-H3 regions were produced by the process of V-D-J recombination, and do not appear later in the development of the B-cell lineage, via processes such as insertions or secondary rearrangement.

The workshop ended with a panel discussion that included all of the presenters and questions and discussion with the audience. The topics that the panel were asked to consider included: (1) Making publicly available expressed VH and VL gene sequence data; (2) Agreement on a "universal" definition of "clonal lineage" based on analysis of expressed VH gene sequence data; (3) Making publicly available the tools for clonal-lineage analysis; (4) A comparative analysis of different approaches to clonal lineage analysis, using shared expressed VH (and associated VL) gene datasets; and (5) Recommending "best practices" for assignment of B-cell clonal lineage based on expressed VH (and VL) gene datasets.

The panel engaged in a vigorous discussion regarding definitions of the terms clonotype and clonal lineage within Ab repertoires. There was no consensus, and some discussion as to the practical necessity of such a definition. In other words, as long as similar approaches and definitions were adopted, comparisons between repertoires of healthy and unaffected individuals, for example, should be valid. The talks showed that most of the Ab analysis pipelines, sets of programs for analyzing large-scale Ab repertoires in terms of V-gene usage or clonal expansion, were available from the developers of the tools. There was little consensus on if it would be possible to share these Ab repertoire data sets, given their large size, and the associated patient identifiers and clinical data. This is clearly a field that will be growing in size of data sets and sophistication of analysis approaches, and it was agreed that there was a need for further meetings to discuss these issues. There are plans to organize such a follow-up meeting for late September 2014 in Vancouver, Canada.

Monday December 9, 2013

The first of the four day Antibody Engineering & Therapeutics meeting began with a morning session featuring three keynote speakers. This event was followed in the afternoon with sessions in two tracks.

System Biology, Systems Medicine and Systems Immunology

Devin Sok, Paul WHI Parren

Chaired by **James D. Marks** (University of California, San Francisco), the keynote session of the meeting focused on a systems approach to basic and translational research. Overall, such an approach extends beyond identifying or engineering new antibodies for antigen targets and focuses instead on better understanding the myriad of components that are fundamentally involved in protein folding and protein-protein interactions. Subsequently, these lessons can be leveraged to engineer protein scaffolds to manipulate or simulate protein interactions with potential therapeutic or diagnostic outcomes.

The first speaker, **Leroy Hood** (Institute for Systems Biology), discussed a cross-disciplinary, systems approach to research that is intended to deconvolute the complexity of diseases such as cancer and neurodegeneration. In particular, he emphasized use of systems strategies, such as family genome sequencing and systems-driven blood diagnostics, and new genomic and proteomic technologies for the exploration of patient data. Dr. Hood noted that, in seeking to explain the behavior of complex systems, systems biology has potentially far-reaching implications for medicine, as well as many other areas, e.g., agriculture, energy production, environmental protection.

Paul Parren (Genmab) delivered a keynote lecture entitled "Generation of bispecific antibodies via Fab-arm exchange." This presentation showed a strong example of how academic and industry groups can work together to both answer basic biology questions and translate such knowledge to advance drug development. The studies started with a general interest in exploring (IgG4) antibody biology. Human IgG4 antibodies were shown to naturally engage in a process termed Fab-arm exchange. This dynamic and stochastic process involves the *de novo* joining of antibody heavy-light chain pairs derived from random IgG4 antibody molecules. Bispecific IgG4 antibodies consisting of Fab-arms with different antigen-specificities are therefore formed in a continuous post-production process. The in vivo dynamics of Fab-arm exchange in humans in the therapeutic setting were studied using the IgG4 therapeutic antibody natalizumab. The molecular requirements for Fab-arm exchange were determined, and it was found that both the hinge region and the CH3 domain play essential roles; more specifically, amino acid residues serine at position 228 (S228) in the hinge and arginine at position 409 (R409) in the CH3 domain were found to be critical residues for Fab arm exchange of human IgG4 antibodies. Notably, the mechanism appeared to be conserved in several mammalian species, but no consensus sequence could be identified. Indeed, for (Chinese origin) rhesus monkey, the ability to engage in Fab-arm exchange was mapped to a distinct position in the CH3 domain and a hinge region containing an alanine at position 228 (A228). Generally speaking, Fab-arm exchange was found to require CH3 domains with reduced non-covalent interaction strength, as well as a hinge region containing inter-chain disulfide bonds with reduced covalent stability, when both compared to human IgG1. The natural process of Fab-arm exchange is thought to represent an adaptation to provide IgG4 antibodies with non-crosslinking and anti-inflammatory properties.

The knowledge of Fab arm exchange gained was exploited to build the DuoBody® platform, which enables the generation of bispecific IgG1 antibodies.[7,8] By scanning the CH3 interface, matched mutations were identified with the characteristic that they preferred the formation of heterodimeric CH3 domain interactions. Thus, under permissive in vitro conditions, homodimeric heavy-light chain pairs were found to dissociate and form heterodimers (with bispecific reactivity) in a unidirectional reaction. These permissive conditions were designed to allow temporal breakage of the highly stable interchain disulfide bonds in IgG1 hinge. Bispecific antibody formation was obtained with $\geq$ 95% efficiency at both laboratory scale production and large scale manufacturing. Bispecific antibodies generated using the Duobody technology retain all regular IgG1 characteristics (structure, stability, Fc-mediated effector functions, PK properties and manufacturability). Functional proof-of-concept was shown using a HER2xCD3 DuoBody molecule, which engages T cells to successfully kill tumor cells both in vitro and in vivo, and a monovalent cMet-targeting DuoBody molecule, which was a full cMet antagonist that effectively inhibited the growth of HGF-dependent xenograft tumors. IgG4 biology knowledge was therefore successfully leveraged to develop an elegant and robust platform for the discovery and development of bispecific antibodies in a human IgG1 format.

Advances and concepts in protein design was first highlighted by the keynote speaker **David Baker** (University of Washington). Professor Baker first presented an overview of Rosetta, which is an algorithm designed to predict the lowest energy fold and lowest energy sequence for a desired protein structure or function.[9] Impressively, Rosetta has demonstrated atomic-level accuracy in predicting protein folds, which has been corroborated by structure-based measurements such as NMR spectroscopy and X-ray crystallography.[10] The strategies used for protein design can also be applied to the design of binding, which in many cases has yielded protein scaffolds that demonstrate affinities and specificities much like conventional antibody-antigen interactions. Indeed, the breadth of this approach is exemplified by its number of successful applications.

In one example, Professor Baker discussed protein scaffolds that bind similarly to broadly neutralizing antibodies (bnAbs) against influenza hemagglutinin (HA).[11] Notably, the molecules show great specificity and high affinity and could be used as a potential diagnostic to distinguish between different HA subtypes with up to a picomolar limit of detection. They also demonstrate potential therapeutic use, and have been shown by intranasal application to protect mice against lethal infection with influenza virus. Professor Baker has extended this application to other viral antigens like Ebola glycoprotein, and to putative cancer antigen targets like PD-1, Mdm4, BHRF1, as well as small molecule targets such as vitamin D, fentanyl, and cortisol. Indeed, the ability to target different antigens mimics the chemical space afforded to antibodies with potential advantages in terms of size, deliverability, specificity, and production.

In addition to designing protein binders, Professor Baker also discussed the design of protein nanomaterials.[12] These designs were based on symmetrical docking and interface design, which resulted in self-assembling nanoparticles with 24 and 12 subunits that are 14 and 11 nm in size. The materials were subsequently analyzed structurally by electron microscopy and X-ray crystallography and closely matched the intended design. These high-order protein structures provide a diversity of utility, among which include the design of nanocages for targeted delivery or vaccine design, of nanolayers for materials and diagnostics, and of nanowires for molecular or electron transport.

Professor Baker concluded his talk by discussing the multiplayer online game FoldIt, which engages non-scientists and scientists alike to solve difficult protein structure prediction problems.[13] Players use direct manipulation tools and user-friendly

components of Rosetta to arrive at low energy folds of different protein structures. Strikingly, top-ranked Foldit players were able to reach design solutions of complex problems, likely because they were better than computers at utilizing visual cues and choosing alternative solutions in non-prescribed ways. Professor Baker encourages the audience at large to try Foldit, and suggests that this hybrid human-computational framework dually encourages the public's interests in science and helps find solutions to difficult scientific challenges.

While Professor Baker focused mainly on the design of proteins to bind antigens in place of antibodies, the subsequent keynote address by **William R. Schief** (The Scripps Research Institute) focused on the design of protein scaffolds for the elicitation of specific antibodies by immunization. This approach, known as reverse vaccinology, or epitope-focused vaccine design, is based on first isolating protective antibodies that bind to a conserved epitope on a pathogen of interest, obtaining structural information on that epitope, and then designing immunogens to elicit antibodies with similar specificity through immunization.[14] One important target of interest is the human immunodeficiency virus (HIV) envelope glycoprotein (Env), a highly antigenically variable protein that is densely covered with N-linked glycans.[15] Potent, broadly neutralizing antibodies (bnAbs) have been isolated from HIV-infected donors, but induction of bnAbs by vaccination has failed thus far, in part due to the high immunodominance of variable regions relative to conserved epitopes and the lack of structural information on the native Env trimer.[15,16] Recently, however, a structure of a native-like Env was reported using both X-ray crystallography and cryo-electron microscopy, which has provided more information on critical antibody-binding angles that are not apparent in crystal structures of the monomeric gp120 subunit.[17-20] Indeed, even with this key information, immunogen design for HIV bnAb elicitation still presents a daunting challenge.

As an initial proof of concept for reverse vaccinology, Dr. Schief presented a case study on respiratory syncytial virus (RSV) and efforts on immunogen design for HIV. Infection by RSV affects 64 million people and results in 160,000 deaths annually, particularly among infants and young children. There is currently no licensed vaccine for RSV and there is only a single marketed prophylactic antibody, palivizumab (Synagis®). An affinity-matured variant of palivizumab, called motavizumab, has been crystallized in complex with its RSV epitope.[21] To build upon this antibody-antigen information, Dr. Schief used Rosetta to design stable protein scaffolds that precisely mimicked the RSV viral epitope, as determined by X-ray crystallography. One scaffold was then presented multivalently on a virus-like particle,[22] which resulted in an amplified antibody response following immunization. The scaffolds induced RSV neutralizing responses in rhesus macaques, a first for scaffold-based vaccines. Furthermore, Schief and colleagues isolated mAbs from an immunized macaque that are ~10 times more potent than palivizumab, and showed by crystallography that these mAbs target the same epitope as motavizumab with high precision. Thus, the concept of reverse vaccinology has proven to be capable of eliciting potent neutralizing antibodies by rational immune design.[23] Accordingly, these principles can be applied to other pathogens such as HIV and influenza, and the precise mAb-targeting achieved in the RSV case study provides encouragement for those efforts.

For immunogen design to elicit HIV bnAbs, Dr. Schief focused on the highly conserved CD4 binding site (CD4bs). BnAbs that utilize the heavy chain gene VH1-2[20,24,25] to mimic CD4 in the interaction with HIV gp120 have been isolated from multiple donors. Strikingly, however, monomeric gp120 or native Env have not been shown to bind to germline precursors of these bnAbs by conventional biochemical measurements, suggesting that immunizations with Env or gp120-derivatives may not reliably elicit such bnAb responses.[26-31] Reasoning that a successful vaccine prime would require binding to both germline precursors and affinity-mature antibodies, Dr. Schief designed immunogens that were capable of binding both with high-affinity.[32] The immunogens were then displayed on self-assembling nanoparticles and were capable of activating bnAb germline B cells in vitro, while similar immunogens lacking germline affinity elicited no response. These germline-targeting immunogens show high promise as a vaccine prime to initiate the elicitation of CD4bs bnAbs by immunization, although this process will likely require multiple iterations to develop a sequence of boosting immunogens to induce appropriate somatic hypermutation. Notwithstanding, this approach exemplifies the integration of multiple disciplines in a systems approach to address significant scientific and biomedical challenges.

Track 1: Antibody Deep Sequencing and Repertoires

Matthias Pauthner

Dennis Burton (The Scripps Research Institute) was Chair of the afternoon session on antibody deep sequencing and repertoires. The first speaker, **William Robinson** (Stanford University, Atreca Inc.), discussed the Immune Repertoire Capture™ technology, which utilizes novel DNA barcoding and NGS to retrieve paired heavy and light chains of antibodies expressed by single B cells, and can be used to unravel functional antibody repertoires generated in response to vaccination, infection and cancer. Developed in the Stanford University laboratory of Dr. Robinson, the technology involves 454 deep sequencing of antibody heavy and light chains, which are barcoded at the single-cell level with unique oligonucleotide primers during cDNA synthesis. This process not only allows correct pairing of immunoglobulin heavy and light chain sequences originating from the same cell, but also enables unbiased and quality-controlled recovery of antibody sequences. Specifically, the use of an universal 5' priming system results in amplification of all antibody cDNA, even cDNA with 5' variable region mutations arising from affinity maturation. Based on Atreca's plasmablast datasets, up to 10% of all identified antibodies would have not been amplified by V-gene specific primers. In addition, the barcodes enable accurate determination of clonal proportions, which in the absence of single-cell barcoding become distorted by the PCR. Further, the incorporated barcodes are used to correct PCR and NGS errors by oversampling, followed by determination of the consensus

sequence, while negative control barcodes identify contaminants. This technology has thus far been primarily used to generate antibody sequences from short-lived plasmablasts, thereby yielding functional repertoires comprised of both immuno-dominant and rare clonal antibody families generated in response to recent physiological events. It is also being used to generate antibody sequences from antigen-specific memory B cells and to sequence T cell receptors.

Applications for Atreca's technology are the generation of monoclonal antibodies, clinical vaccine monitoring, development of autoimmune disease diagnostics, and the discovery therapeutic antibodies and their targets in infectious disease and cancer. The technology was validated by analyzing the antibody repertoire of KLH-NP immunized mice, which showed a large clonal expansion in IGHV1-72 antibodies, as expected for a KLH-NP response. As an example for clinical vaccine monitoring, Atreca sequenced the antibody repertoires of individuals recently immunized against influenza. Notably, the response was dominated by a clonal lineage of hyper-mutated VH1-69 antibodies akin to rarer families, which were likewise comprised of multiple antibody variants per family. Several antibodies rationally selected across somatically mutated clonal families showed in vitro neutralization and up to picomolar antigen binding.

Similarly, Atreca's technology was used to pin-point autoantigen (rheumatoid factor, CCP) binding antibodies recovered from a rheumatoid arthritis (RA) patient. Since RA reactive blood plasmablast levels correlated with disease activity and produce pathogenic autoantibodies, monitoring of RA-reactive plasmablast repertoires is a potential novel diagnostic tool. The technology was further used to isolate plasmablast antibodies of patients suffering from an acute *Staphylococcus aureus* infection, which yielded antibodies binding novel antigens that mediated neutrophil killing of *S. aureus*.

The technology was also used to isolate anti-cancer antibodies from long-term non-progressing patients with metastatic melanoma and lung adenocarcinoma. It identified 106 clonal families of antibodies observed across metastatic melanoma non-progressing patients, and the antibody repertoire evolution over a span of 3 months was examined. This approach yielded an initial hit rate of >25% melanoma reactive antibodies compared to all tested antibodies. An even higher hit rate was observed for multiple melanoma-reactive antibodies encoded by clonal antibody families maintained by a patient over the course of three months. Atreca's technology was also used to isolate lung adenocarcinoma-reactive antibodies from a long-term non-progressing patient. Again, an initial hit rate of >25% was observed, and multiple antibodies binding either cell surface or intracellular targets on a lung adenocarcinoma cell line were identified. In concluding, Prof. Robinson noted that these examples illustrate how Atreca's Immune Repertoire Capture™ process can be used to identify candidate therapeutic antibodies directly from humans, and by extension their targets in various disease relevant environments.

Adrian Briggs (AbVitro) then discussed immune sequencing technologies for pairing of heavy and light antibody chains. In the next presentation, **James Crowe, Jr.** (Vanderbilt University Medical Center) first introduced the concepts of antibody "swarms" and convergence and divergence during antibody evolution, and then went on to investigate the 'long CDRH3 problem' related to HIV-1 broadly neutralizing antibody (bNAb) induction, and the question of how frequently HIV reactive antibodies occur in HIV-naïve individuals.

During affinity maturation, unrelated as well as related B cell clones can converge and identical B cell clones can diverge, creating complex clonal families or "swarms" of inter-related antibodies. For example, the hemagglutinin stem binding antibody 8D4, which was isolated from an influenza-vaccinated individual, was shown by 454 deep sequencing to be part of a large family of slightly divergent clonal siblings. A more extreme example was presented from a dengue virus-infected donor, from whom over 100 dengue mAbs were isolated. Dr. Crowe and colleagues deep sequenced and compared dengue virus binding and unsorted B cells, focusing on a clonal family of V_H3-30 and J_H4 gene-encoded antibodies of which two members have previously been isolated. Interestingly, the V_H3-30/J_H4 family of antibodies, by itself presenting only a fraction of the dengue reactive repertoire, already formed a complex swarm, leading to the conclusion that virus-induced antibody repertoires can be regarded as a "swarm of swarms." Another deep sequencing study of five clonal families of H1 influenza-reactive antibodies emphasized that divergence and convergence can occur simultaneously. While all families diversified themselves during maturation, convergence on certain residues critical for binding was observed across all families. Therefore, functional convergence is an important concept to consider when analyzing antibody repertoires, as analogous developments across families can evolve identical functionally important amino acids, but not necessarily lead to large-scale sequence identity across the variable gene.

Dr. Crowe then focused on the question of how prevalent long (>24 amino acids) heavy chain complementarity determining region 3 (HCDR3)-containing antibodies are in naïve antibody repertoires and what implication can be derived for the induction of HIV-1 bNAbs. Long HCDR3 antibodies populate about 3-4% of the naïve B cell repertoire and HCDR3 length correlates well with N-nucleotide addition and the use of D2/D3 and J_H6 genes, which are the longest of their respective classes. For example, the HIV bNAb PG9 and its clonal relative PG16 use the longest common D gene, D3-3, and J_H6, as do over 80% of all long HCDR3 antibodies, raising the possibility that HIV-naïve individuals already possess HIV-reactive PG9-like antibody precursors. To investigate that question, Dr. Crowe and colleagues deep sequenced the antibody HCDR3 regions of 64 HIV-naïve donors on the Illumina Hi-Seq platform, gathering 130 million unique reads, of which about 19,000 encoded non-redundant >30 amino acid (aa) HCDR3 length heavy chains. They then used position structure specific scoring matrices (P3SM), threading and energy minimization algorithms in Rosetta to computationally predict whether the gathered variable genes could fold into antibodies with PG9-like structure and function and, if necessary, apply minimal sequence design to recover PG9 mimicry. While this is an ongoing effort, Rosetta clustered the top 100 hits into 12 groups of related antibodies, representing sequences from 26 out of 46 donors, of which multiple sequences appeared

in more than one donor repertoire. The preliminary results show that unmodified antibody sequences from several of the clusters bound to HIV Envelope when synthesized and tested in vitro. In conclusion, these data imply that the central problem of inducing long HCDR3 HIV bNAbs by vaccination is the targeting and activating of existing long HCDR3 naïve precursors, rather than a dependency on somatic hypermutation generating long HCDR3 precursors by nucleotide insertions.

Following the afternoon break, **Jiang Zhu** (The Scripps Research Institute) discussed multiple techniques and applications relying on a combination of immunoglobulin NGS and bioinformatics, so called "antibodyomics." Specifically, he depicted how antibodyomics can be applied to identify somatic variants of known antibodies and how to analyze evolutionary convergence between antibodies. He also presented a novel solution for the NGS Ig heavy and light chain pairing problem and demonstrated how antibodyomics can be used for the discovery of novel HIV bNAbs from donor samples.

To demonstrate how the computational analysis of antibody NGS data can be used to identify somatic variants of known antibody clones, Dr. Zhu and colleagues used 454 deep sequencing to amplify Ig genes from two donors of the NIAID and IAVI cohorts, from whom the HIV bNAbs VRC03 and PGV04 had previously been isolated. This analysis yielded >100 new variants for VRC03 and >5000 new variants for PGV04. To verify the integrity of the recovered sequences, they devised a 2D grid-based selection approach and synthesized 35 representative Ig heavy chains, of which 24 paired with the respective light chains and neutralized HIV pseudoviruses in vitro.

To investigate the evolutionary convergence of VRC01-class antibodies across individuals, Dr. Zhu and colleagues analyzed and compared Ig NGS data of five donors from whom antibodies of this class had previously been isolated. VRC01-class antibody light chains show strong convergence on either highly flexible (glycine rich) or short loop length (containing a two aa deletion) LCDR1 regions, and fixed loop length (5 aa) LCDR3 regions. They could show that across all donors, the short LCDR3 feature was found in a majority of sequences with low divergence from the respective germline genes, while LCDR1 deletions only appeared in highly mutated 'mature' light chain reads. These findings indicate a common maturation pathway, in which the characteristic 5 aa LCDR3 is likely created by VJ-recombination, while the LCDR1 mutation/deletion is created by somatic hypermutation (SHM) during affinity maturation.

As a computational solution to the 'chain pairing problem' for Ig deep sequencing, Dr. Zhu presented an analytic strategy that facilitates correct chain pairing.[33] By calculating intra-donor phylogenetic trees of both heavy and light chain reads from Ig 454 deep sequencing of two IAVI donors, Dr. Zhu and colleagues could show that known and novel heavy and light chains clustered in very distinct patterns. Therefore, the correct chain pairings can be inferred based on clustering patterns and assigned by mapping the respective heavy and light chain groups to each other.

Dr. Zhu then focused on how antibodyomics can be utilized for the discovery of HIV bNAbs from infected donor samples. Dr. Zhu and colleagues performed Ig 454 deep sequencing on an HIV infected donor whose serum was previously mapped to have CD4 binding site neutralizing activity. Using iterative cross-donor phylogenetic analysis, a technique the exploits the known phylogenetic segregation pattern of VRC01-like heavy chain genes, they identified 10 novel VRC01-like broadly neutralizing heavy chains that were experimentally verified for neutralization paired with the VRC01 light chain.[34] One of these hybrid bNAbs was able to neutralize over 80% of HIV isolates on a 153 virus panel at intermediate potency. Dr. Zhu closed his talk with an outline of how antibodyomics could be used as a powerful tool to guide vaccine design in the future, by providing differentiated and complex feedback on how antibody repertoires respond to immunization.

During affinity maturation, B cell populations expand and contract, and B cells leave germinal centers and sometimes re-enter that cycle upon later antigen contact. As a result, diverse lineages of related B cell clones and their antigen receptors are created, and these can be studied using next generation immunoglobulin sequencing. In his presentation, **Thomas Kepler** (Boston University) first introduced the statistical tools his group developed to study these lineages, and then presented results from longitudinal studies of individuals infected with influenza and HIV-1.

A common problem when analyzing immunoglobulin deep-sequencing data, is to infer the unmutated common ancestor (UCA) or 'germline-like' antibody of a given clonal family, which requires statistical modeling of two factors that determine antibody maturation: rearrangement of Ig gene segments and somatic hypermutation (SHM). While it is hard to confidently estimate all rearrangement parameters, Baysian probabilistics can be used to estimate posteriors on all the parameters and the summing over these parameters. Further, the chance of SHM events can be estimated as a function of the specific nucleotide exchange and the evolutionary time that passed. Since an UCA must be derived from a pool of ~10^{12} valid Ig rearrangements, the two methods combined allow for calculating posterior probabilities of each sequencing read, including the estimated error of the assignment. For example, the predicted UCA of VRC01, a >32% mutated HIV-1 bNAb, is estimated to deviate 9.9 nucleotides from the true UCA, which was calculated using the sequences of VRC01 and a clonal relative VRC02.

These methods were also used to reconstruct the lineage of five pairs of clonally related anti-influenza antibodies that were recovered from an individual experimentally infected with influenza seven days prior to isolation. The UCA and several clonal intermediates were estimated and experimentally tested for binding to hemagglutinin by ELISA. The K_d of all inferred intermediates, the UCA as well as the isolated antibodies declined in accordance with the predicted evolutionary distance from the origin of the lineage. Similar analysis have been performed with the clonal family of HIV-1 bNAb CH103, which was isolated from a HIV-1 infected donor ~2.5 years post infection. Likewise, binding affinity and neutralization potency of inferred precursors declined with the evolutionary distance from the UCA.

The last part of the talk was focused on the analysis of insertions and deletions (indels), which are very frequently observed in HIV bNAbs. To test the hypothesis whether indel frequencies are a proxy for activation induced deaminase (AID) activity and therefore linked to nucleotide substitution frequency, Dr. Kepler and colleagues used 454 sequencing to amplify and investigate Ig heavy chains from HIV positive bNAb producers, HIV-positive non bNAb producers, influenza vaccinated and healthy individuals. Notably, only the indel prevalence of HIV bNAb producers was strongly elevated above uninfected controls and the observed indel frequency slightly surpassed the prediction based on the substitution frequency. Thus, indels were found to super-linearly correlate with mutation frequency, which could be explained by increased Ig gene destabilization through point mutations and subsequent indel creation by AID. Indels were also found to occur close to antigen binding sites, which given the AID hotspots in complementarity determining regions is not surprising, and are likely contributing to affinity maturation. To exemplify this point, they investigated the clonal family of the HIV bNAbs CH30 to CH34, which inherited a 27nt compound insertion from intermediate ancestors that increased neutralization potency and breadth of the lineage tremendously. Dr. Kepler concluded with the prediction that vaccine regiments able to induce sustained antigenic stimulation and in turn inducing high levels of SHM, might enable the elicitation of bNAbs in a majority of individuals.

Vaughn Smider (The Scripps Research Institute) discussed a thoroughly analyzed subset of bovine antibodies comprising 50-69 aa complementarity determining region (CDR) 3 segments in their heavy chains. With few V, D and J segments, the combinatorial diversity of cow antibody heavy chain genes is strongly reduced compared to humans. However, the majority of the bovine antibody repertoire has a median HCDR3 length that is much longer than in humans (about 25 aa), and additionally comprises an "ultralong" subset of antibodies whose HCDR3 lengths are over 50 aa and are extremely cysteine rich. Dr. Smider and his colleagues solved crystal structures of two of these antibodies, BLV1H12 and BLV5B8, and discovered that those antibodies possess a β-ribbon "stalk" protruding from the center of the Fab structure, which is topped by a disulfide bonded "knob," presenting a novel structural antibody fold.[35] The stalk is formed by a β-sheet that is stabilized by some of the remaining heavy and light chain CDRs, while the knob fold is supported by multiple disulfide bridges. The knobs are divergent in sequence and structure and display different surface charges and geometries, imposing the question of how that diversity is generated.

Since the CDRH3 length is constrained by the lengths of the V, D and J segments, and junctional diversity usually contributes little to CDR3 length, Dr. Smider and his colleagues were able to identify one D gene, D_H2, accounting for the extreme HCDR3 length in all antibodies of this class. Surprisingly, deep sequencing of ultralong HCDR3 antibodies revealed enormous sequence diversity within the CDR3 regions, which were also heavily enriched for even numbers (4, 6, 8) of cysteines, emphasizing the importance of disulfide bridges for the knob fold. Those remarkable features can be explained as follows: first, cows undergo somatic hypermutation before forming their primary repertoire, thereby overcoming the limitations of their combinatorial diversity, and second, the DH2 gene is almost exclusively comprised of codons that can be mutated to cysteines with single base changes. They further showed that ultralong HCDR3 antibodies bind functional antigen upon immunization with bovine diarrhea virus (BVDV), and that antigen binding is mediated by the knob region.

The unique fold of these antibodies might also allow for unprecedented therapeutic applications, e.g., binding of enzyme catalytic sites and other buried epitopes or reaching into the pores of ion channels and trans-membrane proteins. To exemplify this, Dr. Smider described work where Kv1.3 ion-channel targeting peptides were swapped with the knob region, and he showed that these engineered antibodies were able to inhibit T cell activation at nano-molar concentrations. Likewise, ultra-long HCDR3 antibodies with IL-8 engineered into the knob region were able to activate the G-protein-coupled receptors (GPCR) CXCR1 at low nano-molar concentrations. Finally, every part of this novel subset of antibodies but the stalk and knob region can be humanized, paving the way for potential therapeutic use in humans.

Track 2: Antibodies in Cardiometabolic Medicines

Janice M Reichert, Kai Y Xu

The session Chair, **James W. Larrick** (Panorama Research; Velocity Pharmaceutical Development), introduced the topic of antibodies as cardiometabolic medicines by noting that cardiovascular (CV) disease is a leading cause of death, but the lack of relevant targets has delayed development of antibody-based drugs. He also pointed out that the cost for clinical studies of CV drugs is high because large numbers of patients are needed for the study outcome measures required for approval.

The first speaker, **Simon Jackson** (Amgen), discussed proprotein convertase subtilisin/kexin type 9 (PCSK9), which is a serine protease, chaperone and target for antibodies used to treat hypercholesterolemia. He briefly described the research that determined the function of PCSK9, which binds the low-density lipoprotein (LDL) receptor and induces its degradation. In particular, the observation that individuals with loss of function mutations in PCSK9 had low levels of circulating LDL cholesterol, but appeared otherwise to be normal, suggested that PCSK9 might be a suitable target for drug discovery. Dr. Jackson then discussed the preclinical and clinical development of the anti-PCSK9 antibody evolocumab (AMG145), which is currently undergoing evaluation in numerous Phase 3 studies that include patients with hyperlipidemia or mixed dyslipidemia, and patients with CV disease.[36]

Olga Fedorova (National Institutes of Health) presented marinobugagenin (MBG)-induced fibrosis as a novel therapeutic target for immune-neutralization in preeclampsia, diabetes mellitus and chronic kidney disease. In mammals, MBG, which is a potent inhibitor of Na, K-ATPase, participates in water-salt homeostasis. Levels of MBG are elevated with aging and in salt-sensitive hypertension and chronic renal failure. Dr. Fedorova described MBG levels and the effects of MBG neutralization

with the anti-MBG mAb 3E9 in five scenarios: (1) salt sensitive hypertension (Dahl rat model); (2) chronic renal failure (human disease, rat model); (3) preeclampsia (human disease); (4) diabetes (rat model); and (5) aging (rat model). Heightened MBG levels initiated pro-fibrotic signaling in these models. The anti-MBG treatment reduced tissue fibrosis initiated by MBG via pathways that are dependent on TGF beta and Fli-1, a negative regulator of collagen synthesis. Anti-MBG treatment also revealed cross-talk between these two pro-fibrotic pathways. Dr. Fedorova concluded by noting that the next step of her work with the antibody is humanization, which would enable potential clinical applications.

Knut Pettersson (Athera Biotechnologies) introduced Athera as a pre-IND biotechnology company developing first-in-class anti-inflammatory therapeutics for CV disease. A spin-off from the Karolinska Institute, the company's focus is PC-mAb, a human IgG1 antibody that targets phosphorylcholine (PC). The intended therapeutic indication is secondary prevention following revascularization procedures after a CV event. PC-mAb recognizes the PC group on metabolized or oxidized phosphatidylcholine, which are exposed in a damage – and pathogen-associated molecular pattern that is recognized by natural antibodies (IgM) and other innate immune receptors. Dr. Pettersson then reviewed experimental in vivo data indicating the protective role of anti-PC treatment in CV disease, and mentioned that the companion diagnostic kit CVDefine® can be used to measure endogenous IgM antibodies to PC.

Dr. Pettersson presented the selection strategy for PC-mAb, which was done in collaboration with Dyax Corp. Binding to PC, oxidized LDL (oxLDL) and cell membranes was assessed, with positive selection for PC-BSA, PC-transferrin, Cu-oxidized LDL and apoptotic cells, and deselection for linker-BSA, linker-transferrin, native LDL and native cells. Function was assessed via ox-LDL uptake in macrophages, with positive selection for molecules with better inhibition than IgM anti-PC. Anti-inflammatory and anti-restenosis properties were assessed in a mouse cuff model, with positive selection for inhibition of white cell accumulation and neointima formation. Dr. Pettersson noted that recent preclinical data show that PC-mAb reduces infarction size and prevents LV remodeling after experimental myocardial infarction. Toxicology studies are ongoing and the start of a Phase 1 study is planned in 2014.

Shijie Li (Genentech) discussed approaches to target vascular inflammation in atherosclerosis, which is driven by oxLDL. Data presented showed that a human IgG1 derived from malondialdehyde oxLDL reduced plaque size and macrophage content in atherosclerotic mice and reduced monocyte cytokine secretion. Dr. Li indicated that inhibition by anti-oxLDL antibody requires immune complex formation and is antibody Fc-dependent, and that the antibody acts through FcγRII. The anti-oxLDL antibody MLDL1278a was found to modulate NFκB activity and phosphorylation of Syk and p38 MAPK.[37] When MLDL1278a was administered to monkeys with diet-induced obesity, pro-inflammatory cytokines decreased and overall immune cell function increased. Dr. Li also noted that the antibody treatment also improved insulin sensitivity in rhesus monkey. MLDL1278A was evaluated in a Phase 1 and a Phase 2 study. In the Phase 2 GLACIER study (NCT01258907), the primary endpoint was the relative change in inflammatory activity in an index arterial vessel after twelve weeks, as measured by [18F]-2-deoxyglucose positron emission-tomography/computed tomography. No statistically significant reduction between placebo and the two active treatment arms was observed.

Kai Y. Xu (University of Maryland) presented data on potent inotropic antibodies that specifically bind to the activation sites of the $(Na^+ + K^+)$-ATPase (NKA) and markedly promote NKA activity, resulting in increased myocyte contraction in vitro and generating positive inotropic effect in mouse heart in vivo. This is the first time that activation of NKA-regulated cardiac contraction at the enzyme, cellular, and animal level have been discovered. The finding provides direct evidence to establish a new concept that activation of NKA induces positive inotropy.[38-42]

Dr. Xu discussed her group's investigations of the fundamental differences in mechanism between inhibition and activation of NKA induced positive inotropic action. Conventional inotropic drugs-induced positive inotropic effect is dependent on the inhibition of NKA and impairment of the active transport of the enzyme linking to an increase in intracellular Na^+ ($[Na^+]_i$), which in turn initiates the reverse-mode function of Na^+/Ca^{2+}-exchanger (NCX) to alter intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$, leading to Ca^{2+} overload. The experimental results demonstrate that inotropic antibody-induced positive inotropic effect through activation of NKA does not alter $[Na^+]_i$ and is independent of the reverse-mode NCX function. Their studies further reveal a novel mechanism that activation of NKA-induced positive inotropic effect is attributable to a moderate increase of Ca^{2+} influx through cardiac L-type Ca^{2+} channel (LTCC) and a release of SR Ca^{2+} via Ca^{2+}-induced Ca^{2+} release mechanism. Newly discovered molecular pathways reveal five major steps responsible for inotropic antibody-mediated cellular Ca^{2+} movement. First, it is initiated by the activation of NKA through an antibody-protein interaction at the activation site of the enzyme. Second, activated NKA induces signal to activate Src kinase. Third, Src kinase transfers signal to Erk1/2, which leads to the phosphorylation of α_1 Ser^{1928} and subsequently the activation of LTCC. Fourth, Ca^{2+} influx through activated LTCC simultaneously stimulates SR Ca^{2+} release. Lastly, increased Ca^{2+} may soon be up-taken back to SR by SR Ca^{2+}-ATPase and/or transported out of the cell via a standard function of NCX (transporting Ca^{2+} ion out of cell and Na^+ into the cell) to reach an apparent cellular Ca^{2+} ion dynamic homeostasis. The fundamental difference in mechanism between activation and inhibition of NKA-induced positive inotropy suggest that activation of NKA-induced positive inotropic action may have great potential for safer therapy for the treatment of heart failure (HF) than the inhibition of NKA-induced positive inotropy.[43,44]

Dr. Xu emphasized four points relating to in the significance and importance of the work: (1) Heart failure (HF) is a major public health problem and major cause of morbidity and mortality worldwide. The central feature of HF is weakened cardiac contractility. Increasing/strengthening heart contraction is a crucial goal of medical management for HF patients. There are

thus unmet needs for new treatments for HF; (2) Conventional inotropic drugs increase cardiac contraction through inhibition of NKA activity, which links to $[Na^+]_i$ and Ca^{2+} overload and leads to the undesirable toxic side effects. In distinct contrast, NKA activator increases cardiac contraction by protecting NKA activity and cell function. This provides a significant improvement for better and safer treatment options to HF patients; (3) The $[Na^+]_i$ is an important cause of Ca^{2+} overload in HF. It has been demonstrated that inhibition of NKA-induced $[Na^+]_i$ plays an critical role in the genesis of cardiac arrhythmias. NKA activator inhibits $[Na^+]_i$, which provides better options to prevent and treat arrhythmias in HF patients; and (4) To date, the absence or inhibition of NCX reverse-mode is one of the critical approaches to reduce arrhythmias in HF. NKA activator increases cardiac contraction without $[Na^+]_i$, without Ca^{2+} overload, and absence of reverse-mode NCX activity, suggesting that NKA activators may represent an important new generation of therapeutic antibodies that can increase cardiac contractility without causing arrhythmias. She concluded by noting that NKA inotropic antibody (NKA activator)-based immunotherapy has potential to revolutionize the current therapeutic strategies for the better and safer treatment of HF.

Ke Cheng (North Carolina State University) discussed magnetic bifunctional cell engagers (MagBICE) as nano-theranostic agents for regenerative medicine. MagBICE are nanoparticles composed of an iron core (Feraheme®) covered with antibodies, which capture stem cells and redirect them to injured heart tissue. Prof. Cheng described model MagBICE that utilize anti-CD45 and anti-MLC for targeting. In a study in mouse, human stem cells were injected to determine whether the particles directed cells to the heart; some targeting to liver and lung was also observed. Regarding potential applications, Prof. Cheng noted that, in addition therapeutic uses, MagBICE could be used as diagnostic agents.

Track 1: The Interface Between Monoclonal Antibody Therapy and Cellular Immunity in Cancer

Gustavo Helguera

The morning Antibody Engineering session was chaired by **K. Dane Wittrup** (Massachusetts Institute of Technology). In his opening remarks he explained the relevance of T cells in the therapeutic activity of antibodies against tumors and emphasized the important interplay of innate and adaptive immunity in the context of monoclonal antibody therapy of cancer.

The first speaker was **Glenn Dranoff** (Dana Farber Cancer Institute, Harvard Medical School), who discussed mechanisms of protective tumor immunity. He introduced his presentation showing that tumors send "mixed messages" to the body. On one side, tumor cells may be perceived as foreign due to multiple mutations arising from genetic instability and also may be perceived as dangerous due to the disruption of tissue homeostasis. In contrast, they also retain many features of normal self that trigger immune tolerance, creating an immunosuppressive microenvironment that facilitates immune escape and promotes tumor progression. In this context, he identifies opportunities for cancer immunotherapy through the generation of vaccines with the modulation of T cells and of the tumor microenvironment. Examples of cancer immunotherapy include vaccines such as sipuleucel-T (Provenge®), which is marketed for prostate cancer, and PROSTVAC (Phase 3 study in prostate cancer underway), T cell modulators such as ipilimumab (Yervoy®, anti-CTLA-4 mAb marketed for melanoma), and tumor microenvironment modulators such as antibody blockers of the programmed death receptor (PD)-1/PD-L1 axis (Phase 3 studies for lambrolizumab and nivolumab are underway).

Dr. Dranoff presented data showing durable clinical responses in advanced non-small cell lung cancer (NSCLC) via PD-1/PDL-1 blockade.[45,46] But, even though major advances are being made in NSCLC immunotherapy, most patients do not derive durable clinical benefits and he proposed that understanding the basis for immune escape in murine tumor models could help improve on these results. Next, Dr. Dranoff presented a model for the pathogenesis of cytokine deficiency-induced lung cancers, which consist in Balb/c GM-CSF/IL-3/IFN-γ-deficient mice. In this model, the impaired immune regulation triggers persistent inflammation that over time results in the appearance of malignant cells with the capacity for autonomous production of inflammatory cytokines, which contribute to tumor progression. This inflammatory microenvironment and other defects attenuate innate and adaptive protective cytotoxic responses, resulting in an adequate model to evaluate non-small cell lung adenocarcinoma immunotherapy. Later he presented epidermal growth factor receptor (EGFR)-driven lung cancers as a prototype for targeted therapy, where the conditional expression of mutant human EGFR in bronchial epithelial cells induces pulmonary adenocarcinomas in mice. Moreover, expression profiling analysis revealed changes in immune profiles early after mutant EGFR induction and presented a strategy to analyze pulmonary cellular responses. Using this method, it was possible to observe that EGFR-driven tumors are associated with significant alterations in myeloid cells, in particular alveolar macrophages, and with the production of a broad array of pro-inflammatory cytokines and chemokines such as IL-6, TGFβ1, CXCL1 or CXCL10, among others. In addition, could be observed that EGFR-driven tumors trigger natural killer (NK) cell dysfunction, the expansion of PD-1+ T cells and Foxp3+ Tregs, and that both EGFR-expressing tumor cells and associated myeloid cells express PD-L1. These observations were consistent with the possibility that PD-1 pathway could be critical to immune escape in EGFR-driven lung carcinogenesis and Dr. Dranoff presented data showing that anti-PD-1 antibody therapy achieves partial regressions of EGFR tumors and that PD-1 antibody blockade prolongs survival in EGFR-driven murine lung cancers.

Dr. Dranoff also showed that anti-PD-1 mAbs bind to CD4+ and CD8+ T cells in vivo and that anti-PD-1 mAb treatment provokes tumor cell killing. Moreover, PD-1 blockade stimulates an increase in intra-tumoral T cells, primarily CD8+ T cells, diminishing tumor-promoting pathways such as IL-6 and TGFβ1, but does not modify the number of FoxP3+ Tregs. Finally, Dr. Dranoff provided evidence of a direct link between EGFR and PD-L1.[47] Immunocytochemical analysis shows that a majority of

human EGFR-driven lung cancers show increased PD-L1 expression on tumor cells or myeloid elements. Moreover, he showed that EGFR pathway activation is associated with increased PD-L1 expression in humans and that EGFR, but not Kras signaling enhances PD-L1 expression in human bronchial epithelial cells. In contrast, blockade of EGFR signaling in NSCLCs with kinase inhibitors such as gefitinib decreases PD-L1 expression without triggering apoptosis. Dr. Dranoff concluded that EGFR-targeted treatments might work, at least in part, through their effect on immune reactions, suggesting new possibilities for combinatorial therapies.

Robert L. Ferris (University of Pittsburgh Cancer Institute) discussed the role of therapeutic mAbs in triggering cross-priming in NK cells and DC with antitumor T cells in patients with head and neck cancer (HNC). Dr. Ferris began his presentation showing that EGFR is expressed in 80-100% of HNC patients and that the anti-EGFR chimeric mAb cetuximab, in combination with radiotherapy, significantly extend the survival of patients with advanced HNC.[48] Cetuximab's high affinity prevents ligand binding to EGFR, reducing proliferation and preventing angiogenesis. However, its clinical anti-tumor activity is limited to ~20% of patients, and response is not correlated with the level of EGFR expression or gene copy number. Moreover, although cetuximab blocks EGFR activation, it does not kill HNC cells. This variability in response could be explained in part by differences in FcγRIIIa V or F genotype in the patient, since in vitro NK-dependent ADCC activity in PBMC correlates with the FcγR IIIa V genotype: VV > VF > FF. In addition, there is a significant difference in progression-free survival for patients with metastatic colorectal cancer (mCRC) according to the presence or absence of KRAS mutation and to the FcγR IIa and FcγR IIIa polymorphisms combination. Accordingly, cetuximab-activated NK cells with VV genotype secrete significantly higher levels of T cell-attracting cytokines and chemokines, such as IFN-γ, tumor necrosis factor (TNF), IL-8, MIP-1α, or RANTES, compared to the FF genotype, suggesting a role in antitumor lymphocyte activation. In addition, Dr. Ferris showed that cetuximab significantly enhanced tumor antigen-specific cross-presentation by DC after activated NK:PCI-15B cells, and that cetuximab treatment enhances EGFR-dependent activation of NK cells and NK:DC crosstalk. This activity has associated an increase in expression of antigen processing component, IL-12 secretion by DC and enhanced secretion of the Th1 biased chemokines CXCL10 and MIG by NK cells. In addition, EGFR-specific tetramer T cell frequencies are elevated in cetuximab-treated HNC patients compared to cetuximab naïve HNC patients. Dr. Ferris also showed a therapeutic protocol of neoadjuvant cetuximab followed by surgery and standard postoperative chemotherapy and radiation therapy with cetuximab. Using this protocol, a significant induction of EGFR-specific T-cells was observed in cetuximab-treated HNC patients. He concluded by showing data suggesting that mAbs targeting EGFR could restore a type 1 microenvironment, resulting in the reversal of tumor immune escape, reprogramming anti-tumor CTL to reverse inhibitory signals and with CTL recognition of HNC.

The presentation of **Jean-Luc Teillaud** (Cordeliers Research Centre, INSERM) provided insights into the adaptive anti-tumor immunity induced by anti-CD20 treatment. Dr. Teillaud introduced his presentation showing that, although mAbs exhibit clinical efficacy in oncology, their long-term efficacy still remains a challenge. Moreover, to achieve long-term survival of cancer patients, it is important to investigate whether mAb therapy can induce or strengthen an anti-tumor adaptive immune response and how to potentiate this response. Dr. Teillaud evaluated tumor survival by mAbs using the C57Bl/6 lymphoma model EL4-huCD20, which expresses human CD20.[49] After injection of EL4-huCD20 cells in C57Bl/6 mice, treatment with the anti-huCD20 CAT-13 mAb (IgG2a, κ) resulted in more than 80% survivors. The more than 70% survival after tumor rechallenge indicates that long-term protection is elicited in tumor-bearing C57Bl/6 mice after anti-CD20 treatment. Further studies showed that $CD4^+$ T-cells are needed both in early and late phases of the anti-CD20-induced anti-tumor protection, and that this anti-tumor protection can be transferred through $CD4^+$ T cells isolated from anti-CD20 treated animals. In addition, long-term surviving anti-CD20 treated mice exhibit an elevated pool of functional effector memory $CD4^+$ T cells that produce IFN-γ, suggesting that anti-CD20 treatment induces an adaptive immune response with immunological memory.

Next, Dr. Teillaud showed that the anti-CD20 treatment skews Treg expansion towards Th1 polarization with no effect on Th2 and Th17 subsets and that the anti-CD20 mAb treatment requires IL-12 for Th1 polarization. Although in vivo IL-12 neutralization has only a mild effect on survival induced by anti-CD20 treatment, IFN-γ and NK cells are required for survival of tumor-bearing mice. Finally, Dr. Teillaud addressed whether use of immunomodulators such as IL-2 could enhance the anti-tumor protection, showing that the presence of IL-2 significantly enhanced the mAb-induced anti-tumor protection and that this response was associated with high frequency of tumor antigen specific T-cells. These observations suggest that "passive" immunotherapy with therapeutic mAbs may evolve towards an active immunotherapy that could be further manipulated with biological response modifiers such as IL-2.

Yang-Xin Fu (University of Chicago) presented new strategies to target the tumor microenvironment for immune protection. Dr. Fu first discussed major limitations in the field of immunotherapy, including difficulty to break tolerance, poor recruitment to tumor site, strong suppressive environment within the tumor site, fast growing tumor in mouse models and lack of defined antigens and adjuvant for vaccination and T cell transfer. Next, Dr. Fu presented the mouse model TUBO derived from MMTV-Rat neu that he used to evaluate anti-HER2/neu antibody-induced oncogenic blockade and ADCC.[50] Using this model, he showed that anti-HER2/neu tumor protection was dependent on innate immune cells, such as NK cells, and FcγR dependent. Moreover, he also showed that tumor control by antibody depends on adaptive immune responses, and that the antibody-mediated tumor regression was CD8+ T-cell dependent. The immune response triggered by anti-HER2/neu also required signaling dependent on HMGB-1/TLRs and MyD88.

In the clinic, conventional chemotherapy could either enhance tumor eradication or erase this antibody-triggered immunity. Using mouse models, Dr. Fu showed that giving chemotherapeutic drugs such as paclitaxel before the anti-HER2/neu injection could increase short and long term protection, suggesting that the drug dose and schedule might be critical for optimal response.

Dr. Fu also showed data from an anti-EGFR molecule fused at its Fc domain via a flexible linker to IFNβ. This anti-EGFR-IFNβ molecule localizes the cytokine preferentially to the tumor and is effective for controlling tumor growth using a TUBO-EGFR murine tumor model. The anti-EGFR-IFNβ molecule was effective for controlling mAb-resistant B16-EGFR tumor. Although anti-EGFR-IFNβ treatment was not effective in direct tumor killing, it could induce anti-tumor activity through re-activation of CTLs. In addition, protection against tumor depends on DC, responding significantly to the anti-EGFR-IFNβ treatment and increasing the cross-presentation. Moreover, antagonizing PDL-1, a major negative inhibitor of CTL, favor long-term protection by anti-EGFR-IFNβ. Finally, Dr. Fu showed data in which depletion of $CD4^+$ cells enhanced the anti-tumor effect of anti-EGFR-IFNβ, suggesting that T-regs could play a role antagonizing the activity of the fusion protein. He concluded proposing a model of how type I IFNs link innate and adaptive anti-tumor immunity and suggesting many check points for mAbs treatment.

In the session's last presentation, **K. Dane Wittrup** (Massachusetts Institute of Technology) discussed strategies for coordinated tumor growth suppression via synergistic innate and adaptive immunotherapy. He began by introducing the concept of "tumor genetic mosaicism",[51] proposing that directing therapy based on genetic tumor markers is too simple because genetic heterogeneity combined with the polygenic nature of drug resistance results in resistance to targeted therapies, as in the cases of lapatinib, ipilimumab or vemurafenib, among others. He proposes, however, that immunotherapy has the potential to overcome resistance, since an adaptive immune response could counter an evolving pathology such as cancer. Immune-modulatory molecules like the cytokine Interleukin-2 (IL-2) have been show to potentiate the activity of mAbs via improved ADCC in vitro. However, no clinical benefit has been observed in patients when given in combination with trastuzumab or rituximab. This discrepancy seems to be related to the IL-2 exposure time in vitro, where the effective concentration of the cytokine is extended for days. In contrast, the half-life of IL-2 in vivo is in the order of minutes, requiring daily infusions at high concentrations, which results in a very different IL-2 exposure profile between conditions. The PK profile of IL-2 could be significantly improved with the fusion of IL-2 to a mouse IgG2a Fc fragment with the D265A mutation (Fc/IL-2), which exhibits reduced ADCC and complement-dependent cytotoxicity (CDC), but longer half-life. Using the syngeneic murine melanoma tumor model B16-F10, the protective activity of the antibody TA99, an anti-TRP1 murine IgG2a, was evaluated in the presence or absence of murine IL-2 wt or Fc/IL-2. The combination of TA99 and Fc/IL-2 significantly suppressed tumor growth, while wt IL-2 alone or combined to the mAb was ineffective. This activity was fully dependent on $CD8^+$ T-cells and neutrophils, required NK cells, IFN-γ and myeloperoxidase. However, macrophages, $CD4^+$ T cells and TNF were dispensable. Finally, Prof. Wittrup showed that the combination of Fc/IL-2 and TA99 with pmel-1 $CD8^+$ T-cells, used in the context of an adoptive T cell therapy, completely protected against B16 tumor cells, resulting in cure of 14 of 15 animals. These results provide further evidence of the great potential of immunotherapy for the treatment of cancer.

Track 2: The Effects of Antibody Gene Variation and Usage on the Antibody Response

Raiees Andrabi, Ann Feeney, Wayne A Marasco

The morning session on the effects of antibody gene variation and usage on the antibody response was chaired by **Jamie K. Scott** (Simon Fraiser University). **Corey T. Watson** (Icahn School of Medicine at Mount Sinai) presented data on characterization of haplotype variation at the human immunoglobulin heavy chain (IGH) locus and implications for human disease research. Structural variants, including deletions, insertions, and duplications that result in changes in gene copy number (copy-number variants, CNVs), are common features of the human genome and are a significant source of inter-individual sequence variation. This form of variation has been implicated in a broad spectrum of human phenotypes, including adaptive traits, developmental and neurological disorders, and infectious and autoimmune diseases. The study was inspired by early observations of the extraordinary genetic diversity at human immunoglobulin loci, many portions of which could possibly have remained unsequenced (gaps) or poorly represented/described in the human reference genome. This is supported by the fact that 45% of the IGH locus falls within a segmental duplication region (>90% identity) that is known to be associated with structural variation. Consistent with these assumptions, at least 40 new IGHV gene allele variants have been identified in the last decade.

To identify and characterize additional IGH genomic haplotypes, Prof. Watson and colleagues generated a high-quality alternate sequence assembly of the IGH locus by using large-insert bacterial artificial chromosome (BAC) clones from a haploid hydatidiform mole library (CHORI-17);[52,53] the genomes of hyatidiform moles are diploid, but comprise identical chromosomes. Thus, the CHORI-17 hydatidiform mole BAC library (CH17) genome composes a single haplotype, and as a result, any variation observed in assemblies can be attributed to paralogous sequence variation rather than allelic variation. They sequenced 9 BAC clones for IGH by the Sanger method, and identified 12 large structural variants ranging in size from 9 to 60 Kbp, including 222 Kb of novel insertion sequence; also described were 16 novel IGHV gene alleles. Prof. Watson and colleagues proposed that the results generated in this study represent the first map of copy number variants in the human IGH locus at nucleotide resolution, including descriptions of novel deletions and insertions of functional IGH genes. These data should be instrumental in analysis and interpretation of expressed antibody repertoire analyses. Overall, this work has provided a new, exceptionally detailed resource for addressing the potential roles of genetic

diversity among IGH loci in antibody function and expression, disease risk and vaccine design.[52]

Chunguang Guo (Harvard Medical School) addressed the role of a critical V(D)J recombination control region in the immunoglobulin heavy chain locus. B-cell development in the bone marrow involves the rearrangement of gene segments in the immunoglobulin heavy-chain locus (IgH) and immunoglobulin light-chain loci (kappa and lambda). The nature of V(D)J rearrangement primarily defines the lineage, stage and allele specificity of B cells with a critical VH to DJH joining step in IGH regulated by a feedback mechanism. Remarkably, the balance in the utilization of proximal versus distal VH genes is maintained by selection on B-cell receptors and locus contraction.[54] Overall, B-cell lineage, stage, and VH allele specificity are achieved by controlling the accessibly of the substrate to the RAG recombinases, which in turn is highly regulated by many factors, including "non-coding" transcription at germline VH gene segments (i.e., transcription that does not lead to translation into proteins), DNA methylation, chromosome positioning, locus contraction and histone modification.[55,56] It has been previously shown that the VH to D intergenic regions of T cells house important regulatory elements required for VH to DJH rearrangements, and thus are involved in defining B-cell lineage.

To understand the role of these elements, the regulatory role of intergenic control region 1 (IGCR1) during B-cell development was studied.[57-59] IGCR1 is a CTCF (CCCTC-binding factor) that has multifunctional roles in gene regulation, such as enhancer insulation, transcriptional activation/repression and long-range chromosome looping.[60,61] To understand the role of this intergenic element, a transgenic mouse model bearing an IGCR1 deletion and mutations in the CTCF binding element (CBE) was used. It was found that these changes led to a defect in the pro-B to pre-B cell transition, and a reduction in B-cell maturation. These results revealed that an IGCR1/CBE mutation results in over-utilization of proximal versus distal VHs, and that IGCR1 regulates non-coding transcription of proximal VH segments. In addition, it was shown that IGCR1/CBE regulates B-cell lineage and stage specificity, whereas IGCR1 also regulates germline VH gene usage. Dr. Guo concluded by noting that that IGCR1 may regulate B-cell development by mediating chromosomal interactions.

Ann Feeney (The Scripps Research Institute) discussed her deep sequencing analysis of the repertoire of rearranged VH gene regions in pro-B cells. Dr. Feeney and her colleagues have performed the first deep sequencing of IGH V, D, J rearrangements in cell-sorter purified pro-B cells from normal C57BL/6 mice, revealing the entire newly generated repertoire of IGH VDJ rearrangements. The library for deep sequencing was made in an unbiased manner using RACE technology, in which an adapter was ligated to the end of the mRNA and PCR amplification of cDNA was made with primers complementary to the adaptor and to the mu constant region of the heavy chain, Cμ. Each library consisted of a pool of ten independent PCR reactions, and six independent libraries were pooled to obtain over 17,000 unique rearrangements for analysis. This dataset allowed detailed analysis of the factors that influence the frequencies of V, D and J gene rearrangements at the IgH locus. It was observed that the two most frequently rearranging D gene segments (DFL16.1 and DQ52) are located at the two ends of the ~150 kb DH gene region of the IGH locus. Dr. Feeney and her colleagues had previously demonstrated by chromosome conformation capture (3C) that the CTCF sites, which they had identified as being just upstream of the most 5' D gene, interact directly with the CTCF sites at the 3' end of the IGH locus, and with the Eμ enhancer. Since Eμ is directly downstream of the four JH genes, that places DFL16.1 and DQ52 in close proximity to those four J genes in 3-dimensional space, thus likely explaining their high frequency of utilization in VDJ rearrangements. This loop also creates a domain that contains the D and J genes, but excludes the large, V-gene-containing region of the IGH locus, which may facilitate the known ordered rearrangement in which the joining of D and J genes always precedes joining of a V-gene with the rearranged DJ segment.

The ~100 functional V genes are spread over 2.8 Mb in the IGH locus. It has been known for some time from studies on individual V-gene families, that V genes are utilized at very different frequencies, but the reasons for this are unknown. Moreover, until this study was performed, there was no in-depth study of the entire repertoire of rearranged VH gene regions. With the current deep-sequencing data set, it was clearly observed that V genes are rearranging at very different frequencies throughout the IGH locus. The VH dataset allowed a detailed bioinformatic analysis of the factors that could impact V-gene accessibility, and therefore gene rearrangement frequency. ChIP-seq data for several histone post-translational modifications has already been done in the Feeney lab. They also published the first RNA-seq analysis (i.e., deep sequencing analysis of total RNA) of pro-B cells, revealing all of the non-coding "germline transcription" throughout the IGH locus. It had been proposed over two decades ago that one role of this ncRNA was to make the regions being transcribed accessible to VDJ gene rearrangement. In order to determine how the local transcriptional environment and the local epigenetic environment influence V-gene rearrangement, the number of ChIP-seq or RNA-seq reads aligning to a 2.5 kb region surrounding each V gene was computed, and those data were analyzed bioinformatically. It was observed that the IGH locus could be divided into four domains based on these parameters, and each domain had different characteristics. The V genes in domain 1, the domain closest to the DJ genes, are relatively devoid of active histone marks and ncRNA in general, but had highly functional RSSs. Importantly, all of the functional V genes in this domain had CTCF sites close to them. Since CTCF-CTCF homodimerization is hypothesized to create the multi-looped structure of the compacted IGH locus, V genes close to CTCF sites would be close to the base of the loops, and it appears that this proximity to the base of the loop is very important for the V genes in domain 1. In contrast, the two distal domains, domains 3 and 4, had high levels of histone modifications and ncRNA, which may compensate for their poorer quality RSSs and for being distant from CTCF sites. Within domains 3 and 4, the V genes with the highest levels of H3 acetylation, or of H3K4 methylation rearranged most often. Thus, the large IGH locus has evolved a

complex system for the regulation of V(D)J rearrangement that is different for each of the four domains comprising this locus.

Ignacio Sanz (Emory University School of Medicine) discussed VH4-34-encoded antibodies in autoimmune and protective responses. Systemic lupus erythematosus (SLE) is an autoimmune disease that can affect any part of the body including the heart, joints, blood vessels, liver, kidney and nervous system. The generation, maintenance, and diversification of autoantibodies in SLE reflect a complex interplay between self-antigens and B cells in lymphoid organs and peripheral blood. This process eventually gives rise to stable anti-Ro and anti-Sm/RNP autoantibodies originating from long-lived plasma cells in the bone marrow, and to fluctuating autoantibodies against double-stranded (ds) DNA and antibodies reactive with the mAb 9G4, which react with antibodies encoded by germline VH4-34 gene segments. VH4-34-encoded antibodies are intrinsically autoreactive due to a germline encoded framework-1 hydrophobic patch that determines binding to N-acetyl lactosamine glycans. VH4-34-expressing B cells (9G4+) are negatively selected in healthy subjects, but not in SLE patients, nor in a fraction of HIV-infected patients. The recruitment of new, naïve B cells or pre-formed memory cells in active SLE could be predicted by B-cell clonal expansion and repertoire diversity.

Prof. Sanz described a combined approach of deep sequencing, single cell mAb generation and antigenic screening used to gain understanding of the B cell repertoire diversity, molecular patterns and autoreactivity in SLE. Prof. Sanz and colleagues observed that SLE flares are accompanied by large expansions of proliferating (ki67+) plasmablasts (PB), which are CD138-, and their differentiated offspring, the plasma cells (PC), which are CD138+. PB/PC-derived antibody-secreting cells (ASCs) in SLE are largely polyclonal, and are not driven by SLE-associated autoantigens. It was also shown that SLE flares are punctuated by clonal expansions of B cells producing autoreactive VH4-34-encoded antibodies, which recognize major lupus antigens such as apoptotic cell antigens, cardiolipin, dsDNA, and chromatin, and that produce anti-nuclear staining patterns. These newly recruited naïve B cells contribute substantially to the polyclonal expansion of ASCs during lupus flares. Dr. Sanz noted that, overall, the observation of these two types of ASC bear significant implications for our understanding of the mechanisms of action of current anti-B cell therapies, and should help guide the design and testing of new B-cell targeting agents.

Kevin Henry (Simon Fraser University) focused on the biased VH gene usage by antibodies (Abs) of distinct B cell subsets in HIV infection, specifically HIV-associated modulations of immunoglobulin heavy chain gene (IGVH) repertoires. It is widely known that HIV broadly neutralizing (bNt) Abs display a several unique features including: (1) high levels of somatic hypermutation in the heavy- and light-chain gene variable regions (VH and VL), (2) long third complementarity-determining regions of the heavy chain (CDR-H3s) encoded by the V-D-J joint, and (3) biased germline VH gene usage.[62] VRC01-like bNt mAbs recognizing the CD4 binding site (CD4bs) of the HIV envelope accumulate as much as 30% amino-acid divergence from its parental gene reflecting high levels of somatic mutation driven by persistent antigen and affinity maturation.[63-65] The V1/V2 binding class of bNt mAbs, like PG9/16 or the PGT140 series, have extraordinarily long CDR-H3s (up to 32 amino acids).[66] In addition, germline VH gene usage by the HIV bNt Abs tends to be skewed, especially those against three regions on the HIV envelope: CD4bs (IGHV1-2, IGHV1-46), the CD4i (IGHV1-69) and the V3 loop (IGHV5-51).[64,67,68]

Prof. Henry discussed studies designed to investigate questions about whether these unusual genetic features are specific to bNt Abs, or to Env-specific Abs in general. How do these Abs compare with the overall repertoire in HIV positive individuals, and in general with those of healthy seronegative individuals? How are they related to the specific B-cell functional compartments? The questions were investigated using NGS of immunoglobulin heavy chain repertoires from phage display Fab libraries, and of sorted B-cell subsets derived from HIV+ individuals and healthy controls. The results revealed no CDR-H3 perturbation in the overall Ab repertoire of HIV+ individuals as compared to healthy controls. It was also observed, by specifically analyzing the V-D and D-J joints of the VDJ joining regions, that the same mechanisms produced Abs with long CDR-H3 loops in HIV-positive individuals as in healthy individuals. Repertoire diversity generated by V-D-J rearrangement, as revealed by NGS of naïve B cells, was similar between HIV+ and healthy controls and was concentrated in regions encoding CDR-H3s. Somatic mutation levels in VH regions of memory B cells and plasmablasts were higher in HIV+ individuals compared to healthy controls (6-10/VH in HIV-positive versus 1-2/VH in healthy individuals, respectively). The results of B-cell phenotype analysis supported previous studies in showing that, compared to healthy controls, HIV-infected individuals have perturbations in the distributions of naïve B cells (reduced), resting and activated memory B cells (elevated) and plasmablasts (elevated);[69,70] however, NGS of these subsets showed no difference between the HIV+ and healthy individuals on VH gene usage, or CDR-H3 length in the VH repertoire of any B-cell subsets. In addition, there was weak or no evidence of increased IGHV1-69, IGHV5-51 or IGHV4-34 usage among HIV+ individuals. Overall, the data suggest that VH repertoire diversity among memory cells expands by somatic mutation driven by with non-specific immunomodulatory mechanisms associated with chronic infection, whereas features observed both for bNt mAbs and for weakly or non-Nt mAbs cloned from chronically infected HIV+ individuals, such as very heavy somatic mutation levels, long CDR-H3s and biased VH gene usage, may be driven by prolonged antigenic stimulation associated with persistent infection.

Wayne A. Marasco (Harvard Medical School, Dana Farber Cancer Institute) started his presentation by mentioning to the audience that reignited efforts to develop a "universal" influenza vaccine can be attributed to the recent discoveries of rare human broadly neutralizing antibodies (sBnAbs) that bind to a highly conserved hemagglutinin (HA) stem epitope. Interestingly, several studies have reported on the biased usage of *IGHV1-69* germline gene in discovered sBnAbs and several co-crystallographic studies have shown that only the VH chain alone makes contact with HA. Prof. Marasco sought to better define the V-segment

amino acid substitutions and CDR-H3 amino acids within rearranged *IGHV1-69* germline genes to allow an *IGHV1-69* germline based antibody to become a potent HV1-69-sBnAb.

Analysis of 38 HV1-69-sBnAbs recovered from 8 laboratories indicates that broad-spectrum binding and neutralization is conveyed by a triad of critical anchor residues composed of two CDR-H2 residues, including a hydrophobic residue at position 53 and Phe54, and properly positioned CDR-H3 tyrosines. In addition, he showed the occurrence of distinctive V-segment mutations within the CDR H1/H2/H4 loops. Analysis of the distinctive substitutions showed that they occur in positions that are sparse in activation-induced cytidine deaminase (AID) and polymerase eta (pol η) consensus "hot-spot" motifs, suggesting that HV1-69-sBnAbs generation mainly relies on phase 2 long-patch error-prone repair or random non-AID somatic hypermutation (SHM) events. Screening of a semi-synthetic *IGHV1-69* phage-display library that was designed to bypass these normal physiological restrictions resulted in the high-frequency isolation of sBnAbs that were characterized by a minimal number of VH-segment substitutions and that bare these same distinctive HV1-69-sBnAb substitutions and CDR-H3 98/99Tyrs. Mutagenesis studies and structural analysis focused on one of the sequence solutions suggested that the distinctive mutations which occur in the CDR-H2 loop function facilitate the insertion of 2 key contact/anchor residues, CDR-H2 Phe54 and CDR-H3-Tyr98/99, into adjacent pockets on the stem of HA. Prof. Marasco summarized that these studies provide new information on the SHM requirements for *IGHV1-69*-encoded B cells to produce HV1-69-sBnAbs and suggest that there may exist alternative routes to B cell germinal center reentry/recycling that could be exploited as a pathway for HV1-69-sBnAb elicitation by vaccination.

Tuesday December 10, 2013
Track 1: Directed Evolution, A Tribute to Pim Stemmer and Michael Neuberger

Robert Mabry

Andreas Plückthun (University of Zurich), Chair of the session, briefly discussed the effect of directed evolution on protein therapeutics, diagnostics and on preparing the targets for generating antibodies and other binding proteins. He noted that Michael Neuberger and Pim Stemmer, two energetic scientists who were pioneers in directed evolution, passed away in 2013. Prof. Plückthun celebrated Dr. Neuberger's work, who had originally been scheduled to speak in this session, by providing a short biography on his achievements in the fields of biochemistry and immunology.

Michael Neuberger was the youngest of four sons in a very gifted family. With his siblings, he attended Westminster where he won a scholarship to Trinity College. He went on to obtain his PhD in Biochemistry in Dr. Brian Hartley's lab at Imperial College London, focusing his work on phage and enzymes in the bacterium of *Klebsiella aerogenes*. Dr. Neuberger trained in Immunology in Cologne under Klaus Rajewsky, and he then returned to Cambridge to begin a fruitful partnership with Cesar Milstein. By the age of 40, Dr. Neuberger was elected fellow to the Royal Society. His contributions included the generation of knockout mice to investigate effects of recombination, elucidation of the mechanism of somatic hypermutation, the evolution of enzymes and antibodies, and the discovery of activated-induced cytidine deaminase (AID) as the key enzyme for somatic hypermutation. This latter work led to the founding of AnaptysBio. Dr. Neuberger's scientific contributions are truly remarkable, reflecting not only breadth in his contributions to several fields, but also his ability to breed innovative science at every turn of his career. Prof. Plückthun described Dr. Neuberger as an understated and very friendly fellow. His scientific prowess was matched by his affable character, and his passing at the early age of 59 cut short a brilliant career.

In the first presentation, **Volker Schellenberger** (Amunix) discussed XTEN, a protein polymer for half-life extension and drug conjugation, but began his talk by highlighting his partnership with Pim Stemmer. He described Dr. Stemmer as an intense scientist with a hands-on approach to industry. As a founder of both Maxygen and Avidia, Dr. Stemmer was very comfortable working in the limelight of young companies. Yet, despite his financial success following those ventures, he preferred to operate on a level that allowed him to maintain full exposure to all activities within small organizations. While Dr. Stemmer proved himself as a viable entrepreneur with an impressive track record, he continued to explore models that would both satiate venture capital investment and maintain sustainability as a professional workforce within a startup architecture. Dr. Stemmer favored a lean company model and always valued a bargain. Dr. Stemmer tended to apply directed evolution to everyday planning, recognizing that plans should be altered to adapt to the ever-changing events that steer us from one direction to the next. His candor, attention to detail, and scientific integrity will be missed by Dr. Schellenberger and his colleagues.

Dr. Schellenberger then began his scientific presentation with an introduction to the XTEN technology created at Amunix. The platform is based on a protein-based unfolded polymer employed for half-life extension that is similar to PEG, but provides additional advantages.[71] XTEN is composed of natural amino acids and thus the proteins are biodegradable, and, avoiding risks associated with kidney vacuolation and potential toxic metabolites. The technology brings the precision of recombinant production to polymers. Dr. Schellenberger also pointed out that, in contrast to traditional PEG conjugation, XTEN has a non-immunogenic profile. Predictive T-cell epitope software reported a non-detectable immunogenicity profile, and other methods for antibody discovery (phage panning and animal immunizations) yielded no or extremely low responses from either synthetic or naïve antibody repertoires.

Dr. Schellenberger mapped out several different strategies for employing XTEN for therapeutic design. He provided a case study that showed homogenous XTENylated products can be obtained via conjugation to XTEN, compared with heterogeneity observed with PEGylated pharmaceuticals such as pegfilgrastim (Neulasta®). Chemical conjugation of molecules was presented as an additional option because Amunix has now moved into the drug conjugate field. A folate-XTEN drug conjugate generated

by click chemistry exhibited very good conjugation efficiency as monitored by HPLC.

Frances Arnold (California Institute of Technology) began her presentation "Innovation by Recombination" by addressing some of the obstacles to the design of enzymes with evolved function. She gave insight into the efficiency of evolution, stating that naturally-occurring enzymes are the products of changes that occur over millions of years. Yet, the natural mechanism for generating diversity is limited, as she pointed out, and does not include all design space that could be explored. Directed evolution may make something impossible possible through the exploration of sequence space that is large, but also mostly "empty," i.e., unexplored. She added that mutations, conceptually, are made one at a time, and, through iterative cycles, we can arrive at mutations that achieve altered function. Directed evolution exploits the smooth paths in the fitness landscape by consecutive iterations until the "problem" is "solved".

Dr. Arnold supported her commentary with examples of enzyme evolution performed by her group's work. Multiple generations of mutagenesis and screening can generate large changes in function, further underscoring that enzymes (and other proteins) are highly "evolvable." One interesting observation, however, is that many of the mutations identified through these expedited evolutions are distant from active sites.[72]

Recombination is another effective strategy for creating new molecular functional diversity. This strategy covers novel diversity by introducing gene fragments to produce chimeras that are substantially different from predecessors. By applying concepts from the schema theory, Dr. Arnold and her group developed a computational algorithm to cut genes into blocks to minimize disruption of folded subdomains. The blocks were then reassembled to produce chimeric libraries that were screened for functional hybrids. This method has been applied to the evolution of beta-lactamases,[73] cytochrome p450s,[74] cellulases,[75] arginase,[76] and other enzymes.[77] Dr. Arnold emphasized that the innovation that recombination captures for creating novel diversities may reap molecules with potentially new reactions.

Prof. Plückthun then discussed the directed evolution of stable GPCRs for use as drug and antibody targets and for structure determination, and focused on the hurdles related to the production of GPCRs as soluble protein. There is a strong demand for the generation of stable constructs that recapitulate the structure and function of transmembrane receptors for drug discovery. However, GPCR molecules are encumbered with inherent stability problems when solubilized in detergent micelles, resulting in aggregates not suitable for screening processes. There is a need for the generation of GPCR preparations that possess improved stability yet faithfully represent native functional receptor in a format conducive to discovery processes.

A library approach was presented by Prof. Plückthun in which GPCRs were engineered for improved properties on the inner membrane of *E. coli*.[78] Stochastic diversity was introduced into the rat NRT-1 gene to generate a library of mutants that were then screened for binding to fluorescence-labeled ligand. Through iterative cycles of mutagenesis and screening, mutants were identified that express 10-fold better in bacteria than wild-type and maintain functional ligand binding. Improved expression was also observed in *Pichia* and mammalian HEK293 cells, and corresponded with improved thermal stability. While this library approach was successful, there exists a much larger diversity space that could be explored.

To further push the exploration of larger diversities, libraries were generated in which all possible codons for each amino acid were changed one at a time.[79] Improved mutants captured via selective pressure possess improved expression both in prokaryotic and eukaryotic hosts, which translated to greatly improved yields as solubilized and purified receptor preparations. This method was expanded upon by both recombination and a synthetic binary library generated using the Slonomics technology.[80] "Shift" positions were identified where the selected mutants all converged on residue types that are different from wild type, i.e., where the wild type sequence encodes problematic residues. Mutants retrieved from the selections expressed 50-fold over wild-type, and showed greatly improved stability in harsh detergents yet maintained function in G-protein signaling.

While the selection for functional expression was strongly correlated with stability in harsh detergents, Prof. Plückthun and colleagues sought to focus selective pressure directly based on stability in detergents. He discussed a method that encapsulates single *E. coli* cells of a library to form detergent-resistant nanocontainers.[81] This method permits the solubilization of GPCR proteins in situ while maintaining genotype/phenotype linkage. Combined with fluorescence-activated cell sorting (FACS), mutants were identified that possess the highest stabilities for GPCRs reported in short-chain detergents. This strategy, which Prof. Plückthun referred to as CHESS (cellular high-throughput encapsulation, solubilization and screening), may also be applied to other proteins to improve expression and stability. These evolved receptors have been crystallized, and their structure confirmed that the evolution maintains structure and function of the GPCRs, allowing them to be used for drug design and as targets for antibodies.[82]

In the final presentation, **David R. Liu** (Harvard University) discussed the continuous directed evolution of proteins, focusing on the streamlining of iterative engineering cycles. While successful in the laboratory setting, directed evolution can be improved by addressing time and labor devoted to the engineering process. His work confronts the limitations of in vitro engineering by tackling certain rate-limiting steps to produce a continuous process for evolving proteins in a fraction of the time required for traditional methods. The initial work Dr. Liu presented is built upon earlier research in which RNA ligase ribozyme was engineered through ~300 successive rounds of catalysis and selective amplification in less than 3 days.[83] This was the first report of continuous in vitro evolution made possible through the culturing of *E. coli* with a "hands-off" platform.

Dr. Liu reported the ability to adapt this continuous process to overcome the limitations of conventional phage display.[84] To achieve this, he and his colleagues sought to link phage production to the desired activity using a system called phage-assisted continuous evolution (PACE).[85] His team devised a system in which *E. coli* cells containing both a mutagenesis plasmid and

an accessory plasmid continuously flow through a fixed-volume vessel (the "lagoon") where they are infected with selection phage encoding a library of mutants hosted on a third plasmid. Focusing on the pIII protein that mediates F pilus binding and host cell entry, gene III encoding pIII was deleted from the phage vector and inserted into the accessory plasmid hosted in *E. coli* to make the production of pIII dependent on the activity of the evolving gene of interest. Phage vectors capable of inducing sufficient pIII production for infectivity will propagate and persist in the lagoon. This system leverages the speed of the phage life cycle, where up to 16 rounds of PACE selections can be performed in a single day. Using PACE, T7 RNA polymerase was engineered to recognize a distinct promoter and initiate transcripts with ATP and CTP rather than GTP all within one week of continuous evolution. Dr. Liu's presentation also highlighted one case in which 200 rounds of PACE were executed in eight days.

Dr. Liu also discussed a study in which his team investigated evolving populations subjected to first divergent selection pressures, and then convergent selection pressures.[86] Two selection pathways subjected the T7 RNA polymerase to evolve recognition to either the T3 or SP6 promoter. They observed the evolutionary convergence of at least two clusters of local fitness peaks identified by the different selection pathways. Despite the incorporation of convergent selection pressures, populations subjected to the T3 selection pathway were not able to achieve higher-activity cluster discovered by populations exposed to the SP6 pathway. The results from this study demonstrate how the evolution trajectory of proteins can be influenced by stochastic occurrences and prior selection history.

Dr. Liu then expanded on the repertoire of applications with PACE.[85] RNA polymerases were engineered to increased specificity over 100,000-fold compared to wild-type in just 3 days of PACE. His lab is currently applying PACE other proteins, such as monobodies and DARPins, with potential to expand to other classes.

Track 2: Antibody Pharmacokinetics, Distribution and Off-Target Toxicity

Lubna Abuqayyas, Janice M Reichert, Stefan Barghorn

Following the lunch break, the session on antibody PK, distribution and off-target toxicity was chaired by **Trudi Veldman** (AbbVie). The first speaker, **Lubna Abuqayyas** (Amgen) discussed work she had done as a graduate student at the State University of New York at Buffalo. In particular, she presented an investigation of the PK, tissue and tumor distribution for therapeutic IgG, via modulating determinants of disposition with drugs and knockout animal models. Such determinants include the role of Fc receptors (FcR), FcγR and FcRn, on IgG PK and tissue disposition, and uptake into the brain. She also discussed evaluation of the effects of co-administration of agents that alter the tissue vasculature (e.g., anti-VEGF mAb) on PK and tumor uptake of an anti-cancer antibody.

To evaluate the role of Fc receptors (FcR) on IgG disposition, FcRn α-chain knockout mice, FcγR knockout mice, and wild-type mice were used. Two model antibodies were also used in this investigation: (1) a model monoclonal IgG1 antibody with high affinity for a soluble ligand, and (2) a model monoclonal IgG with specific affinity for cell surface proteins. The results demonstrated identical plasma and tissue disposition in wild-type mice and in FcγR knock-out mice for each model antibody, indicating that mAb PK are not substantially influenced by FcγR expression.

To examine the role of FcR on IgG distribution to the brain, the disposition of 8C2, a murine monoclonal IgG1 antibody, was also evaluated. The brain/blood areas under 8C2 concentration vs. time curves ratios were comparable across all strains of mice. These findings indicate that FcRn and FcγR do not contribute to the "blood–brain barrier" that limits mAb uptake into the brain.

Dr. Abuqayyas also discussed the effect of administration of anti-VEGF antibody on the plasma, tissue, and tumor disposition of an anti-carcinoembryonic antigen (CEA) antibody in SCID mice bearing CEA-expressing human colorectal cancer. The experimental results showed that anti-VEGF therapies may lead to a substantial reduction in the delivery of mAbs to tumor tissues. The finding is especially important because no alterations in anti-cancer antibody disposition were visible based on assessment of plasma PK alone.

Naoka Hironiwa (Chugai Pharmaceutical Co.) discussed calcium-dependent antigen binding as a novel mechanism for generation of recycling antibodies. She first described the isolation of a clone from a naïve library. The antibody derived from the clone binds to its antigen only in the presence of calcium and dissociates the antigen within the endosome in vitro. The calcium-dependent antigen binding capacity was found to accelerate antigen clearance; a unique calcium ion binding structure that underwent conformational change in the presence or absence of calcium ions was observed. Dr. Hironiwa then described a synthetic library approach that allowed identification of a relevant calcium-binding motif can be grafted without loss of the calcium-binding property. She concluded by noting that antibodies with calcium-dependent antigen binding were efficiently obtained from a predesigned synthetic library, antibodies targeting several antigens have been generated, and these were found to accelerate antigen clearance.

Jean Lachowicz (Angiochem Inc.) discussed the creation of peptide-antibody conjugates that cross the blood-brain barrier (BBB) to achieve therapeutic concentrations in the central nervous system (CNS). She explained that the BBB functions to protect the brain via tight junctions and P-glycoprotein activity that prevents almost all foreign molecules from penetrating the brain, thereby preventing damage. The BBB also regulates brain homeostasis; receptors must actively transport essential molecules, including glucose, insulin, and growth hormones, into the brain. The low-density lipoprotein receptor related protein (LRP)-1, one of the most highly expressed receptors on the BBB, is known to transport small and large molecules across the BBB. Dr. Lachowicz noted that Angiochem has developed 'Angiopeps', which are peptides derived from human Kunitz domains that target LRP-1 and engage in receptor-mediated transcytosis.

Dr. Lachowicz then presented data for ANG4043, which is a chemical conjugate of trastuzumab with Angiopep-2. The conjugation does not affect target affinity, and ANG4043 retains anti-proliferative efficacy in BT-474 breast cancer cells (IC_{50} = 4.99 ±76 for trastuzumab and 3.16 ±97 for ANG4043). In a PK study in C57 mice, trastuzumab and ANG4043 demonstrated a similar profile. T1/2β was 11.4 and 8.5 days, Cl was 0.43 and 0.97 ml/day, and Cmax was 166 and 91.5 μg/ml for trastuzumab and ANG4043, respectively. In vivo studies in mice showed that animals treated with 15 mg/kg ANG4043 had intracranial BT-474 tumor shrinkage, as assessed by near-infrared imaging. Dr. Lachowicz concluded by noting that ANG1005, a conjugate of paclitaxel with the Angiopep-2 peptide, is undergoing evaluation in Phase 2 studies of patients with glioma, non-small cell lung cancer with brain metastases or breast cancer with brain metastases.

Louis Weiner (Georgetown University Medical Center) discussed manipulating ADCC to promote anti-tumor responses. His talk focused on points related to whether antibody therapy induces host protective adaptive immunity and, in preclinical studies, what happens when animals 'cured' by antibody therapy are rechallenged with tumor.

Srinath Kasturirangan (MedImmune) presented data on the enhanced clearance of interleukin (IL)6 by a chelating bispecific antibody. The targeting of two distinct epitopes allows each antigen molecule to bind two antibodies, thereby driving complex formation. Antibody/antigen complexes can bind to FcγR-expressing cells with high avidity, potentially leading to fast clearance. The combination of ligand neutralization and fast in vivo clearance may result in improved therapeutic potency of chelating antibodies compared to canonical mAbs, which can prolong half-life of soluble antigens through a 'buffering' effect.

Dr. Kasturirangan provided a brief background on IL6, which is a multifunctional cytokine that regulates immune response, hematopoiesis and inflammation. The elevated production of IL6 contributes to the pathogenesis of various autoimmune diseases, and it is a growth factor for multiple myeloma and renal carcinoma. IL6 exerts its biological activities through the IL6 receptor (IL6R) and gp130. Binding to IL6R is low affinity (5.5. nM); upon induction of homodimerization of gp130, a high affinity (50 pM) functional receptor complex of IL6, IL6R and gp130 is formed. Dr. Kasturirangan then described generation of a bispecific antibody, Bis3, comprising the IgG CNDY120, which blocks IL6 binding to IL6R, with CAN119 scFv, which blocks IL6/IL6R complex binding with gp130, attached via the Fc. The affinities to IL6 (K_D) were 0.324 nM for scFv CAN119, <10 pm for IgG CNDY120, and 5 pM (apparent) for the bispecific construct Bis3. Dr. Kasturirangan showed data indicating that Bis3 can bind both IL6 epitopes simultaneously (Biacore analysis), and it generates predominantly large complexes with IL6 (SEC analysis). The chelating complexes showed enhanced FcγR binding, and binding of the complex to FcγRII on the surface of Daudi cells was observed. Internalization of the chelating complex in PMA-stimulated U937 cells was observed, indicating phagocytosis of the complex. Dr. Kasturirangan also indicated that the Bis3 antibody blocked IL6-induced proliferation of DU145 cells to a greater extent compared to CAN119 or CNDY120. Fast alpha phase clearance of injected complex was observed in mice, while the parental mAbs showed a buffering effect. In concluding, Dr. Kasturirangan noted that future work and concerns include binding of chelating complex to mouse FcγRs, inhibition of human IL6-induced haptoglobin response, effect of kidney function/vasculitis/type III hypersensitivity, and determination of complex size.

Brooke Rock (Amgen) discussed the chemistry of ADCs. She explained that combining chemical potency with antibody selectivity promises superior performance over current cancer therapy. Three general mechanisms are used for conjugation: (1) lysine linkage (~50 possible sites); (2) cysteine linkage (eight possible sites); and (3) site-specific engineering (only defined sites possible). She noted that the properties of the intact ADC, catabolites and the cytotoxin are distinct, and that it is imperative to characterize ADC functional and structural activity, as well as ADC catabolism and deconjugation of the cytotoxin. Regarding the functional integrity of ADCs, she mentioned that, in theory, lysine labeling has the potential to directly and indirectly block the antigen binding site and FcR binding, but, in reality, this is rarely observed in ADCs with drug:antibody ratio (DAR) <5. Assessment of cell-based activity provides a tool to evaluate mechanism(s) of action and compare the potency of cytotoxin with identified catabolites.

Dr. Rock then discussed the linker chemistry, noting that the labeling functionalities commonly used, N-hydroxy succinimide or maleimide, introduce either an amide or thioester bond, respectively, in coupling the drug to the antibody. Chemical properties of the linker may affect labeling selectivity, as evidenced by experiments showing more hydrophobic linkers tend to modify fewer lysine residues. For in vitro stability characterization of new amides, she noted that researchers should consider the potential biological sites for catabolism, such as the target cell, non-target cells and subcellular fractions, identify appropriate buffer conditions, and use multiple in vitro matrices to evaluate ADC and catabolite stability. She pointed out that NHS can react with Cys, Tyr and Ser residues and the resultant NHS ester products have variable stability. Dr. Rock indicated that direct analysis of ADC stability in plasma can be achieved using LC/MS, which provides the ability to directly measure the cytotoxin. She cautioned that the capture reagent also needs to be evaluated to ensure that there is no bias in the capture. In concluding, Dr. Rock emphasized that studies to characterize ADC stability in vivo should be designed to define the stability of ADCs in preclinical species and humans, characterize ADC catabolism in preclinical species and extrapolate catabolism in preclinical species to humans.

The common view on therapeutic antibodies is that they have a high specificity, a precise mode of action and thus an improved risk-benefit ratio over low-molecular-weight drugs. However, a number of target-related, but also off-target adverse antibody effects, have been described. In the final presentation of the session, **Stefan Barghorn** (AbbVie) discussed the biochemical identification of an unexpected cross-reactivity of ABT-736, an

anti-amyloid β antibody with potential as a Alzheimer disease immunotherapy.

ABT-736 is a humanized monoclonal IgG1 antibody that detects its target Aβ-oligomers in Alzheimer disease brain, yielding a broad preclinical efficacy profile. The high selectivity of ABT-736 versus other Aβ species results in a beneficial PK and safety profile. In preclinical toxicological studies, no potential signs for adverse effects were observed. Unexpectedly, in a cynomolgus monkey 13-week GLP toxicological study at low doses (≤ 60mg/kg/week), thrombocytopenia, vasculature changes including thrombosis, neuron loss and brain microhemorrhages occurred. At high doses (120 and 200 mg/kg/week) acute infusion reactions (5-10 min post-dose) with lethal consequence occurred. Extension of infusion time to 1h did not ameliorate adverse effects in a follow-up study. The conclusion derived from the fast onset, which occurred already after the first dose, was that the toxicity was not driven by a classical cytokine storm, but rather triggered by binding of ABT-736 to an already present plasma antigen. This is in line with the finding that ABT-736 triggers some cytokines in human and cynomolgus monkey whole blood, but not washed human PBCs. Abrogating FcR binding by an Fc mutation also prevented cytokine induction. Due to the above findings, ABT-736 development was stopped.

To identify the cross-reactivity partner, an ABT-736 immunoprecipitation of cynomolgus monkey serum was performed. The immunoprecipitate was analyzed by silver-stained SDS-PAGE and revealed a specific cross-reactivity partner of ~8 kDa. The molecular weight was further specified with 8,144 Da by MS. This mass could be assigned by database searches to the cytokine platelet factor-4 (PF4), which was confirmed by positive anti-PF4 Western blot staining and ESI-MS-MS peptide sequencing of the ABT-736 immunoprecipitate. Physiologically, PF4 acts as a strong chemo-attractant for neutrophils and fibroblasts and also neutralizes the anticoagulant effect of heparin. Pathophysiologically, PF4 acts as antigen in heparin-induced thrombocytopenia (HIT), an autoimmune reaction that occurs upon heparin administration, resulting in thrombocytopenia and a worsening of the thrombosis. The hypothesis was that ABT-736 binds to PF4, which resembles the pathological function of the anti-heparin/PF4 complex autoantibody, and thus leads to HIT-like symptoms. Dr. Barghorn noted that an important point for toxicological studies is that ABT-736 cross-reacts with human and cynomolgus monkey PF4, but not with mouse, rat or dog PF4.

Dr. Barghorn stated that a PF4 specific ELISA was developed, and a number of anti-Aβ-oligomer antibodies were found to show PF4 cross-reactivity. However, humanized back-up antibodies A1 and A2 without PF4 cross-reactivity and cytokine induction were successfully identified. Importantly, no adverse effects of A1 were observed in a cynomolgus toxicological study. Structural analysis revealed that the PF4 cross-reactive ABT-736 paratope shows marked differences to the A1 paratope with respect to their binding mode of the target Aβ-oligomer epitope. Dr. Barghorn emphasized that, overall, this study demonstrates the importance of testing safety of therapeutic antibodies in a species closely related to human, and in a range of relevant human cell types and body fluids.

Wednesday December 11, 2013
Track 1: Knowledge-based Design to Guide Development of Cdrs and Epitopes to Engineer or Elicit The Desired Antibody

Gustavo Helguera

The morning session was chaired by **Gregory P. Adams** (Fox Chase Cancer Center), who made opening remarks, then transitioned to being the first speaker in the session. Dr. Adams explained the difficulty in obtaining antibody variable regions with high affinity against some conserved targets. For example, some functional epitopes that initiate signaling events are often the most attractive targets for therapeutic antibodies. However these are often highly conserved between species, making them particularly difficult targets for antibody generation via in vivo immunization or combinatorial phage display approaches. Müllerian inhibiting substance type II receptor (MISIIR) is a transmembrane receptor expressed in more than 60% of gynecologic malignancies, and it is a relevant functional target for the treatment of cancer. The ligand is Müllerian inhibiting substance (MIS) and ovarian cancer cell lines undergo apoptosis following treatment with MIS in vitro and in vivo. Moreover, primary ovarian cancer cells respond to MIS treatment, suggesting that inducing signaling through MISIIR represents a promising approach to treating ovarian cancer. However, MISIIR is highly conserved between human, mouse, rat and rabbit. After panning three naïve human phage display libraries and testing hybridomas from immunized mice, over 30 phage-derived scFv and all hybridoma clones failed to induce signaling or kill MIS-responsive cells, suggesting that the high degree of conservation could be limiting the ability to identify functional mAbs.[87] ADCs composed of MMAE conjugated to the mAbs, however, were capable of specifically killing MISIIR+AN3CA endometrial cancer cells, with an IC50 of ~250 nM.

Next, Dr. Adams explained that agonistic antibodies could induce MISIIR signaling if the antibody CDRs mimic the MIS structure, and proposed to use structure prediction and homology modeling to design a MIS mimic. Modeling the MIS/MISIIR interaction, it was possible to generate a hybrid MIS-mAb with MISIIR. The hybrid mAbs were modeled based upon variable domain frameworks selected to accommodate and properly orient MIS loops predicted to interact with the MISIIR ligand-binding site. scFvs were constructed based upon these models, and, using a MISIIR signaling reporter gene assay, two anti-MISIIR scFvs candidates inhibited MIS signaling, while another seemed to induce signaling. Further refinements in the structure promise to induce apoptosis in cancer cells.

Dr. Adams then described current work focused on turning the antagonist antibody that blocks signaling into an agonist by simultaneously engaging the type I receptor MISIR. The approach consists of creating a multifunctional MISIIR/MISIR antibody that would impart anti-ALK2/3 (MISIR) specificity onto the light chain. This involves redesign of the irrelevant light

chain using computer algorithms to identify docking combinations of the light chain CDRs that bind ALK2 or ALK3. Dr. Adams concluded that protein design represents a promising approach for developing antibodies against difficult to target functional antigens.

Roland L. Dunbrack Jr. (Fox Chase Cancer Center) presented new methods for computational design of antibodies. Dr. Dunbrack first explained that several different antibody design scenarios are possible, depending on the available data. A database of clustered antibody CDRs from known structures was generated providing germline assignment for PDB antibody structures. Many antibodies in the PDB are heavily engineered, with protein sequences poorly annotated, and sometimes with the wrong species. In this process, species assignments were corrected by the PDB after notification by Dr. Dunbrack. In the new clustering of antibody CDR conformations, all VH and VL domain structures in PDB were identified, including scFvs, anti-idiotypes, and all asymmetric unit copies; CDR definitions were chosen and CDRs identified in all VH/VL chains in PDB. The PDBs were filtered for structure quality and redundant sequences, clustered with a dihedral angle metric and an affinity propagation algorithm was applied.

Next, Dr. Dunbrack discussed new antibody-specific methods for computational protein design developed by utilizing their recent clustering of antibody CDRs into conformational clusters, which can be predicted from sequence.[88] He showed basic principles for antibody design using the structure and sequence information from CDR "canonical" clusters from the PDB database with a two-stage computational design: Stage 1 is called GraftDesign and allows CDR sampling, grafting and docking based on input "rules" for CDR lengths and clusters. Stage 2 is called SequenceDesign and allows antibody-specific sequence design of top-scoring hits from GraftDesign using observed sequence variation in antibody structural clusters. This intensive structural bioinformatics analysis of antibodies enables computational design to improve the affinity of a given existing Ab/Ag complex, switching affinity to a homologous antigen, and adding new CDR contacts in one mAb by grafting longer CDRs. Dr. Dunbrack concluded that this database of antibody CDRs from known structures could serve as a source of design templates, guiding design in a highly reliable and predictable manner.

After the morning break, **Brian Weitzner** (Johns Hopkins University) discussed the latest developments in Rosetta-based approaches for structure prediction, docking and design of antibodies. Computational structure prediction provides a fast and inexpensive route to obtain structures that are unavailable or difficult to generate via crystallography. However, downstream protein engineering applications require high-quality homology models to properly predict the formation of antibody-antigen complex (docking) or to improve the mAb affinity (design). RosettaAntibody is a protocol for homology modeling of antibody variable regions. Starting from an antibody sequence, the RosettaAntibody protocol follows a series of steps to achieve the homology model: a) selects V_L and V_H frameworks; b) assemble β-barrel; c) graft canonical loops (CDRs L1-L3, H1 and H2); d) H3 loop modeling (low resolution); e) refine-barrel and optimize CDRs; f) H3 loop relaxation (high resolution); g) store decoy; h) iteration X2000 between d and g; and i) sort decoy by full atom score to finally get the homology model.[89] This protocol was used in the Antibody Modeling Assessment II, that was presented during the pre-meeting 3D Structure Antibody Modeling session. In this assessment, the RosettaAntibody performed well, with 42 of 55 non-H3 CDRs within 1.0 Å RSMD, and with sub-Ångström CDR H3 predictions on a homology framework. But still there is limited predictive power for underrepresented species and for λ light chains due to the limited availability of these templates in PDB. In addition, the presence of non-native contacts can bias V_L–V_H orientation. Moreover, it was not possible to make an accurate prediction H3 CDR, but progress is being made.

Next, Dr. Weitzner discussed the RosettaDock antibody-antigen docking protocol, in which the model is adjusted from a random start position with a low-resolution Monte Carlo search, followed by high-resolution refinement, and clustering to achieve a prediction. The prediction quality of this docking homology model was rather poor, but a significant improvement was observed with the ensemble docking algorithm, a multi-scale Monte Carlo-based structural refinement algorithm that samples the local conformation space for alternate low-energy structures using small backbone torsion angle perturbations, side-chain packing and energy gradient-based minimization in torsion space. Using a different approach named SnugDock, it was possible to predict high-resolution antibody-antigen complex structures by simultaneously structurally optimizing the antibody-antigen rigid-body positions, the relative orientation of the antibody light and heavy chains, and the conformations of the six complementarity determining region loops.[90] However, a major improvement was observed in the prediction quality after the combination of SnugDock and EnsembleDock, suggesting that these new methods are a significant step forward in the state of the art of protein-protein docking towards general making homology models useful for further high-resolution predictions.

Dr. Weitzner then showed a protocol using the RosettaAntibody package that was used to create a homology model to "supercharge" antibodies while considering energetic implications of each mutation.[91] This supercharged antibody displayed thermal resistance and unanticipated improved binding affinity, with implications for the development of more stable reagents. Dr. Weitzner concluded by discussing recent progress in the development of a new method that uses structure-based definitions to model CDR H3 loops.

Stephen Demarest (Lilly Biotechnology Center) presented structure-based design of novel bispecific molecules with varying valency and geometry. Dr. Demarest described the combination of computational and rational design approaches paired with directed engineering to support the development of novel bispecific antibody platforms with high stability, multiple geometries and multiple valencies. Applications enabled by these bispecific molecules were also discussed.

David A. Pearlman (Schrödinger) described recent validation studies using structure-based approaches to predict antibody structure and to identify residues that represent mutational hot spots. The Schrödinger approach has had particular success in

predicting the structure of the important H3 CDR loop from some sequences, when an acceptable model for the remainder of the CDR is available.

Track 2: Optimizing Antibody Formats for Immunotherapy

Juha Laurén

The morning session on optimizing antibody formats for immunotherapy was chaired by Dr. **Paul W.H.I. Parren** (Genmab). The speakers discussed several approaches to engineer designer antibody scaffolds for optimized safety and efficacy, as well as emerging insights into antibody mechanism of action.

Randall Brezski (Janssen R&D, a Johnson & Johnson company) discussed the inherent protease sensitivity of IgG1 antibodies and a protease-resistant IgG platform technology. He noted that many microbial and tumor-associated proteases are capable of cleaving IgG1 within the lower hinge region, and that this phenomenon could serve as an evasion tactic against host immunity. In this regard, formation of single-cleaved IgG appears more prevalent than formation of double-cleaved IgG.

Dr. Brezski described an anti-hinge antibody that specifically detects cleaved IgG molecules. Using this antibody as a detection reagent, he showed that single-cleaved IgG1 accumulates on the surface of mAb-opsonized tumor cells in the presence of proteases, and are detected in tumor-stroma – interfaces.[92] The observed localization pattern raises the possibility that the partial cleavage could influence effector functions.[93] Consistent with this, single cleavage of trastuzumab resulted in reduced effector function and in vivo efficacy.[94]

In the second part of this talk, Dr. Brezski outlined chimeric IgG constructs combining properties of both IgG2 (protease resistance) and IgG1 (effector functions). These constructs possess an IgG2-derived hinge segment, but also several point mutations in the CH2 domain to restore a range of effector functions.[95] These alterations did not affect plasma half-life in normal BALB/c mice compared to IgG1. Furthermore, the protease-resistant antibodies targeting CD20 were effective in depleting B cells in *Cynomolgus* monkeys in vivo, suggesting that engineered protease resistance does not impair other functional properties of the antibodies. These antibody variants could prove to be useful in a range of proteolytic microenvironments, such as cancer or infectious and inflammatory conditions.

Peter Sondermann (SuppreMol GmbH) discussed ongoing efforts to develop antibodies targeting low-affinity Fc gamma receptors (FcγRs) for the treatment of autoimmune diseases. The focus of Dr. Sondermann's talk was on SM201, a humanized antibody that binds to and potentiates the endogenous inhibitory function of FcγRIIB, and thereby down-modulates antibody-mediated immune responses.

Dr. Sondermann noted that FcγRs are expressed on virtually all cells of the immune system except T cells. They recognize the Fc part of IgG antibodies, thereby triggering effector functions that depend on the subtype of the bound, and thereby recruited FcγR, as well as on the expressing cell type. Furthermore, maintaining the proper balance of activating and inhibitory FcγR signaling appears important for health, and this homeostasis is perturbed in at least certain immunological conditions.[96] Importantly, FcγRIIB is the only inhibitory Fcγ receptor.

SuppreMol generated SM201, an IgG antibody bearing a N297A mutation by which binding of the Fc to complement or FcγRs is eliminated. SM201 specifically recognizes amino acids 27–30 of the inhibitory FcγRIIB and does not cross-react with the closely related but activating FcγRIIA. At the same time, as noted by Dr. Sondermann, SM201 does not interfere with IgG-Fc binding to FcγRIIB, making SM201 the only non-blocking antibody solely specific for FcγRIIB. According to the intended mode of action, both properties are required for SM201 to amplify the inhibitory effect mediated by IgG-containing immune complexes.

In vitro results presented by Dr. Sondermann indicate that SM201 appears to inhibit B cell activation by acting synergistically with immune complexes to promote phosphorylation of intracellular immunoreceptor tyrosine-based inhibition motif (ITIM) in FcγRIIB. In a vaccination model in which white blood cells of healthy donors were grafted to SCID mice, SM201 was able to control the anti-tetanus toxoid (TTd) titer after challenge with the antigen, demonstrating the in vivo efficacy of SM201. Dr. Sondermann also presented data on SM211, a variant of SM201 that does not bear the N297A mutation. This IgG1 antibody can homo-polymerize FcγRIIB by binding back with its Fc to already Fab-bound FcγRIIB. According to the presented data, SM211 works immune complex-independently, and may therefore recruit the inhibitory potential of FcγRIIB disease-independently, suggesting a different therapeutic profile for SM211.

Finally, Dr. Sondermann noted that both SM201 and SM211 do not deplete B cells in mice grafted with human white blood cells nor in mice transgenic for human FcγRIIB, suggesting promising safety profiles for these preclinical development candidates.

George Georgiou (The University of Texas at Austin) focused in the first part of his talk on the construct IgGA.3, which combines properties of IgG and IgA. Prof. Georgiou noted that IgA is able to engage with FcRαI, which is expressed, e.g., on neutrophils and macrophages. Furthermore, IgA anti-EGFR antibodies have been shown to mediate tumor killing in vivo.[97] However, from a manufacturing and stability perspective, IgA possesses suboptimal properties, and IgA does not engage FcγRs, thereby limiting its therapeutic potential. The IgGA.3 construct possesses an IgG backbone with IgA CH3 domain and portions of IgA CH2 domain, and retains affinity, albeit somewhat reduced, to FcRαI and FcγRs.

A proof-of-concept study demonstrated that in a HER2 high-expressing SkBr3 breast cancer in vitro model system that uses human neutrophils as effector cells, IgGA.3-trastuzumab mediated more effective ADCC than trastuzumab alone. Similarly, Prof. Georgiou demonstrated an analogous proof-of-concept for enhanced ADCP (antibody-dependent cellular phagocytosis) by using MDA-MB-453 cells with low to moderate HER2 expression, and human macrophages as effector cells. Finally, IgGA.3-rituximab appeared to perform at least as well as rituximab alone in mediating CDC in a Raji CD20+ cell assay.

Prof. Georgiou then discussed a set of aglycosylated Fc mutants that display very high affinity and selectivity for various Fc receptors, and are thus capable of imparting high ADCC activity. He noted that the absence of the N297 glycan normally results in high flexibility in the CH2 and CH3 portions of IgG. Prof. Georgiou's group applied bacterial display to identify a palette of engineered aglycosylated Fc domains displaying high selectivity toward specific Fcγ receptors or C1q, potentially by stabilizing a conformer that binds only to a particular Fcγ receptors or C1q with high selectivity.[98] Finally, Prof. Georgiou noted that he is looking for a partner to further the development of the described antibody platforms.

Ronald P. Taylor (University of Virginia School of Medicine) discussed a series of in vitro experiments and clinical studies indicating that saturation or exhaustion of cytotoxic effector mechanisms mediated by anti-CD20 mAbs rituximab and ofatumumab can compromise their therapeutic efficacy. Prof. Taylor noted that numerous reports have now demonstrated that rituximab and ofatumumab require host effector mechanisms for cytotoxicity, and do not trigger direct apoptosis of CD20+ B cells to any measurably important degree in chronic lymphocytic leukemia (CLL) therapy.

Early studies by Prof. Taylor's lab demonstrated that typically over 90% of CD20 expression on B cells of CLL patients is lost immediately after the usual 375 mg/m^2 rituximab infusion, thus rendering the CLL cells refractory to subsequent rituximab doses.[99] Prof. Taylor noted that his studies reveal that mechanistically CD20 is lost ("shaved") via uptake by effector cells expressing Fcγ receptors, in a specialized example of trogocytosis.[100]

Prof. Taylor then discussed a pilot clinical study in which CLL patients were treated with either intermediate or very low doses of rituximab (~35 mg three times per week; ~420 mg total in four weeks). Even at these very low doses, rituximab was able to clear the CD20+ CLL cells. One of the best outcomes was observed with a patient receiving the very low doses rituximab, suggesting that lower, more frequent mAb doses may provide a superior treatment paradigm.[101]

Next, Prof. Taylor reviewed his recent study suggesting that complement exhaustion may also be in part rate-limiting for the efficacy of ofatumumab. In a clinical study in CLL, an initial 300 mg dose of ofatumumab effectively depleted complement, suggesting that the efficacy of subsequent doses could be limited by complement exhaustion, as well as by substantial loss of CD20, which was also demonstrable in this study.[102]

In concluding, Prof. Taylor noted that, taken together the results suggest that the substantial and protracted loss of CD20 mediated by infusions of large doses of mAb, coupled with the loss of potency of cytotoxic effector systems, may allow malignant cells to continue to proliferate for extended time periods, even after infusion of gram quantities of mAb. The findings caution against the maximum tolerated dose regimens often sought in clinical development. Finally, Prof. Taylor suggested that new clinical trials should be undertaken to rigorously test whether lower, more frequent dosing (for example 40–50 mg, subcutaneously 3x per week) with rituximab or ofatumumab could increase clinical efficacy by limiting CD20 loss and also preserving or allowing for more rapid restoration of complement and other effector functions.

Janine Schuurman (Genmab B.V.) started by presenting her team's recent results suggesting a model for explaining how antibodies engage complement C1q. By using cryo-electron tomography she showed that IgG1 is capable of forming hexamers on cell surfaces upon cellular target binding. The IgG hexamer then provides a docking station for hexameric C1q, thus forming what she aptly described as an Eiffel Tower-like structure. Dr. Schuurman then demonstrated that IgG mutants that cannot form hexamers are impaired in interacting with C1q on cells and compromised in triggering CDC. In contrast, a mutant with enhanced ability to form hexamers potentiated IgG1 mediated complement activation and could also convert IgG2, IgG3, and IgG4 into potent activators of complement.

Dr. Schuurman then discussed exploiting the above-mentioned IgG mutant with an enhanced ability to hexamerize on the cell surface to generate antibodies with potentially superior therapeutic efficacy. She explained that most IgG therapeutic antibodies are rather poor complement activators; epitope, target density and fluidity of the target on the plasma membrane may influence the ability of target-bound antibodies to form hexamers, and thereby the engagement of complement. Genmab's HexaBody™ mutant with an improved capability of forming hexamers on cell surface appears to help overcome these issues. She demonstrated that Hexabody™ variants of antibodies were more potent in killing cell lines and primary CLL cells, and more effective in preventing tumor growth in Raji-luc lymphoma model. The ability to enhance the potential of antibodies to activate complement could allow biosuperior product development, as well as rescue of previously failed antibody drug candidate projects.

Tariq Ghayur (AbbVie) presented a case study on the discovery and early development of ABT-981, a dual-specific engineered antibody construct capable of neutralizing both IL-1α and IL-1β. Dr. Ghayur noted that IL-1 signaling plays an important role in inflammation, for example in arthritic joints. IL-1 family consists of two agonistic ligands, IL-1α and IL-1β, as well as an endogenous antagonist, IL-1Ra, which binds to the same receptor as the other ligands, but does not activate it. Dr. Ghayur reviewed data demonstrating that in collagen-induced arthritis (CIA) and destabilization of medial meniscus (DMM) osteoarthritis mouse models, the blockade of both IL-1α and IL-1β offered a superior therapeutic effect compared to blockade of either ligand alone. With regard to the currently available therapeutic options targeting the IL-1 family, canakinumab (ILARIS®) blocks only IL-1β. The need to neutralize specifically both agonistic ligands led Dr. Ghayur's team to develop the dual variable domain immunoglobulin (DVD-Ig™) concept.

Dr. Ghayur provided an overview of the DVD-Ig concept: In a DVD-Ig construct each Fab arm is designed to bind two different targets. This is achieved by adding an extra pair of variable domains to both heavy and light chains of a mAb. In the case of IL-1 α/β DVD-Ig™, the outer and inner V domains of both arms bind specifically, and with sub-nanomolar K_D, to IL-α and IL-β, respectively. The inner and outer variable domains are connected by a linker peptide. The final construct has a MW of ~200

kDa. Finally, in the above-mentioned CIA and DMM models, IL-1 α/β DVD-Ig had an efficacy comparable to that of a combination of IL-1α and IL-1β targeting mAbs.[103] Furthermore, Dr. Ghayur reviewed a set of experiments demonstrating that the linker length, as well as the relative position of the variable domains presented in tandem, may affect the potency and manufacturability of DVD-Ig constructs.

In concluding, Dr. Ghayur noted that in PK analyses IL-1 α/β DVD-Ig (ABT-981) behaved as a typical antibody; it has completed Phase 1 clinical study; and is now anticipated to move to Phase 2 for arthritis indication. It will be of interest to see how the DVD-Ig platform compares to bispecific antibody and oligoclonal antibody platforms.

The views expressed herein are those of the author and do not necessarily reflect the views of Regeneron Pharmaceuticals, Inc.

Track 1: Antibodies in a Complex Environment

Mathieu Bléry

The session on antibodies in a complex environment was co-chaired by **Sally Ward** (University of Texas Southwestern Medical Center) and **Sergio Quezada** (University College London Cancer Institute). Professor Ward opened the session with a tribute to Philip Thorpe who, during his career, developed immunotoxins, worked on VEGF and developed bavituximab, an antibody recognizing exposed phosphatidylserine through a β2-glycoprotein 1 bridge, to target tumor vasculature. Bavituximab is now entering Phase 3 clinical trials in non-small cell lung carcinoma.

Professor Ward presented an overview of FcRn biology, showing impressive microscopy images that allow dissection of recycling pathways. The data demonstrated that transport carriers can take multiple different itineraries on the endocytic recycling pathway.[104] She then focused on tools to target FcRn-IgG interactions, and in particular on "Abdegs," which are antibodies that enhance IgG degradation). Abdegs contain Fc regions that are engineered to bind to FcRn with high affinity at both acidic and near neutral pH. Abdegs inhibit FcRn function by accumulating to high levels in cells through receptor (FcRn)-mediated uptake, driving (auto) antibodies into the lysosomal degradation pathway rather than recycling. In vivo efficacy of Abdegs was demonstrated in arthritis and EAE models in mice, both antibody-mediated inflammation models.[105] Abdegs are being developed by arGEN-X for their therapeutic potential in autoimmune diseases.

Dr. Quezada presented the dissection of the mechanism of action of anti-CTLA-4 antibodies to understand the basis of Treg disappearance observed both in humans and mice. His team used the B16 melanoma and CT-26 colon carcinoma experimental systems in mice to investigate the importance of the interaction of anti-CTLA-4 antibodies with Fc receptors. Through the use of knock-out mice for different Fc receptors, the role of FcγRIV, primarily expressed on tumor infiltrating macrophages, was demonstrated as one of the mechanisms of Treg clearance by anti-CTLA4 antibodies. Dr. Quezada then discussed several points underlying Treg clearance in the tumor microenvironment. First, Treg clearance by anti-CTLA-4 occurs only in the tumor and not in the draining lymph nodes, correlating with the fact that CD11b+ macrophages expressing FcgRIV are in much higher numbers in the tumor compared with the lymph nodes. Second, Tregs are preferentially killed in the tumor because they express more CTLA-4 at their surface compared with other CTLA-4-expressing tumor infiltrating lymphocytes. The point was raised during the discussion that Fc engineering of anti-CTLA4 therapeutic antibodies may result in the killing of effector T cells expressing lower levels of CTLA-4 compared with Tregs. Elimination of Tregs in the tumor thus seems to be related to the effector/target ratio and to the level of CTLA-4 expression. Indeed, when these criteria are not met, as is the case outside the tumor, anti-CTLA-4 seems to promote Treg expansion.[106] However, Dr. Quezada pointed out that other modes of action are also required for the therapeutic efficacy of anti-CTLA-4, such as effector T cell expansion by the anti-CTLA-4 antibodies. Whilst FcgRIV is not expressed in humans, the human orthologues are FcγRIIA and FcγRIIIA, and it remains to be determined whether the presence of these receptors can influence the activity of anti-CTLA4 mAbs in the human setting. Dr. Quezada's group is currently performing immunohistochemistry studies on ipilimumab-treated patients to investigate Fc receptor expression, Treg and macrophage infiltration in order to determine whether similar modes of action occur in humans. In summary, Dr. Quezada proposed that the tumor microenvironment and Fc receptors are relevant to the efficacy of immune-modulatory therapies.

Holbrook Kohrt (Stanford University) presented strategies to enhance ADCC mediated by therapeutic antibodies. Prof. Kohrt's focus is on NK cells and he presented elegant translational research work that led from an initial in vitro observation to Phase 1 clinical studies. The starting observation was that primary human NK cells in the presence of a CD20-positive cell line and rituximab for 24 h upregulated CD137 (4-1BB) at their cell surface, and this upregulation was dependent on effective ADCC. The same observation was made with trastuzumab and cetuximab in the presence of HER-positive and EGFR-positive tumors, respectively. Using an agonistic anti-CD137 antibody, Prof. Kohrt demonstrated that triggering CD137 on NK cells enhanced the anti-tumor activity of rituximab, trastuzumab and cetuximab in vitro. Moving to various in vivo efficacy models in mice, Dr. Kohrt group showed that the combination of an anti-CD137 antibody with either rituximab, cetuximab or trastuzumab greatly enhanced anti-cancer activity of these therapeutic antibodies.[107] The next step was to study CD137 expression on NK cells in patients with head and neck cancer, NHL, or breast cancer treated with cetuximab, rituximab or trastuzumab. Overall, CD137 is heterogeneously upregulated on circulating NK cells in these patients. This heterogeneity is accounted for by tumor histology, tumor burden, extent of prior antibody treatment and CD16 polymorphism. In addition, only circulating NK cells were studied and the extent of CD137 upregulation on tumor infiltrating NK cells was not investigated in these studies. Time post-treatment was also very important, as observed in preclinical experiments, as CD137 is transiently expressed on circulating NK cells. Prof. Kohrt also presented various additional

means to enhance ADCC by blocking inhibitory signals, such as CD47 on macrophages, IL-15 on monocytes or DCs, KIR or PD-1 on NK cells. He concluded that the future is bright for combination therapies that enhance immune responses.

Jon Weidanz (Texas Tech University Health Sciences Center) discussed T cell receptor mimicking (TCRm) antibodies. Over 50 such TCRm were made for use in research, diagnostics and therapeutics. These antibodies mimic TCR binding to a specific MHC/peptide complex. Two examples of TCRm generation were presented by Prof. Weidanz. The first example discussed was the TCRm RL1B, which was generated by immunizing mice with the HLA-A2 molecule loaded with a Her2 peptide. RL1B specifically stained human breast, endometrial and metastatic ovarian Her2+ cancers by immunohistochemistry. RL1B also has direct effects on the viability of Her2+ cancer cell lines and reduces growth of low Her2-expressing tumors in mouse models. The second example came from a so-called "Bottom-up strategy" in which cancer cells are transfected with secreted HLA-A2, followed by identification of the peptides associated with HLA-A2. This approach led to the identification of peptides derived from macrophage migration inhibitory factor-1 alpha (MIF-1a), against which TCRm RL21A was generated. Immunohistochemistry studies demonstrated that RL21A specifically stained breast and metastatic ovarian cancer tissue, but not normal human tissue. RL21A demonstrated therapeutic efficacy in a model of syngeneic tumors in HLA-A2 transgenic mice. Prof. Weidanz proposed that RL21A was inducing apoptosis of cancer cells upon binding to HLA-A2. RL21A is now humanized for Phase 1 studies in breast and ovarian cancer. Prof. Weidanz also discussed a TCRm that induces internalization and recognizes a peptide derived from p68, a RNA Helicase/DeadBox protein. This RL6A TCRm was coupled to saporin and can control the growth of BT-20/HLA-A2 human breast carcinomas in HLA-A2 transgenic mice. Notably, the RL6A TCRm (but not RL21A) is capable of crossing the BBB in HLA-A2 transgenic mice. Professor Weidanz concluded that TCRms are effective against tumors expressing low density targets, can trigger internalization, directly kill tumor cells or be conjugated, and may have interesting properties for the delivery of drugs across the BBB.

Thierry Wurch (Laboratoires Servier) presented research that his company is pursuing on targeting immune checkpoints. They are developing an anti-B7H3 therapeutic antibody originating from Macrogenics, Inc. B7H3 is a member of the B7 family identified as a poor prognostic factor in the clinic. The receptor for B7H3 on immune effector cells is currently unidentified. A panel of anti-B7H3 antibodies was generated, and one of them, BRCA84D, was Fc-engineered for improved ADCC and humanized.[108] No toxicity was observed in cynomolgus macaques at doses up to 250 mg/kg with the resulting S80169/MGA271 candidate, although B7H3 is widely expressed on normal tissues. S80169/MGA271 is currently in a Phase 1 clinical study in multiple major cancer types and is well tolerated. Dr. Wurch suggested during the discussion that the therapeutic anti-B7H3 antibody is specific for a tumor epitope. The second part of the presentation focused on the bispecific DART (Dual Affinity ReTargeting) format, also originating from Macrogenics. The anti-CD123/CD3 DART (MGD006/S80880) is scheduled to enter clinical development in early 2014.

Carl June (University of Pennsylvania) ended the session by presenting a cell therapy approach using autologous T cells engineered with chimeric antigen receptors (CARs) directed against CD19 (CART19 cells). Data were presented for adult patients with incurable chronic lymphocytic leukemia (CLL). In the first three treated patients, a single infusion of CART19 cells elicited a durable remission to date (up to 3 years). Each CAR T cell or its progeny killed more than 1000 tumor cells. Pharmacology and PK of the CART19 cells showed an initial expansion in the patient upon tumor encounter. In one patient, 27% of the CD8 T cells were CART19 cells at day 58 and were functional. CART19 cell expansion in vivo is a good prognostic indicator of response, with higher levels of peripheral CART19 cells being detected in patients with complete responses (CRs). Prof. June also suggested that the effector:target ratio may dictate the proliferation rate. The second part of the presentation focused on pediatric acute lymphoblastic leukemia (ALL). A total of 22 children with relapsed refractory ALL were treated; CART19 cell infusion led to 86% CR. Examples of deep remission in 23 days without chemotherapy were observed. CART19 cells divided more rapidly in children than in adults, resulting in up to 75% of T cells being CART19.[109] CART19 cells were detected in cerebrospinal fluid (CSF) indicating a potential trafficking to the CNS, and providing a rationale for the use of CARs in neuro-oncology. Long-term persistence of CART19 cells occurred in blood, bone marrow, and CSF. On-target toxicity was observed, such as B cell aplasia, tumor lysis syndrome, cytokine release syndrome, and macrophage activation syndrome. The later was not observed in mouse preclinical models. This toxicity was severe in some cases, but clinically manageable with intravenous gammaglobulins and anti-IL-6 antibodies. The upcoming challenge is to scale up this therapeutic approach by automating CAR/T cell production so that this technology is made widely available. Prof. June called for large pharmaceutical companies to provide engineering solutions to scale up this promising approach.

Track 2: When Two or More Antibody Binding Sites Are Better Than One: Polyclonal, Oligoclonal and Bispecifics

James E Voss

Andrew Bradbury (Los Alamos National Laboratories) chaired the session on polyclonal, oligoclonal and bispecific antibodies, and gave the first presentation, which focused on recombinant polyclonal antibodies as research reagents. These reagents comprise multiple antibodies recognizing different epitopes that are usable in different assays, like polyclonal antibodies, but they also have the advantage of being renewable. Dr. Bradbury emphasized that properties of the polyclonals can be easily improved through use of phage and yeast display, by, for example, eliminating undesirable specificities, or improving affinities. This format, recombinant renewable polyclonal antibodies generated in vitro, is likely to be extremely useful as research reagents.

James D. Marks (University of California, San Francisco; San Francisco General Hospital) discussed antibody combinations for the treatment of botulism. These antibodies were generated using a combination of phage and yeast display, and affinities significantly improved to the sub-nanomolar range for most antibodies. Furthermore, specificity of some of these antibodies could be significantly broadened by yeast display, so that antibodies originally recognizing only one isotype of botulinum toxin (e.g. A1), could effectively recognize and neutralize two, or more (e.g., A1 and A2). Dr. Marks described the use of defined antibody mixtures as far more effective than monoclonal treatments for botulism, and also significantly more effective than the currently used anti-toxin produced in horses. In general, the combination of three antibodies appeared to provide the greatest synergistic improvement, with the mechanism of action of such antibody combinations being due to increased clearance of the toxin.

David Humphreys (UCB Pharma) discussed use of an oligoclonal mAb mixture to treat symptoms of *Clostridium difficile* infection. This spore-forming organism can be asymptomatic in carriers and activated by certain antibiotics. Symptoms are mainly caused by toxins produced by the organism (TcdA and TcdB) and include mild-to-severe diarrhea, fever, and gut inflammation. Currently, the best 'global cure' rates are achieved with vancomycin (64%) and fidaxomicin (74%). TcdA and TcdB each have enzymatic, cleavage/translocation and cell-binding domains. Toxins A and B differ in their cell-binding domains, but both are composed of 50 aa long repeats (long CROPs) that are separated by 20 aa short intervening repeats (short CROPs). These CROPs form short B-hairpin repeats. Peptides based on the TcdA and TcdB cell-binding domains were synthesized by UCB Pharma and used as immunogens to raise TcdA – and TcdB-specific mAbs. Three hIgG1s were used together as a therapy in the hamster infection model and were compared directly with antibiotic treatment and mAb therapy (CDA1 and MDX-1388) developed by Merck.[110] Doses of 50 mg/kg of the mAb therapy protected ~80% of hamsters from lethal infection, while all vancomycin-treated animals died and Merck's mAbs protected ~20%. Protection was associated with healthier tissue in the small intestine (caecum) and better body-weights compared with Merck's mAb therapy. The UCB mAbs neutralize multiple key TcdA ribotypes 003, 027 and 078 at high toxin concentrations. One of the antibodies in the UCB cocktail (CA997), has the ability to protect against loss of transepithelial electrical resistance in an in vitro surrogate assay for diarrhea; it thus prevents disruption of the tight junction between Caco-2 cells. In vitro toxin binding shows both high affinity and high valency, which improves toxin clearance times. Three Fc binding events per antigen appear to be the critical level for rapid clearance and improved neutralization. MAb:antigen complexes can educate and vaccinate the immune system both locally and systemically in an antigen-specific manner through a mechanism where antigen is transported via FcRn across epithelial cells for improved presentation to APCs.

After a networking break, the session continued with a presentation by **Robert Mabry** (Adimab) on the directed evolution of full-length bispecific antibodies on the surface of yeast. Dr. Mabry began his talk by emphasizing the obstacles for development of bispecific antibodies related to propensity of aggregation and other manufacturing issues. These are problems that require engineering solutions for manufacturing bispecifics as clinical candidates. To address these problems, Dr. Mabry presented several case studies demonstrating the discovery of IgGs and their conversion to several final bispecific formats on the surface of yeast. Large libraries of full-length bispecific antibodies were generated by incorporating diversity in variable regions, as well as structural elements to select for significant affinity and biophysical improvements using flow cytometry. By applying stringent pressures to address developability and specificity, enriched populations were assessed in real-time and selected for improved progeny over assembled bispecifics. Characterization of yeast-produced bispecific progeny revealed improved properties that correlated to assessment on the surface of yeast during pressured selections. More importantly, detailed biophysical characterization of progeny expressed from mammalian hosts also corresponded to activity observed for yeast-produced protein. The engineering of improved constructs in several formats demonstrated a consistent pattern of generation of stable bispecific molecules by appropriately pressuring selections when interrogating diverse libraries. The ability to engineer bispecific antibodies in the final therapeutic format increases the confidence of generating developable molecules by overcoming pervasive issues associated with antibody assembly.

Giovanni Magistrelli (NovImmune SA) then discussed how exploiting light chain diversity can support the development of multiple therapeutic antibody formats. An antibody library comprising varying kinds of VL diversity (variable CDRL3, CDRL1+CDRL2, all CDRs or the entire natural VL repertoire) coupled to a fixed VH with a diversity of 7×10^{10} was used to select binders against oncology targets, including CD47, CD19, HER2, EpCAM and EGFR. As the antibodies all share the same VH gene, expression of bispecific antibodies through expression of various VL genes along with their common VH gene could be easily carried out. It was shown that bispecifics of this nature could be very useful for binding of ubiquitously-expressed targets on subsets of target cells. In the case of a CD19/CD47-targeting bispecific molecule, higher affinity binding to CD19 guides the antibody to CD19+ cells first, then subsequent binding to the CD47 target occurs. This bispecific antibody showed superior ADCC/ADCP and significant ability to inhibit tumor growth in mice compared with a monoclonal targeting CD47 only.

The session concluded with a presentation by **G. Jonah Rainey** (MedImmune) focused on bispecific antibodies as a new weapon in the fight against antibiotic-resistant bacterial pathogens. Dr. Rainey described the use of a novel scFv-based bispecific antibody format as a potential treatment of *Pseudomonas aeruginosa*.

Special Session: The Antibody Society

Janice M Reichert

The Antibody Society's special session was conducted by **Janice M. Reichert** (Reichert Biotechnology Consulting LLC;

Editor-in-Chief, *mAbs*), President of the Society. She first introduced newcomers to the Society and described member benefits. The Society provides an international forum for everyone with an interest in recombinant antibody-based molecules, antibody engineering and related areas, including all targeted protein therapeutics. Society members organize the sessions of the Antibody Engineering and Therapeutics meeting, publish previews and proceedings of the meeting in *mAbs*, organize the October special issue of *Protein Engineering, Design and Selection* (*PEDS*) dedicated to antibody-related articles, and maintain resources on the Society's web page (www.AntibodySociety.org). Member benefits include a 25% discount on standard registration for the Antibody Engineering and Therapeutics meeting, 20% discount to registration for numerous other meetings, and discounted subscriptions to *mAbs* and *PEDS*. Dr. Reichert invited the audience to visit the Society's web site for additional information.

Dr. Reichert then discussed 'Antibodies to watch in 2014', which is the latest of a series of articles designed to provide an overview of antibody-based therapeutics in Phase 3 clinical studies, those in regulatory review and recently approved products. At least one 'Antibodies to watch' article has been published in *mAbs* each year since 2010.[36,111-115] Dr. Reichert noted that two mAbs received marketing approval in the US and EU in 2013, the anti-HER2 ADC ado-trastuzumab emtansine (KADCYLA®) and the glyco-engineered anti-CD20 obinutuzumab (Gazyva™). Possible first marketing approvals in 2014 may go to vedolizumab, siltuximab, and ramucirumab, which are undergoing regulatory review for ulcerative colitis/Crohn disease, Castleman disease, and gastric cancer, respectively. She noted that submission of a marketing application for secukinumab was likely by the end of 2013, and submission of marketing applications for onartuzumab, necitumumab and gevokizumab were possible in 2014 based on plans announced by the sponsoring companies. In addition, she pointed out that mAbs with breakthrough therapy designations, including lambrolizumab, bimagrumab and daratumumab were likely to have faster than average clinical development periods, and so these were also 'antibodies to watch in 2014'.

Dr. Reichert then discussed the biopharmaceutical industry's clinical pipeline of mAbs. She noted that there are currently a substantial number of mAbs for immune-mediated disorders in Phase 3 studies, despite the fact that the traditional focus for mAb development is on cancer. In particular, she mentioned 11 mAbs in Phase 3 that target interleukins and three that target PCSK9 (alirocumab, bococizumab, evolocumab). In the cancer area, she noted two mAbs in Phase 3 that target CD22 (inotuzumab ozogamicin, moxetumomab pasudotox) and two that target PD1 (lambrolizumab, nivolumab). In concluding, Dr. Reichert stated that the biopharmaceutical industry's clinical pipeline of ~360 molecules and preclinical pipeline of over 200 molecules certainly appears sufficient to supply 2–4 first marketing approvals per year. She also noted the trend toward development of enhanced antibodies, such as ADC, glyco- or Fc engineered, bi- or multi-specific, radioimmunotherapy and immunoconjugates, and congratulated attendees on their creativity in generating these innovative molecules.

Thursday December 12, 2013
Track 1: Polyreactive Antibodies and Polyspecificity

James S Huston, Jamie K Scott, James E Crowe, Jr., Eric Krauland, Stephen Albert Johnston, Eshel Ben-Jacob, Fridtjof Lund-Johansen

James S. Huston (Huston BioConsulting, LLC; Chairman of The Antibody Society) was Chair of the session on "Polyreactive Antibodies & Polyspecificity," and presented a talk on "Insights on Ig Domain Binding Sites of Low Intrinsic Affinity." Low affinity polyreactivity is an attribute of antibodies (Abs) at early stages in their B-cell lineage, as well in broadly neutralizing antibodies that develop in some patients after years of exposure to chronic HIV-1 infection. Insights from this area are being exploited in next-generation vaccines that target germline B cell receptors, which has led to strategic advances for vaccine development.[116-118] Their maturation in the immune system is directed in specific ways, governed by antigen interactions with a given lineage of B-cell receptors to progressively improve antigen-binding properties of the displayed antibody binding site. This process has been analyzed in detail by the combination of cellular immunology and x-ray structure determination of progressively matured antibodies for a given lineage.[119,120] These data revealed that the long CDR-H3 in the given B-cell lineage becomes increasingly more rigid as the binding site achieves higher affinities with greater specificity. Recent studies[121,122] were noted that describe results of a statistical analysis of the 200 distinct antigen-antibody crystal structures in the Protein Data Base. Quantitative structural comparisons verified these specific complexes involve Ag-Ab contacts with both V_H and V_L domains.

A useful model for combinatorial Ab libraries is high affinity polyclonal Fab specific for a discrete antigen or hapten. Separation of the component chain populations from the affinity-purified polyclonal Fab (into Fd and L chains) or IgG (into H and L chains) can give valuable insights on the V_H and V_L domain components of all the binding sites that were originally present. In order to rigorously compare Ag association with the isolated domains *versus* intact parent binding sites, their association must be analyzed in terms of the unitary free energy change that occurs upon antigen binding. This important concept of solution thermodynamics was emphasized by Kauzmann[123] in his landmark paper on the importance of hydrophobic effects in protein folding, applied to antibody binding sites by Karush.[124] The unitary free energy eliminates the entropic contribution to the Gibbs free energy change, termed the cratic free energy, and leaves the unitary free energy change which is strictly additive for the separate V_H and V_L domain contributions to antigen binding in the corresponding intact Fv antigen-binding site.

A classic example of this analysis is the investigation of Painter, Sage, and Tanford[125] involving binding studies of the native, polyclonal H and L chain dimers derived from rabbit IgG (or Fab) specific for anti-dinitrophenyl (DNP) hapten. Scatchard plot analysis of binding data yielded the average association

constants ($K_{0,av}$) and negative change in average unitary free energy [$-\Delta G_{u,av}{}^{0}$] upon association with the DNP hapten. The results revealed the striking observation that low affinity Ab or V domain association with Ag can elicit substantial changes in unitary free energy. The isolated H and L chains dimerized at the concentrations used for these experiments, and both H_2 and L_2 dimers bound a single hapten per dimer (n = 1 on their Scatchard plots), presumably because the bound Ag blocks association with the adjacent site. Ag association exhibited a substantial [$-\Delta G_{u,av}{}^{0}$] and the average binding contributions for the separate V_H and V_L associations with DNP adding up to the corresponding average [$-\Delta G_{u,av}{}^{0}$] value for the intact parent Ab combining sites (within modest experimental error). This was observed for the polyclonal H and L chain binding site populations despite measurements by Scatchard plots yielding average association-constant values. In contrast, the negative unitary free energy change [$-\Delta G_{u,av}{}^{0}$] upon binding DNP was 8.3 +/ – 0.3 kcal/mole for V_H sites and 5.4 +/ – 0.6 kcal/mole for V_L sites , indicating the low affinity V_L binding site population complexed DNP with 65% of the [$-\Delta G_{u,av}{}^{0}$] measured for the V_H population binding to DNP.

Clearly, component V domain binding sites that are quite weak according to association (or dissociation) constants can exhibit thermodynamically significant association with antigen as an optimally paired intact site. This is likely a reflection of the integrity of their separate domains enhanced as properly paired V_H-V_L combining sites;[126] this was also studied as free Fd[125-127] interacting with light chains, coupled with the impact of the hydrophobic effect upon Ag binding.[123] Despite their polyspecificity, weak binding sites early in a B-cell lineage appear to provide a structural basis that facilitates the progression of B-cell affinity maturation to form high affinity Ab binding sites of exquisite specificity.

The final section reviewed an investigation by Huston, et al. on engineering low affinity Fc-binding agents that are highly specific as multivalent binding resins for immune complexes (ICs).[128] The normal 58 residue B domain of staphylococcal protein A (FB 58) was truncated in stages from a 3-helix bundle protein to 29 AA (residues 8-36, termed FB 29), forming the minimal pair of helices that contact the Fc region at the junction between their C_H2 and C_H3 domains. Using radioimmunoassays to measure apparent association constants (Ka,app), FB 29 exhibited reduced human Fc binding affinities; for FB 58, Ka,app = 2.5×10^7 M^{-1} , while FB 29 affinity was only [2×10^2 M^{-1} < Ka,app < 1×10^3 M^{-1}]. These FB species were genetically fused with Cys peptides at their N – termini, to yield a Met-Ala-Cys-FB 58 and Met-Pro-Ala-Cys-FB 29 that allowed them to be separately coupled to diaminodipropylamine-Sepharose CL-4B after reaction with m-maleimido-benzoyl sulfosuccinimide (sulfo-MBS). The resin conjugates were used to remove ICs from human serum spiked with a known concentration of covalent IgG tetramers as model ICs, by providing a multivalent scaffold with the FB proteins spatially positioned randomly. FB 58 could stably complex IgG and ICs with even a single binding event, whereas the immobilized FB 29 should require at least a triple binding event to retain ICs on the resin. The human IgG only has 2 binding sites for the FB proteins *versus* 8 sites on the model ICs. Thus low affinity multivalent binding was engineered into the immunoaffinity resin to differentiate between monovalent Fc and multivalent Fc complexes. Experimental data are abbreviated as the resin-bound ratios of [IC (mg Eq/ml)/HuIgG (mg/ml)], measured as follows for [FB 58] vs FB 29: No resin control – both yielded 21.0/4.03; resin control without FB-both 18.3/3.70; 4 mg FB per ml resin, IC/huIgG: [FB 58] 8.0/0.90 vs [FB 29] 7.0/3.55.

More results and detailed FB-Fc molecular models may be found in the original publication.[128] This investigation emphasizes how specificity and high affinity complexation are readily achieved by IgM and other multivalent complexes of low affinity binding sites, including polyclonal polyreactive Abs.

Jamie K. Scott (Simon Fraser University) presented "Antibody polyspecificity and auto-reactivity: their relation to health and disease." Using the example of HIV, she reviewed the dynamics of the antibody response during early-stage and chronic infection, including production of neutralizing antibodies. Serum antibodies from the early stages of infection neutralize HIV but are strain-specific, with broadening emerging over time. Broadly neutralizing serum antibodies arise after 2–3 years of HIV infection, and only in a small subset (~10%) of individuals. Broadening of the anti-HIV antibody response involves recruitment of new B-cell clones and shifting of the epitopes on the HIV envelope protein (Env) that anti-HIV antibodies recognize. There has been a controversy over the degree to which broadly neutralizing antibodies are polyreactive and autoreactive (e.g., ref 129), with some suggesting that tolerance for autoantigens must be broken for neutralizing antibodies to emerge.[130] Given that neutralization breadth evolves over time, and the postulation that neutralization is related to autoreactivity, Dr. Scott's lab set out to characterize the reactivity profiles of sera from HIV-infected individuals over time, and whether there was a correlation between neutralization breadth and auto- or poly-reactivity.

Epitope mapping studies were performed using a panel of 15-AA overlapping peptides covering regions of the Env known to be antigenic. It was found that patients who were "rapid progressors" (i.e., untreated patients who died from AIDS within 5 years of diagnosis) had the broadest reactivity profile against the peptides, whereas "slow progressors" (i.e., untreated patients who had not succumbed to AIDS after >15 years' infection) and especially those who had broadened neutralization profiles had the narrowest reactivities. Moreover the rapid progressors had the most polyreactive sera (as assayed by dried milk and ovalbumin). Thus auto- and poly-reactivity appeared directly related to control of viral load. The serum panel was also assayed on an autoantigen microarray, developed for analysis of sera from the autoimmune disease, systemic lupus erythematosus (SLE) (similar to the mAb study described in ref. 131). Comparison between sera from SLE patients and from HIV patients with different clinical outcomes and neutralization breadth revealed that the rapid progressor sera behaved most like SLE sera in their autoreactivity profiles. Sera from slow progressors and sera with broadened neutralizing activity had the lowest similarity to SLE sera, again indicating that broad neutralization is likely not associated with enhanced poly- or auto-reactivity.

Several studies have used unusual ELISA conditions to study poly- and auto-reactivity of monoclonal antibodies derived from HIV+ individuals. Primary mAbs tested in this ELISA are diluted in saline solution in the absence of detergent or protein blocking agents, whereas the secondary antibodies used in the assay are not. Thus ELISAs were carried out on HIV+, SLE and healthy-control serum samples in which the sera were diluted in (1) saline; or in saline containing blockers, including (2) 5% BSA, (3) 50% fetal bovine serum or (4) 5% dried milk. As expected, HIV+ sera under all conditions reacted against HIV-Env proteins (gp120 and gp41), and SLE sera under all conditions reacted against double-stranded DNA. The reactivity of serum, diluted in saline alone, against antigens unrelated to HIV was highest for SLE and HIV+ rapid-progressor sera, and was lowest for slow progressors and serum with broadened neutralization profiles. Interestingly, the reactivity of saline-diluted sera from healthy-control women was more strongly polyreactive than those from healthy-control men. Thus ELISAs that leave out blocking agents in their antibody diluents revealed "intrinsic polyreactivity" that was not apparent under "normal" conditions. These results may help to clear the controversy and conflicting results surrounding the auto- and poly-reactivity observed for HIV-neutralizing mAbs.

Taken together, the results indicate that the breadth of Env reactivity, as well as polyreactivity and autoreactivity arising during HIV infection are most pronounced in rapid progressors, and is probably related to uncontrolled viremia. In contrast, slow progression and neutralization breadth appear correlated with reduced breadth of Env reactivity and very low auto- and poly-reactivity. Thus, it is unlikely that "breaking of tolerance" is required for neutralizing antibodies to emerge. This is good news for the prospect of an HIV vaccine.

James Crowe, Jr. (Vanderbilt University) delivered a summary of computational analysis of the molecular and genetic basis for polyspecificity in a presentation entitled, "Human germline gene segments encode polyreactive antibodies." Antibodies made by humans following bacterial or viral infection comprise an important component of the acute immune response associated with pathogen clearance, and antibodies are the principal mediators of resistance to reinfection. Immunoglobulins are encoded initially by recombined germline antibody variable gene segments that are generally unmutated prior to antigen exposure, constituting a set of B cell receptors and corresponding antibodies that has been termed the naïve repertoire. The number of potential epitopes in pathogens to which humans might be exposed is enormous, and by comparison the size of the naïve human B cell repertoire is relatively small. Investigators have been interested in how a relatively restricted repertoire has the capacity to recognize such a diverse universe of foreign epitopes. This presentation reviewed evidence to support the hypothesis that antibodies encoded by germline gene segments exhibit structural flexibility, and thereby accommodate varied binding modes and recognition patterns. The phenomenon of a single antibody retaining the capacity to bind to diverse targets is known as polyspecificity. Dr. Crowe presented computational experiments performed with the Rosetta software suite that supported a conformational flexibility hypothesis by showing that the antibody protein variable regions observed in nature that are encoded by germline gene segments are nearly ideal in sequence for encoding polyspecific binding proteins. The group used computational design algorithms in Rosetta to explore antibody sequence repertoires in order to predict immunoglobulin sequences that would exhibit capability for polyspecific binding. The resulting Rosetta designed antibody sequences identified residues critical for achieving polyspecificity. Remarkably, the computationally designed amino acid residues at positions critical for flexibility match those in the germline gene segment sequences to a high degree.

Specifically, the computational experiments examined groups of somatically mutated antibodies from antigen-antibody complexes in the Protein Data Bank that were encoded by a common V_H gene, but bound to different antigens. The information for the specific amino acid residues from somatically mutated positions in matched sets of antibodies was removed, and the antibody heavy chain variable regions were redesigned in Rosetta to predict antibodies that could engage the diverse antigens in the common V_H panels all at once. The Rosetta multi-state design process predicted antibody sequences for the entire heavy chain variable region, including mutated residues in framework, CDR1 and CDR2 regions. The "multi-state design" protocol predicted sequences matched the germline gene sequences to a remarkable degree, revealing by computational design the residues that are predicted to enable polyspecificity. Conversely, when Rosetta designed antibodies to bind a single antigen ("single-state design"), the program revealed a preferred sequence similar to that of the affinity matured antigen-specific antibody. These results reveal how a limited set of antibody germline gene segments encodes immunoglobulins that bind to diverse antigens. Aspects of this work are published.[132]

Eric Krauland (Adimab LLC) provided an update on work at Adimab to assess the developability of therapeutic antibodies at the earliest stages of the discovery process. He opened by suggesting that assessments performed downstream in development, often on a reduced set of leads and at higher cost, introduces added risk in the process. An earlier developability assessment, however, is challenging due to the lack of reagents or tools that accurately predict late-stage properties, while being compatible with limited amounts of antibody, high-throughput, or active selection.

To this end, Dr. Krauland introduced a new flow cytometry-based poly-specificity assay.[133] The assay uses a biotin-labeled cell extract, referred to as poly-specificity reagent (PSR), that when mixed with IgG-presenting cells, can be a measure of IgG poly-specificity. The assay was validated by correlating PSR signals on a panel of greater than 300 antibodies to medium throughput assays such as differential scanning fluorimetry (DSF), Baculovirus particle (BVP) ELISA,[134] and cross-interaction-chromatography (CIC).[135] While antibody poly-specificity level did not correlate with thermal stability by DSF, a quantitative positive correlation could be demonstrated with both the BVP and CIC assays, which have been demonstrated to serve as surrogates for IgG clearance and aggregation, respectively. He then presented preliminary work showing the correlation between PSR values to a few theoretical models of antibody desolvation. Dr. Krauland

sees the future of this work as driving towards a physical basis for poly-specificity, and in turn designing away from it in next generation naïve and optimization synthetic antibody libraries.

He concluded with two examples of how the PSR assay is uniquely compatible with active selection from very large and diverse antibody mixtures. In the first example, a pre-enriched set of target-specific IgGs (diversity > 10^7) underwent an additional round of PSR counter-selection. The antibodies subjected to this selection were shown to have reduced poly-specificity in the PSR, CIC and BVP assays over a population without the counter-selection. Implemented more broadly, this PSR counter-selection method was shown to positively affect multiple therapeutic antibody programs. In the second example, Dr. Krauland demonstrated how the PSR assay can be employed to repair a poor poly-specificity profile. In this case, a starting IgG with high PSR was mutagenized and subjected to rounds of positive target selection followed by negative PSR selection. The resulting optimized IgG had both improved affinity and reduced poly-specificity as measured by the PSR, BVP, and CIC assays. In summary, Dr. Krauland and colleagues believe that implementation of the PSR assay in selections represents a paradigm shift from selecting for target biology then screening for developability, to selecting for both at the earliest stages of discovery, thereby increasing the efficiency of the antibody drug development process.

Stephen Albert Johnston (Center for Innovations in Medicine, Arizona State University) presented a talk on the unique features of the immunosignature technology. The immunosignature diagnostic technology was developed specifically to enable early diagnosis of disease. To meet this goal, a technology would have to meet the following specifications: (1) comprehensive (detect any disease on the same platform); (2) sensitive (to detect early); (3) simple + cheap (so people use it frequently); and specific (so we know what is wrong and if it is serious).

Early diagnosis poses a particular problem in diagnosis since any biomarker is diluted in 5 L of blood. Immunosignatures take advantage of the amplification that naturally occurs when a B cell is activated by an antigen, amplifying this signal up to 10^{11}-fold in a week. The immunosignature diagnostic procedure is quite simple. One drop of blood (0.1 ul) is diluted 5000 fold in buffer and applied to a peptide chip. The chip is washed, leaving only the antibodies. The chip is treated with a labelled secondary antibody, washed, dried and scanned to reveal the signature of bound antibodies.

A unique aspect of this chip is the nature of the peptides on it. The peptides are not chosen from life space, but by evenly sampling random peptide sequence space. The chip they currently make has 350K peptides/0.5 cm^2. The promising features of the immunosignature technology are: (1) the same chip is used to diagnose any disease in any species; (2) it detects all types of antibodies; (3) it can use old sera samples; (4) no sample preparation is required; and (5) it is very sensitive.

In the course of developing the clinical applications, Prof. Johnston's group has noted some interesting features he wanted to discuss with the group of experts present at the session. He reported finding that any monoclonal antibody has a unique signature on the chips. Antibodies developed against linear epitopes, complex epitopes, sugars, haptens or post-translational modifications all bind >100 peptides on the arrays and have unique signatures.

Prof. Johnston presented surprising results from an antibody dilution experiment. A mouse monoclonal antibody was diluted into human sera at various concentrations. Remarkably, a 1000 fold excess of sera over the monoclonal antibody did not significantly dilute the monoclonal signal. He concluded that the high affinity monoclonal antibody competes much better than the low affinity general antibodies for random peptide space.

He noted that, as might be expected for mimotopes, the antibodies have low affinities (~10^{-6}) for the peptide targets. There are two likely ways to explain the retention of the antibodies on the arrays through the washes. Since the peptides are ~2 nm on average apart, the antibody could make cooperative binding connections. The other idea is that the high density of peptides retains the antibodies by avidity. He described an experiment that compared the binding of a complete antibody and its single arm form to the array. The results support that avidity is the dominant force.

Prof. Johnston then described results that indicated that the sensitivity on the arrays is ~10–100x more than ELISAs. He proposed a model to explain this where the "buffering" effect of the off-target peptides increases signal from the relevant antibody. A useful aspect of this effect is that an infection can be detected much earlier than commonly possible.

He also showed data demonstrating an unexpected phenomena. The immunosignature of Type I diabetes in children consisted of features with more antibody binding in the TI sera than the normal controls. But, surprisingly, there were more features where the binding in the normal controls was higher than in the TI patients. He also showed that the signature peptides in both the up and down pools mapped back by sequence to the known autoantigens in TI.

Finally, Dr. Johnston presented a new format for producing the arrays using the computer-industry based photolithography technology for in-situ synthesis of the peptides. This could produce arrays of higher peptide density, more reproducibility, higher volume and lower cost. He thinks these new arrays may open whole new avenues of investigating the antibody population.

Eshel Ben-Jacob (Rice University, Tel-Aviv University) discussed exosome-mediated communication that occurs within and between the immune system and cancer cells in the talk, "Coaching the immune system to outsmart cancer." Despite much-improved understanding of its biology, it is not clear how cancer cells take advantage of and thrive in the body while dodging both innate and adaptive immune responses, responses that evolved to recognize and stop aberrant cells from damaging the host. It is also not clear how cancer manages to exploit multiple stromal cell types (e.g., fibroblasts, epithelial lymphatic, vasculature, specialized mesenchymal cells), turning their normal processes to its benefit, while preventing autoimmunity from eliminating the abnormally behaved enslaved cells. Still more surprising are observations that the immune system can sometimes be tricked into promoting cancer development.[136]

The emerging picture is that cancer, like the immune system, is a networked society of sophisticated cells that master and use all the communication modalities that are also employed by the immune system. Cell-to-cell communication is a fundamental process in multicellular organisms. Many aspects of intercellular communication are mediated through "sending" and "receiving" packets of information via the secretion and subsequent receptor-mediated detection of biomolecular species, including cytokines, chemokines, and metabolites.[137]

An important new area of cell-cell communication involves the exchange of extracellular lipid vesicles called exosomes. These nanovesicles of about 30-200 nm are formed in the multivesicular bodies and released into the extracellular space.[137] Exosomes can carry a broad range of cargo, including proteins, microRNAs, and mRNAs. Membrane markers assign the exosomes to specific targeted cells. Upon entering the target cell, exosomes can modulate cell function and phenotype.[138]

Exosomes are now recognized for their special role in intercellular communication of the immune response, in particular by assisting dendritic cells (DCs) to perform their important control functions by bridging innate and adaptive immune responses[139] For example, DC-derived exosomes can carry antigenic peptides derived by proteolysis of antigens that were endocytosed by the parent DC. These peptides can be presented as MHC-peptide complexes that activate antigen-specific T cells. More recently, exosomes have been shown to play a crucial role in the immune system's battle with cancer. For example, DC exosomes can directly kill tumor cells and activate natural killer cells. However, cancer cells also use exosomes to fight back – cancer exosomes can inhibit differentiation and maturation of bone marrow DCs. In fact, tumors make extensive use of exosomes for survival. They can trick the immune system by pretending to be normal cells under stress. For example, tumor-derived exosomes can promote metastatic niche formation by educating bone marrow-derived DCs toward a pro-metastatic phenotype through upregulation of c-Met.[140] Tumors can also use exosomes to harness the body's repair mechanism: Hypoxic tumor cells secrete exosomes with enhanced angiogenic and metastatic potential, suggesting that tumor cells adapt by secreting exosomes to stimulate angiogenesis or facilitate metastasis to more favorable environments.[141]

The idea of cancer as a networked society of cooperating cells has led to the proposal to develop new therapeutic strategies targeting the communication and control of cancer.[137] Understanding the mechanisms of exosome-mediated transport may provide opportunities to prevent cancer from targeting DCs, and to coach the immune system to outsmart cancer in the cancer-immunity communication battle.[137] Promising directions include (1) the use of engineered exosomes to control and reprogram cancer, and (2) the utilization of exosomes for vaccination against cancer.

Fridtjof Lund-Johansen (Oslo University Hospital) discussed antibody array analysis using labeled antibody microspheres, with resolution of protein size, to define specificity. Antibody array analysis of labelled proteomes has high throughput, and it is simple to perform. Thus far, the technology has mainly been used as a screening tool for complex samples such as serum and total cell lysates.[142] This is a paradox because label-based detection requires mono-specific capture, which is very difficult to obtain in complex samples.[143] To overcome the problem, Dr. Lund-Johansen and colleagues developed a new assay format referred to as microsphere affinity proteomics (MAP).[143-145] MAP arrays consist of microspheres with fluorescent bar codes. By using different concentrations of five dyes, 1728 differently colored microsphere subsets are routinely generated.

MAP arrays have two critical advantages over planar arrays. First, a single person can produce 10,000 copies of a 1728 plex in one week. Second, the arrays can be processed in microwell plates using liquid handling robots. This allows a new assay format where the sample proteins are separated into fractions, and the fractions are measured with copies of the array. Using this approach, different targets of each capture antibody are detected independently like bands on a western blot.

Commercially-available antibodies often contain stabilizers such as albumin or gelatin. This complicates chemical coupling of the reagents to microspheres. The MAP microspheres are therefore first coupled with immunoglobulin-binding proteins such as protein G. The use of Fc-binding proteins also ensures optimal orientation of the capture antibodies. The antibody-coupled beads are incubated with biotinylated sample proteins. Captured proteins are labelled with phycoerythrin-conjugated streptavidin, and the microspheres are analyzed with a flow cytometer that reads the fluorescent bar codes and measures fluorescence from the bound protein.

Dr. Lund-Johansen and colleagues currently obtain four subcellular fractions using a newly developed chemical. The proteins are biotinylated and subjected to size exclusion chromatography to separate monomeric proteins and multi-molecular complexes. A mixture of color-coded microspheres is added to 96 fractions. After overnight incubation, the microspheres are labelled with phycoerythrin-conjugated streptavidin and analyzed with a flow cytometer equipped with a device that harvests samples directly from microwell plates. The data obtained by analysis of 24 size fractions are cumulated to generate size distribution profiles for the antibody targets. These profiles may be complex if the antibody target occurs both as a monomer and in one or more complexes. To identify targets that correspond to specific binding, different antibodies to the same protein are used as internal reference.[143-145] Intended antibody targets are detected as reactivity peaks that are observed with all the antibodies.

The utility of MAP depends on the extent and precision of protein separation and on the coverage of the antibodies in the arrays. During the past five years, 5000 commercially available antibodies have been tested by Dr. Lund-Johansen and colleagues. Only 20-25% are useful, and only 10% are optimal.[144] Their experience is that it is difficult to predict antibody performance from the data specification sheets in the manufacturer's catalogues. This problem is well-known to antibody users.[146] It is therefore common to purchase antibodies that have been used in many publications, and therefore have an "antibody impact factor." Websites such as Abcite.com and Biobrea.com are useful tools to identify antibodies with high impact factors. However, Dr. Lund-Johansen explained that in his experience these

reagents are often not the best because suppliers who produce better reagents have little chance in the competition against well-established reagents. Thus, the current market can be described as "survival of the first," rather than "survival of the fittest".

Dr. Lund-Johansen then emphasized the point that the most important problem with commercially-available antibodies is not that there is a lack of reagents, but that the market fails to select for the best ones. The reagents are largely used one at a time, and the conditions are optimized for individual experiments. Thus, two antibodies with radically different performance may in fact look similar on the product specification sheets. In MAP, thousands of antibodies are used in parallel, and the dynamic range of fluorescence detection in flow cytometry is at least 3 logs. Thus, MAP opens completely new possibilities. It is now realistic to compare thousands of antibodies side by side under exactly the same conditions. However, it is important to note that MAP is a multiplexed form of immuno-precipitation, and there is reason to believe that antibody performance is application-dependent. Currently, it is not known to what extent performance in immune-precipitation predicts performance in other assays such as western blotting, immunohistochemistry and immunofluorescence. Dr. Lund-Johansen and colleagues have therefore started a project to address this question. If they can find ways to compare thousands of reagents side-by-side at low cost, the antibody market may change fundamentally.

Track 2: Great Antibody Discovery Platforms of the Past, Present And Future

Juha Laurén

Ian M. Tomlinson (GlaxoSmithKline) chaired this session focused on antibody discovery platforms of the past, present and future. The title begged the question as to which of the platforms presented were platforms of the past and which held future potential. Dr. Tomlinson offered a reconciling note by suggesting that at the end of the day discovery platform technology may not matter, but one needs to have access to multiple platforms and choose the approach that best suits a particular situation. The session speakers seemed to share his view to a varying degree, but it was also noted that some platforms appear to have more successful historical track records than others.

Jane K. Osbourn (MedImmune) discussed the discovery of adalimumab (Humira®) using the phage display system at Cambridge Antibody Technology in the 1990s. She started out by outlining the critical role that TNF exhibits as a master regulator of a range of cytokines that play roles in rheumatoid arthritis pathophysiology, and highlighted the importance of having good animal models for allowing preclinical development. She noted that the decision to launch a project aiming to discover a fully human TNF antibody was in part driven by a desire to differentiate the product candidate from the emerging competition by minimizing the risk of immunogenicity in long-term use.

In the case of adalimumab, phage display screenings were employed to humanize the murine parental antibody clone (MAK 195) by, in a sequential fashion, swapping murine VL and VH domains to human VL and VH chains in such a way that affinity to TNF was retained to a large extent – a process called guided selection. The fully human lead discovered was further improved by affinity maturation by targeting VH and VL CDR3 loops. Finally, the optimized scFv discovered was converted to IgG1 having a K_D of less than 0.09 nM to TNF.

Dr. Osbourn further remarked that in a way TNF inhibitors redefined rheumatology as a specialty with transformative treatment options, and redefined rheumatologists as pioneers of the biologic revolution. Further, she pointed out that adalimumab foreshadowed the emergence of human antibodies as marketed therapeutics.

Following her presentation, a question came from the audience as to what may have made adalimumab the world's best-selling drug. Dr. Osbourn noted that not only did adalimumab turn out to be very efficacious, but the entire class of TNF inhibitors is broadly utilized; furthermore, self-administration and dosing every two weeks offered improved patient convenience compared with the competition. Curiously, the first biosuperior is now the best-selling drug.

K. Dane Wittrup (Massachusetts Institute of Technology; Adimab, LLC) provided an overview of the yeast cell surface display platform in antibody discovery. Prof. Wittrup discussed the features that set antibody yeast display apart from phage display: First, yeast display allows the use of flow cytometry / FACS, and thus enables quantitative measurement of critical selection parameters (K_D, k_{on}, k_{off}, epitope, T_m), as well as normalization for cell surface expression level. Of note, magnetic bead-based affinity selections are typically used in the first 1 – 2 selection rounds to reduce the complexity of the library to a level manageable with FACS. Second, antibodies discovered using yeast display must have undergone eukaryotic secretory processing; this is expected to select for antibodies with good "developability" characteristics. Finally, yeast display allows creation and screening of libraries at relative ease—as Prof. Wittrup put it: "*A single postdoc made our yeast display antibody library with ~10^9 clones in a week.*"

Prof. Wittrup then described in further detail Adimab's yeast display platform. He emphasized that Adimab has developed a fully integrated and optimized platform for antibody discovery. The screening, expression, hit characterization, and exclusion of poly-specific binders can all be conducted within a month. An average Adimab project generates mAbs binding to seven different epitopes (cross-blocking bins) with a median K_D of 3 nM. He indicated that the yeast strain used by Adimab contains ~20 undisclosed genetic modifications; furthermore, a change of cell culture media allows switching from surface-displayed full-length IgG display format used in selections to secreted IgG format for functional testing. The libraries utilize germline VH sequences and naturally recombined VDJ-segment sequences.

Finally, Prof. Wittrup remarked that certain reports and anecdotal comments suggest that phage display-derived antibodies appear to have historically faced "developability" issues related to physicochemical properties, and perhaps therefore experienced a higher level of attrition than antibodies derived from transgenic mice or mice with humanized immunoglobulin genes selected in vivo. Prof. Wittrup noted that given the properties of yeast display, it is expected to mimic nature's own solution to

developability issues. In this regard, Dr. Osbourn, who gave the earlier talk on phage display, commented in the Q&A part of the talk that in the early days of phage display, efforts were focused on optimizing the efficacy of antibodies, whereas there is currently a much greater appreciation on the importance of considering the developability of the discovered antibodies.

Nils Lonberg (Bristol-Myers Squibb) provided an overview of the development of the concept of using transgenic mice comprising germline configuration human immunoglobulin genes as a platform for discovering therapeutic antibodies. Dr. Lonberg noted that he and his team at GenPharm developed such a mouse comprising four distinct genetic modifications, and that this mouse enabled isolation of antigen-specific human antibodies.[147] In 1997 GenPharm was acquired by Medarex, which was in turn acquired by Bristol-Myers Squibb in 2009. He further noted that many of Bristol-Myers Squibb's immuno-oncology antibody candidates were discovered by using the transgenic mouse platform.

Dr. Lonberg then provided a brief overview on the development of ipilimumab for melanoma as well as an update on Bristol-Myers Squibb's efforts to develop additional cancer immunotherapeutics. Of note, he remarked that BMS has made mAbs targeting most of the immune checkpoint regulators, and is now evaluating them alone and in combinations in a range of preclinical models. In this regard, he emphasized the need to utilize a range of assays, as well as to interpret the results judiciously.

Laurent Jespers (GlaxoSmithKline) opened his presentation by providing a brief overview of human domain antibodies (dAbs), an antibody fragment platform to which GlaxoSmithKline gained access through its acquisition of Domantis, Ltd in 2007. He further explained that at GlaxoSmithKline, biology and patient access primarily drive the selection of an appropriate therapeutic modality. In that regard, dAbs lend themselves to applications where their small size may provide an advantage in accessing and blocking difficult targets/epitopes. Likewise, he noted that domain antibodies held promise for topical delivery. Finally, domain antibodies offer flexible building blocks that can be re-engineered to work as bispecific antibodies.

Dr. Jespers commented that domain antibodies have historically suffered from aggregation issues; he then went on to demonstrate the various quality-by-design selection approaches (high temperature; low pH; presence of trypsin) applied at Domantis and later at GlaxoSmithKline to select domain antibodies with favorable physicochemical properties.

Dr. Jespers then provided a brief overview of the AlbudAb™ platform. AlbudAbs are albumin-binding domain antibodies with an improved plasma half-life. They bind to an epitope on albumin domain 2, which is distinct from FcRn and metabolite binding sites.

The majority of Dr. Jespers' talk covered a case study of applying the AlbudAb platform to discover and develop exendin-4 AlbudAb / PYY (3-36) AbudAb combination. Exendin-4 and PYY are peptide hormones with potential in treating obesity and metabolic diseases. Dr. Jespers shared in vitro results indicating that exendin-4 and PYY could be fused with albumin-binding domain antibody without compromising their potency and selectivity.

Exendin-4 AlbudAb and PYY AbudAb induced weight loss in diet-induced obese (DIO) C57BL/6 mice in two weeks. However, after 3–4 weeks of continuous treatment, the animals' food intake returned to baseline levels, but weight loss appeared to be maintained as compared to vehicle-treated group. Interestingly, the combination of exendin-4 AlbudAb and PYY AbudAb had a synergistic effect in the same set of experiments. Histologically, the combination treatment caused a decrease in cytoplasmic lipid droplets in liver, brown adipose tissue and kidneys. Furthermore, the combination regimen induced significant weight loss and lowering of blood glucose and HbA1c in db/db mice.

Finally, Dr. Jespers reviewed the results of an exendin-4 AlbudAb (GSK2374697) human Phase 1 study. This two month double-blinded, randomized, and placebo-controlled study enrolled 82 subjects to receive escalating doses of exendin-4 AlbudAb or placebo. For exendin-4 AlbudAb, the plasma half-life in humans was ~6.3 days. Of note, the half-life of naked exendin-4 peptide in humans is ~33 minutes. Pharmacodynamic effects on glucose and insulin levels were studied as secondary endpoints. At the highest doses tested, exendin-4 AlbudAb appeared to reduce blood glucose levels, as well as blood insulin levels, in a statistically significant manner. Vomiting and nausea were reported as adverse events on high doses, whereas low doses appeared to reduce appetite.

Bo Yu (Larix Bioscience, LLC) discussed a technology with potential to streamline CHO antibody production cell line development. With Antibody Membrane Switch (AMS) technology, the same cells employ a switchable membrane-anchored antibody expression and secreted expression of the antibody of interest. This is achieved by introducing an alternatively spliced exon flanked by site-specific DNA recombinase recognition sites (e.g., LoxP, FRT, aatB/aatP). To create an antibody production cell line, cells expressing high levels of the antibody of interest on cell surface are initially isolated by FACS. Next, removal of the exon encoding the membrane-anchor from the chromosome is triggered by transient introduction of the recombinase. This results in 100% antibody secretion. According to Dr. Yu, this three-month process can save over 3–4 months of time in antibody production cell development. Yields currently achieved by using AMS varied from 300 mg/L to 750 mg/L in non-fed shaking culture (at seven days), but Dr. Yu noted that he expects to obtain 3–10 times greater yields in bioreactor production format.

In the second part of his talk, Dr. Yu discussed a double-allele knock out (DAKO) method by multiple CRISPR targeting. By transfecting CHO cells with plasmids encoding multiple (optimally 3–4) fut8-targeting CRISPR small RNAs, a double-allele knock out frequency of ~5% was achieved, and a panel of fut8-/– CHO cell lines was generated within five weeks. Antibodies produced from fut8-/ – CHO cells confirmed lack of fucosylation and enhanced ADCC activities.

Geoff Yarranton (KaloBios Pharmaceuticals, Inc.) started his presentation by providing an overview on the KaloBios' Humaneering® platform, which allows "*discovery of antibodies that are more human than many human antibodies*," as Dr. Yarranton put it. In the Humaneering approach, a starting material is provided by any antibody, such as mouse or rabbit

antibody, that binds to the antigen of interest. First, the residues critical for determining specificity are identified. Second, these critical residues, along with the FR4 region, are introduced into human near-germline VH and VL libraries. Third, the libraries are screened using KaloBios' proprietary *E. coli* strain that has high transformation efficiency and allows secretion of functional antibody fragments. Next, antigen-positive clones are identified by using a colony lift binding assay. Finally, an optimal antibody is selected by using a range of physicochemical and functional assays.

In the second part of his talk, Dr. Yarranton provided an overview of KaloBios' clinical stage antibody programs, which focus on serious respiratory diseases and cancer. KB001-A is a PEGylated Fab' fragment targeting *Pseudomonas aeruginosa* PcrV Protein. KaloBios is developing KB001-A for chronic *Pseudomonas* infection in cystic fibrosis. Furthermore, KaloBios is working with Sanofi Pasteur to develop KB001-A for ventilator-associated pneumonia.[148] A single dose of KB001 decreased CF lung inflammatory markers in sputum.[149] Phase 2 cystic fibrosis trial top line data is expected by the end of 2014.

KB003 is an IgG1 neutralizing antibody against GM-CSF and intended for inadequately controlled severe asthmatics. A fully recruited Phase 2 multiple dose study in 160 subjects with severe asthma is currently ongoing. KaloBios is also developing KB004, a non-fucosylated EphA3 agonistic IgG1 antibody. Dr. Yarranton noted that EphA3 is expressed on leukemic stem cells and stem cell niche, and KB004 is capable of inducing apoptosis of EphA3 positive malignant bone marrow cells derived from a CML patient. KB004 is currently in a Phase 1 "all comers" trial for hematologic malignancies. KaloBios has also developed an Immunohistochemistry test to identify patients with EphA3-positive tumors.

The views expressed herein are those of the author and do not necessarily reflect the views of Regeneron Pharmaceuticals, Inc.

Antibody-Drug Conjugates

Robert Mabry

Chaired by **Louis M. Weiner** (Georgetown University Medical Center), the final session of the meeting focused on the development of ADCs as novel cancer therapeutics. The first speaker, **Peter Senter** (Seattle Genetics), began his presentation on generating ADCs for cancer by commenting on the number of antibodies that do not make it to the clinic, and the incentive to empower antibodies to make more effective therapeutics. While ADCs give promise to a novel approach by combining antibody specificity and potent drugs, he pointed out that early clinical results offer insight into why this generation of ADCs failed and where attention was directed to make improved versions. Low potency and linker instability were two areas for improvement, as reflected in the early results of BR96-Dox and gemtuzumab ozogamicin (Mylotarg®). High drug loading is now also known to be of importance as accelerated ADC clearance and reduction in exposure to conjugated auristatin appear to be consequential. Dr. Senter described the underlying cause of accelerated clearance as due to uptake in liver for overloaded drug (outside of hepatocytes). The type of conjugate employed must also be considered when generating ADCs, as evidenced by an example of hydrophobic conjugate localizing much more poorly in tumors than the naked antibody.

Dr. Senter then focused his presentation on what lies ahead in the ADC field. Results from clinical trials with multiple agents were shared against a wide panel of malignancies. He discussed the example of brentuximab vedotin (Adcetris®) combined with standard frontline regimen in a Phase 1 study (ClinicalTrials.gov NCT01060904). The regimen, which included doxorubicin, vinblastine, and dacarbazine with or without bleomycin, was combined with brentuximab vedotin for treatment of advanced stage Hodgkin lymphoma.[150]

Additional improvements to current ADCs strategies at Seattle Genetics were also discussed.

Pyrrolobenzodiazepines (PBDs) are a promising chemotype of ADCs under investigation as part of their partnership with Spirogen. These small molecules are very potent as DNA minor groove-binding molecules, and Dr. Senter reported that the linkage of PDBs *via* engineered cysteine in the CH2 region of the Fc improved stability over other methods. Seattle Genetics currently has an anti-CD33 ADC in Phase 1 that had previously showed improved activity in AML models as conjugated to PBDs over auristatin.

Dr. Senter ended his talk by providing some commentary on linkers employed for ADCs. Traditional maleimide linkage results in fragmentation with half-life in 7 days in plasma and in vivo as a result of unstable bonding. Seattle Genetics has initiated a program to generate self-stabilizing maleimides. They are currently investigating alternatives by stressing conjugates for hydrolysis to identify new/better conjugates (more stable) as part of technology development.

John Lambert (ImmunoGen) discussed how advances in linker and effector chemistry can be used to expand the reach of ADC therapeutics. He began with an overview of ADCs currently in the clinic, and then reviewed the four main components of ADCs: (1) Target selection, (2) Antibody optimization, (3) Linker technology, and (4) Killing agents. While all four components are of paramount significance, he focused his presentation on the latter two topics. Dr. Lambert addressed several aspects of linker technology by centering on ImmunoGen's ADC approach employing lysine residues as the site of attachment. He highlighted an advantage of this approach in that native antibody structural activity and function, including target binding, PK, and effector functions of the Fc region (CDC/ADCC), are retained. He went on to stress the importance of the linker and how the connection between antibody and drug can affect therapeutic activity.[151] To exemplify these points, Dr Lambert described the research to identify IMGN853, an anti-folate receptor (FOLR1) antibody conjugated, via the disulfide-containing cleavable linker sulfo-SPDB, to maytansinoid DM4. The ADC has an optimized linker component to release DM4 post-internalization which binds tubulin and disrupts microtubule assembly to inhibit cell division of FOLR1 over-expressing tumor cells. IMGN853 is currently in Phase 1 clinical trials for ovarian cancer and other FOLR1-positive solid tumors.

Advances in linker technology now employed by ImmunoGen include peptide linkers that yield maytansinoid metabolites that are carboxylates. This approach results in a form of the drug that upon release in the acidic environment of the lysosome, readily gains access to the cytoplasm due to protonation of its carboxyl group. However, at the near neutral pH of the cytoplasm, the carboxyl group is charged and, presumably, hampered in its ability to cross plasma membranes and diffuse into neighboring cells, thus altering its "by-stander effect." Data presented by Dr Lambert displayed evidence that the novel peptide linker-maytansinoid format improves both in vivo efficacy and tolerability in xenograft models.[152] This affords a path to improve tolerated doses and enhance potency of ADCs, and shows that the chemistry of the linker-payload system profoundly influences both antitumor activity and safety of ADCs. Dr Lambert noted that only about 0.01% of the injected dose per gram of tumor of an antibody (or ADC) actually is localizes to tumor, further reinforcing the significance of linker-payload chemistry for the safety profiles for ADCs because catabolism in normal tissues accounts for the ultimate elimination of the majority of the administered ADC in humans.

Dr Lambert then closed his presentation by describing a new class of DNA-alkylating cell-killing agents designed for use as payloads for ADCs. These novel agents, called IGNs, display potent antigen-specific activity against both hematologic and solid tumor xenograft models.[153,154] When a stable disulfide linker was utilized for conjugation of one such DNA-alkylating payload, the resulting ADC was shown to have a good tolerability profile, with a wide therapeutic index in animal models comparable to that of the maytansinoid class of ADCs.

Tom Hawthorne (Celldex Therapeutics) discussed the development of CDX-011 (glembatumumab vedotin) as a treatment for breast cancer and melanoma. CDX-011, an ADC containing an antibody that was derived from the Abgenix transgenic mouse technology, is currently in clinical trials. The targeting antibody (CRO11) is conjugated to monomethyl auristatin E (MMAE) using Seattle Genetics' technology. CRO11 is specific to the oncology target transmembrane glycoprotein NMB (GPMNB), which is also designated osteoactivin. Dr. Hawthorn provided background on the GPNMB expression profile in various tumors,[155,156] with a focus on melanoma specimens in which over 80% of samples tested positive for GPNMB over-expression. Data presented included pre-clinical profiling for CDX-11, which exhibited impressive activity in a mouse melanoma xenograft model using SK-MEL-2 human melanoma cell line.

Dr. Hawthorne then delivered an update on the efficacy of CDX-011 in clinical trials. The Phase 1 qualitative results showed CDX-011 clearance is comparable to EGFR inhibitors at an equivalent dose. Observed dose-limiting toxicities were higher than expected, and Dr. Hawthorne noted they were currently evaluating modified dosing regimens. In Phase 2 melanoma trials, Celldex is examining the tolerability of CDX-011. They have observed skin rashes common to EGFR therapy, but in different areas of patients. Dr. Hawthorne then discussed several of the activity parameters by dosing schedule and GHPNMB expression. Higher dosing of CDX-011 led to elevated toxicity that continues to be a concern. He then presented several waterfall plots of Phase 1/2 tumor shrinkage. Dr. Hawthorne described the overall response rate of >15% as promising. Yet, increase in activity for dosing schedules was accompanied with an increase in toxicity. Observed rashes were also associated with a greater objective response and progression-free survival.

Breast cancer is an additional indication for treatment with CDX-011. GPNMB over-expression was reported for more than 40% of breast cancers. Dr. Hawthorne provided some additional background on the role that GPNMB plays in breast cancer, for which expression is observed within epithelial tumor cell lines as well as stromal cells. CDX-011 is currently in a Phase 2b randomized study for advanced breast cancer. Patients were tested for GPNMB expression using a validated centralized IHC method. Observed partial responses for triple-negative (TNBC) cancer patients was 21%. However, TNBC patients who tested positive for GPNMB exhibited partial responses of 36%, thereby associating GNMB expression with greater activity of CDX-011. Celldex plans to expand clinical studies in TNBC and GPNMB-positive patients during 2014.

Following the afternoon break, **Edward Reilly** (AbbVie) discussed ABT-414, an EGFR-targeting ADC. His presentation opened with an overview of anti-EGFR therapies and the development of tumor resistance observed among patients. Dr. Reilly then noted ADC therapeutic strategies using approved mAbs that inhibit signaling are generally limited by toxicity profiles. The ability to prevent antigen-dependent liabilities would be a significant gain for the approach.

Dr. Reilly then introduced one of the lead ADCs in AbbVie's pipeline. ABT-414 is an anti-EGFR antibody conjugated to the MMAF toxin *via* non-cleavable maleimidocaproyl linker (Seattle Genetics' technology). The ADC binds to a unique epitope on domain III of wild-type EGFR which is not a target for approved anti-EGFR agents. The epitope is only available for binding when the receptor has undergone a conformational change. Due to the conformational change that exposes the ABT-414 epitope primarily in tumor and not normal tissue, ABT-414 is likely to exhibit minimal binding to normal tissue. ABT-414 also binds to EGFRvIII, the most common form of truncated EGFR, which is expressed on over 30% of glioblastoma tumor samples. The antibody component of ABT-414, ABT-806, displays substantially reduced IHC staining on human skin compared to cetuximab, which further supports the notion that the unique epitope is not robustly exposed on non-tumor tissue. Another characteristic distinguishing ABT-806 from other anti-EGFR antibodies is the observance of linear PK, also consistent with its reduced binding to non-cancerous tissue.

Preclinical data was presented by Dr. Reilly in several xenograft models in both cancer cell-line derived (NCI-H1703) and patient-derived (SN-0199 and SN-0207) xenograft models. ABT-414 was potent in both WT EGFR models as well as EGFRvIII over-expressed models (U87MG glioblastoma forms). Dr. Reilly also reported on results from the biomarker strategy for patient selection using EGFR gene amplification and RTPCR for EGFRvIII, which allowed AbbVie to conclude that, preclinically, ABT-414 activity/efficacy correlates to EGFR expression.

Additional pre-clinical studies in cynomolgus monkeys showed the PK for ABT-414 is very similar to the ABT-806 naked antibody. In the toxicology studies, ABT-414 was well-tolerated at a single dose up to 30 mg/kg without any skin rashes typical of conventional anti-EGFR antibody therapies.

ABT-806 has also been employed as an imaging agent following labeling with ^{111}Indium to produce ABT-806i. ABT-806i was applied to monitor selective uptake of the imaging agent in the U87MG xenograft model. In this model, Dr. Reilly presented data showing the inhibition of the ABT-806i with cold-ABT-806. Dr. Reilly also presented SPECT images from a Phase 1 clinical trial with ABT-806i demonstrating binding to intracranial tumors of glioblastoma patients. These results indicated that ABT-806i (and ABT-414) can penetrate the blood-brain barrier, which may have been compromised by previous treatments.

Dr. Reilly concluded by sharing the development plan for ABT-414. He spoke of the desperate need for new therapies in glioblastoma and noted there may be synergy among ABT-414 and the current standard of care treatment of radiation and temozolomide combination therapy. ABT-414 is currently being evaluated as a treatment for EGFR-refractory metastatic solid tumors (Phase 2 study NCT01741727), and glioblastoma (Phase 1 study NCT01800695). Dose-escalation is currently underway and early reports indicate no skin rash has been observed.

Jagath R. Junutula (Genentech) discussed site-specific conjugation as a way to build next-generation ADCs with improved therapeutic indices. He began his presentation with a review of the function of ADCs[157] and the need for a balance between efficacy and toxicity. He provided a short overview of different platforms and the range of DARs observed with different conjugation methods. Dr. Junutula then focused on engineered THIOMAB drug-conjugates (TDCs) as a novel method for generating ADCs.[158] This strategy results in a 2-DAR that can improve tolerability for TDCs over conventional ADC DARs. In one study, an anti-MUC16-vc-MMAE TDC demonstrated improved tolerability by 2-4 fold compared with the ADC version of the same antibody in both rats and cynomolgus monkeys. Dr. Junutula shared his hypotheses that the TDC approach would likely improve the toxicity profile of the anti-MUC16 ADC, DMUC5754A, currently in a Phase 1 study. This ADC has a small therapeutic window due to observed toxicities at maximum tolerated dose (MTD) (2.4mg/kg).[159] The TDC approach have an advantage in achieving better efficacy because it may allow dosing beyond the MTD dose of a conventional ADC, based on preclinical studies.

Drug-release and its influence on ADC activity were also discussed by Dr. Junutula. A report on the effect of conjugation sites and in vivo stability of TDCs was recently been published.[160] Several sites within the heavy and lights chains of an antibody were explored for integration of cysteines for thiol-reactive linker conjugation. A 2-fold increase in efficacy was observed with cysteines engineered into the light chain (LC-V205C) of an anti-TenB2 antibody compared to heavy chain conjugation (HC-A114C).[160] This study further showed the influence of conjugation site on linker or drug-independent drug loss from TDCs. The optimal conjugation site (LC-V205C) was partially solvent-exposed and was reported to result in a positively charged environment that promoted hydrolysis of a succinimide ring in the linker. Dr. Junutula also discussed the influence of 4-DAR vs 2-DAR for TDCS. The 4-DAR TDC showed 2-fold improvement in efficacy compared with conventional 2-DAR. However, the therapeutic index may likely be the same for 2-DAR and 4-DAR TDCs.

In the last presentation of the session, **Henry Lowman** (CytomX Therapeutics) provided an overview of the Probody™ platform, which comprises a novel strategy for improving tissue targeting and selectivity. This technology employs "masks" that bind to the antibody paratope to prevent binding to healthy tissues and, thereby, avoiding toxicities. Dr. Lowman discussed the possibility of applying the Probody concept to ADCs and other non-traditional antibody-based therapeutics to improve selectivity among different classes of therapeutics.

The Probody technology has been applied to EGFR targeting by employing cetuximab as the antibody backbone. Dr. Lowman reported the identification of a masking peptide that binds to cetuximab at the paratope of the antibody to prevent binding to EGFR. The peptide is fused to the *N*-terminus of the light chain *via* a linker containing a proteolytic cleavage site. The masking peptide is cleaved by distinct proteases expressed on the surface of tumors, which convert the prodrug form of the antibody to the active form by removing the "mask" that ablates binding to EGFR. Dr. Lowman shared protease activity data in diseased tissue demonstrating cell staining that was not observed in normal tissue. He also provided data exhibiting proteolytic activity by H292 tumor tissue (ex vivo) that resulted in binding of the Probody to tumor cells. Additional data displayed the localized activity of the EGFR Probody and the evasion of the signature rash observed with conventional EGFR antibody therapy.[161]

CytomX has now leveraged the promise of improved specificity to generate an ADC with the EGFR Probody. CTX-023-MMAE is the cetuximab Probody CTX-023 conjugated to monomethyl auristatin E to produce a Probody-drug conjugate (PDC). He reported a DAR of 4 for CTX-023-MMAE, and Dr. Lowman noted that the drug conjugation to the Probody does not alter its ability to be activated or its binding to EGFR. In vivo studies revealed that efficacy and duration of response was improved for CTX-023-MMAE compared with antibody alone in xenograft models. Dr. Lowman concluded by providing a preclinical update on the CTX-033 PDC that is also conjugated to MMAE. This antibody targets both JAGGED 1 and 2 with sub-nanomolar affinity. Safety and efficacy data were shown for multiple tumor models, and cell staining specific to pancreatic tumors supported the on-target specificity offered by the Probody approach.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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