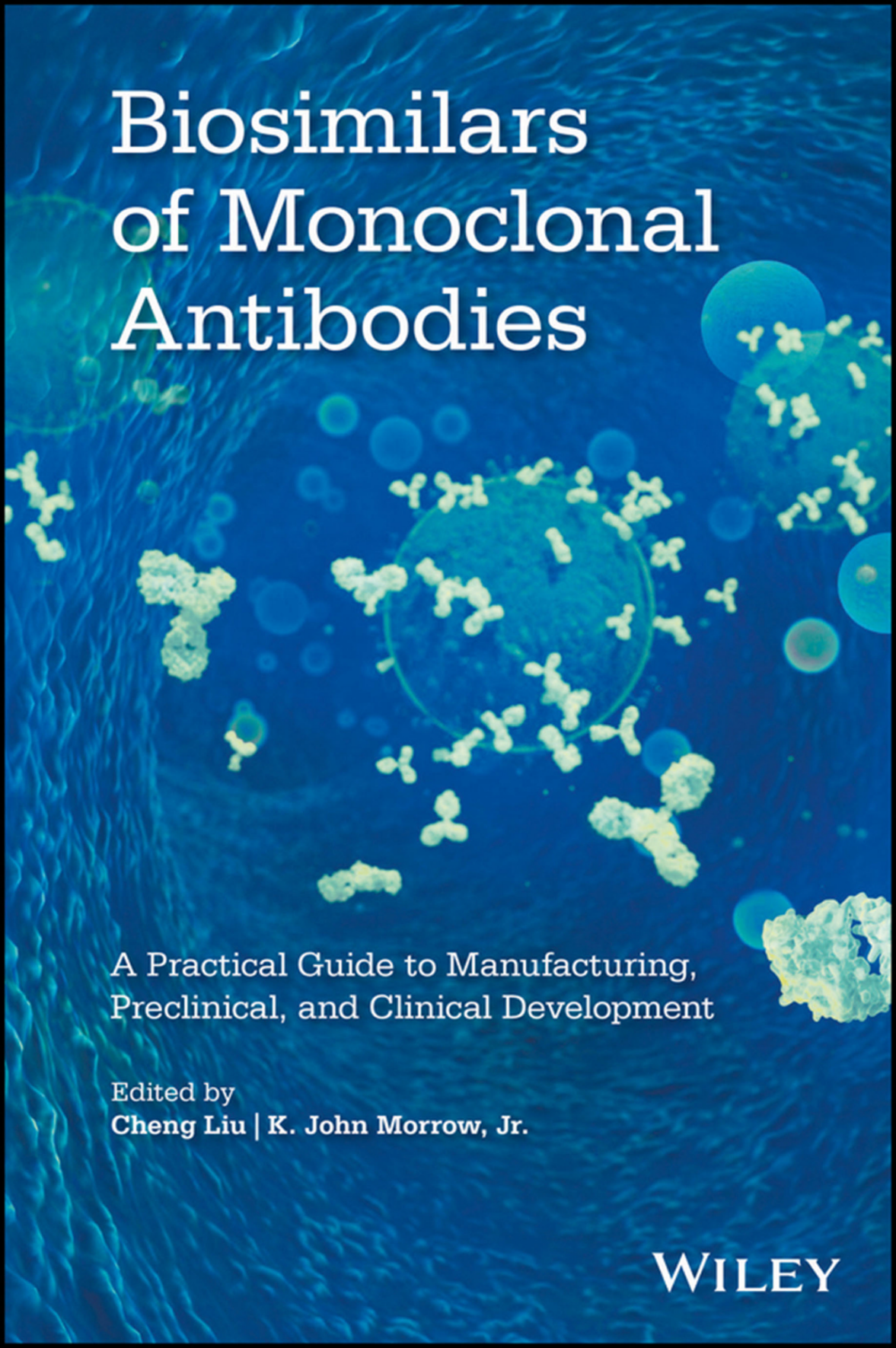
Biosimilars of Monoclonal Antibodies



A Practical Guide to Manufacturing, Preclinical, and Clinical Development

Edited by
Cheng Liu | K. John Morrow, Jr.

WILEY

Biosimilars of Monoclonal Antibodies

Biosimilars of Monoclonal Antibodies

A Practical Guide to Manufacturing, Preclinical, and Clinical Development

Edited by

Cheng Liu, Ph.D.
K. John Morrow, Jr., Ph.D.

WILEY

Published by John Wiley & Sons, Inc., Hoboken, New Jersey

Published simultaneously in Canada

Library of Congress Cataloging-in-Publication Data:

Names: Liu, Cheng, 1966– editor. | Morrow, John, Jr. 1938– editor.
Title: Biosimilars of monoclonal antibodies : a practical guide to manufacturing, preclinical, and clinical development / edited by Cheng Liu, K. John Morrow, Jr.
Description: Hoboken, New Jersey : John Wiley & Sons, Inc., [2017] | Includes bibliographical references and index.
Identifiers: LCCN 2016019958 (print) | LCCN 2016020707 (ebook) | ISBN 9781118662311 (cloth) | ISBN 9781118940624 (pdf) | ISBN 9781118940631 (epub)
Subjects: | MESH: Antibodies, Monoclonal | Biosimilar Pharmaceuticals
Classification: LCC QR186.7 (print) | LCC QR186.7 (ebook) | NLM QW 575.5.A6 | DDC 616.07/98–dc23
LC record available at https://lccn.loc.gov/2016019958

Printed in the United States of America

Cover image: Courtesy of the Editors

Set in 10/12pt Warnock by SPi Global, Pondicherry, India

10 9 8 7 6 5 4 3 2 1

Contents

Notes on Contributors

K. Lance Anderson focuses his law practice at Dickinson Wright PLLC on intellectual property, business and information technology related to emerging business. A registered patent attorney, he has wide-ranging experience in licensing and transactional matters, including negotiation and due diligence for licensing, mergers and acquisitions, asset transfers, and strategic planning. He often counsels on pharma and life science transactions, including research and development, in the developing anticancer and immunotherapeutic industries. His experience also includes patent prosecution, patent litigation, trade secrets, employment, corporate governance, electronic records management, and financial matters.

Lance earned his B.S. in Entomology and M.S. in Crop Science from Texas Tech University and his J.D. from the Texas Tech University School of Law.

Jonathan D. Ball, Ph.D., is a shareholder at Greenberg Traurig, LLP, with nearly 15 years of experience representing clients in intellectual property matters, with an emphasis on patent litigation and contentious Patent Office proceedings. He also has extensive experience in patent portfolio management, prosecution, and licensing and helps companies develop and implement worldwide IP strategies. He has experience in a variety of biopharmaceutical matters, including Hatch–Waxman litigation, pre-ANDA diligence, and counseling on pharmaceutical patent life cycle management.

Jonathan earned his B.A. from Vassar College, his Ph.D. in Organic Chemistry from the University of North Carolina at Chapel Hill, and his J.D. from the University of Richmond School of Law.

Maurizio Chiriva-Internati An author of 146 peer-reviewed publications, Maurizio Chiriva-Internati, Ph.D., D. Bsci., acts as the chief scientific officer at Kiromic, a biotechnology company specialized in immunotherapy, and serves as an associate professor of medicine and director of translational research at the Division of Hematology/Oncology in the Department of Internal Medicine at Texas Tech University Health Sciences Center in Lubbock, Texas. He received

his Ph.D. in Immunology from the University of Nottingham and his doctorate in biology from the University of Milan. He has clinical and teaching experience in M.D., Ph.D., and M.S. school settings as well as experience as a reviewer for a variety of journals. He is an associate editor for the *Journal of Translational Medicine*, a section editor for the *International Reviews of Immunology*, and an associate editor member for both the *Open Hematology Reviews* and the *Open Hematology Journal*. For the past 14 years, he has investigated tumor antigens as therapeutic and diagnostic tools. His research is focused on developing new therapies for a variety of diseases, namely, cancers including multiple myeloma, ovarian, lung, prostate, melanoma, and breast. Using recombinant adeno-associated viruses, he is developing vaccines that direct T-cell immune responses toward unique tumor-specific or tumor-associated antigens.

Everardo Cobos, M.D., is president of Kiromic, LLC, and director of Hematologic Malignancies at Grace Health System in Lubbock, Texas. For the past 24 years, he served as a professor of medicine and oncology and chairman of the division of hematology/oncology at Texas Tech University Health Sciences Center (HSC) in Lubbock. He has also served as medical director of the Southwest Cancer Center at the University Medical Center (UMC) and as program director and founder of the Stem Cell Transplantation Program at TTUHSC/UMC. He is board certified in internal medicine, hematology, and oncology and is an expert in the treatment of hematologic malignancies, stem cell transplantation, and thrombosis and hemostasis.

Dr. Jose A. Figueroa is currently director of Solid Malignancies in the Blood Disorders and Cancer Therapeutics Center at Grace Health System in Lubbock, Texas, and vice president and chief medical officer of Kiromic, LLC, a clinically oriented biotechnology company. He earned his undergraduate and medical degrees at the University of Puerto Rico and completed his fellowship in medical oncology, hematology, and translational research at the University of Texas Health Science Center in San Antonio in 1993. He is board certified in medical oncology. He has been a recipient of the Young Oncologist Award from the American Radium Society and the American Cancer Society Research Grant and has served as medical director and director of the clinical research program of the Southwest Cancer Center/University Medical Center at Texas Tech University Health Sciences Center, School of Medicine. He is the author of many scientific abstracts, peer-reviewed publications, a book chapter on breast cancer, poetry, and a fictional novel.

João Eurico Fonseca is head of the Rheumatology Research Unit at the Instituto de Medicina Molecular and professor of rheumatology and biomedical engineering, both part of the Faculty of Medicine, University of Lisbon,

Portugal. He is also head of the Ambulatory Unit of the Rheumatology Department of the Lisbon Academic Medical Centre. He completed his M.D. and Ph.D. in Rheumatology at the University of Lisbon and has obtained a qualification in health management from the AESE School of Management and Business in Lisbon. His research focuses on bone and inflammatory joint diseases. He is also the president of the Portuguese Society of Rheumatology and a member of the editorial board of several medical journals. He has authored more than 150 scientific papers.

João Gonçalves is head of the Biopharmaceutical and Molecular Biotechnology Unit at the Institute of Innovative Medicines and professor of immunology and biotechnology, both part of the Faculty of Pharmacy, University of Lisbon, Portugal. He is also head of Antibody Engineering Laboratory at iMed—Faculdade Farmacia Universidade Lisboa. He has a pharmaceutical degree and Ph.D. in Infectious Diseases/Immunology at Harvard University and University of Lisbon and has postdoctoral experience at Centocor, Genentech, and Scripps Research Institute, La Jolla. His research focuses on therapeutic antibody discovery and development ranging from autoimmune diseases to oncology. He is a member of the Portuguese Pharmacopoeia Commission and Portuguese Commission of Medicines Evaluation. He is also a member of the editorial board of several scientific journals. He has authored more than 80 scientific papers.

Patrick G. Holder is a native of Cleveland Heights, OH. He received his Ph.D. in Chemical Biology in the lab of Matthew Francis at UC Berkeley. While there, he used bioconjugation to integrate optoelectronic materials into a scaffold of self-assembling viral proteins, including single-walled carbon nanotubes, arrays of phthalocyanines, and thermostable virus-templated polymers. As an NIH postdoctoral fellow in the lab of Daniel Nocera at MIT, in a collaboration with JoAnne Stubbe, he leveraged site-specific bioconjugation of phototriggers to perform time-resolved spectroscopy of radical transport in the enzyme ribonucleotide reductase. In 2012, his work was awarded the Energy and Environmental Science Prize from the Royal Society of Chemistry. He graduated with an A.B. in Chemistry from Columbia University, where he received the William H. Reinmuth Chemistry Scholarship. Formerly at Redwood Bioscience and currently at Bristol-Myers Squibb, he is helping advance antibody-drug conjugates from the benchtop to the clinic.

Dr. Nattamol Hosiriluck is currently a second-year resident in the Department of Internal Medicine of Texas Tech University Health Sciences Center in Lubbock, Texas. She graduated from Phramongkutkloa College of Medicine in Bangkok, Thailand, in 2012. Since then her interest has been on hematology and oncology, and she is planning to pursue her fellowship in this area.

Clarinda Islam is currently the director of Quality Assurance and Regulatory Affairs for Somru BioScience, Inc., an emerging biotechnology company based in Charlottetown, PE, Canada. In this role, she is responsible for the quality management, project management, process improvement, product development life cycle methodologies, and quality management systems. Previously she served as a senior group leader for the Immunochemistry Department at PPD (VA, USA) where she was responsible for leading a team of scientists dedicated to supporting biopharmaceutical development. Prior to joining PPD, she has held positions of increasing responsibility in the Bioanalytical Group at Covance, Inc. She has over 14 years of experience in the development of biologics in a highly regulated environment under FDA and other global regulatory guidelines.

Rafiq Islam is the senior director of Bioanalytical Services at Celerion, Inc. (NE, USA). In his current role, he is responsible for the scientific and operational leadership of both small- and large-molecule bioanalysis, which includes the development, validation, and execution of sample analysis to support pharmacokinetic, immunogenicity, bioequivalence, and biosimilar studies. Previously, he served as the scientific director for Biopharma Services at EMD Millipore. He held similar positions as the head of a bioanalytical department at Covance, Inc. (AZ, USA), and Huntingdon Life Sciences (NJ, USA). He also held several positions of increasing responsibility with CuraGen Corporation (New Haven, CT).

He has a B.S. degree in biology and M.Sc. in data mining. He has 15 years of industry experience. He is an active member of the American Association of Pharmaceutical Scientists (AAPS) and Global CRO Council (GCC).

Roy Jefferis After receiving his B.Sc. and Ph.D. in Chemistry, Jefferis moved to the Medical School (Birmingham) to study the structure and function of antibody molecules. A particular interest has been the influence of glycosylation on the expression of biologic activities, *in vitro* and *ex vivo*. Extension of these findings to glycoengineering and protein engineering of recombinant antibody therapeutics resulted in interactions with the biopharmaceutical industry. A current focus is the potential for immunogenicity of immune complexes, in contrast to aggregated forms of IgG. He is the author of more than 280 publications, with 207 of these referenced in PubMed. He is a member of the Royal College of Physicians (MRCP) and a fellow of the Royal College of Pathologists (FRCPath).

Maria de Jesus Trained as an environmental engineer in Portugal, De Jesus completed her Ph.D. in Plant Cell Culture Technology at the Swiss Federal Institute of Technology in Lausanne, Switzerland (EPFL). She joined the Laboratory of Cellular Biotechnology at the EPFL as a postdoctoral researcher

and led the development of several projects as scientist and senior scientist, directed toward the establishment of manufacturing processes for recombinant proteins from CHO cells. In 2002 she initiated a self-funded, privately owned company, ExcellGene SA, acting in the role of founder and first employee. In subsequent years she established a laboratory and administrative unit of this company while simultaneously assuring the profitability and growth of the company as a CRO and CMO in the field of protein manufacture and process development for high-value therapeutic products from CHO and other cell lines. Currently she is chief operations officer, a member of the board of directors, and a significant shareholder of the company.

She has published over 40 peer-reviewed papers in the field and has lectured on process development and scale-up with animal cells in bioreactors. She is inventor of a small-scale bioreactor system, based on orbitally shaken tubes which are used in high-throughput approaches for suspension culture process development.

Dr. Weidong Jiang is a cofounder of Henlius Biopharmaceuticals, Inc. in United States. He is also senior vice president and chief scientific officer of Shanghai Henlius Biotech Co., Ltd., a joint venture formed by Henlius and Fosun Pharma in 2009 to develop antibody therapeutics. He has more than 20 years of working experience in many biotech companies, including Catalyst Biosciences, Inc.; Vasgene Therapeutics, Inc.; Applied Molecular Evolution, Inc. (subsidiary of Eli Lilly); Microcide; and ChemGenics (acquired by Millennium). He has expertise in the field of drug screening and development, particularly in research and development of antibody therapeutics and other protein biologics. He holds a Ph.D. degree from Giessen University in Germany; a master's degree from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences; and a bachelor's degree from Zhejiang University.

Cheng Liu is the founder and CEO of Eureka Therapeutics, a California company dedicated to the development of innovative cancer immunotherapy. He worked at Chiron (now Novartis), where he championed the development of first-in-class therapeutic antibody against CSF-1, a key immune regulator, for cancer metastasis treatment. He has multiple issued US and international patents and scientific publications, including in journals of *Science* and *Nature*, in the area of therapeutic antibody discovery and engineering. In 2007, he was awarded Special US Congressional Recognition for his contributions to improving human health. He received his B.S. in Cell Biology and Genetics from Beijing University and a Ph.D. in Molecular Cell Biology from the University of California, Berkeley.

Dr. Margaret Liu is an assistant professor in the Department of Chemical and Biological Engineering at the University of Alabama. Her research areas include

mammalian cell engineering, microorganism metabolic engineering and systems biology, and bioreactor process development. Specifically, her research projects focus on (i) improving the production and quality of biopharmaceuticals (e.g., biosimilars and other recombinant proteins) by host cell engineering and integrated process development via omics technologies, (ii) developing novel therapy to treat cancer and heart diseases, and (iii) producing next-generation bioenergy (e.g., biobutanol) by metabolic cell-process engineering (MCPE) using systems biology. She had worked in biopharmaceutical and biotechnology industries for six and half years before joining academia research. She was a senior scientist and team leader of Life Technologies Corporation (LTC) and a scientist of Lonza and Merck KGaA (EMD biopharmaceuticals). She has years of diverse industrial experiences in mammalian cell culture, biopharmaceutical production cell line development, omics technology, and therapeutic protein (including both innovator biologics and biosimilars) production process development in pharmaceutical and biotech companies. She accomplished her Ph.D. research under the instruction of Prof. S.T. Yang in the Department of Chemical and Biomolecular Engineering at the Ohio State University, United States. Her Ph.D. thesis work dealt with bioprocessing of high-value and biofuel products from metabolically engineered microbial mutants using a novel bioreactor production bioprocess. She earned her master's degree in bioengineering from Tianjin University and bachelor's degree in chemical engineering (major) and computer science (minor) from Shandong University, China.

Meimei Liu is a research scientist at Thermo Fisher Scientific, Inc. in Grand Island, NY. She obtained her B.S. and M.S. in Chemical Engineering from Tianjin University, China, and Ph.D. in Chemical and Biomolecular Engineering from the Ohio State University. While working at Tianjin University, she studied drug delivery and biopolymer development. While working at the Ohio State University, she investigated cell culture, cell therapy, and bioreactor process development. At Thermo Fisher Scientific, Inc., she works on cell culture and media development.

Dr. Scott Liu is a molecular biologist by training and holds a Ph.D. degree from Purdue University. As the founder, president, and CEO of Henlius Biopharmaceuticals, Inc. and Shanghai Henlius Biotech Co., Ltd., he takes overall responsibility for strategic planning and implementation, as well as operations management. Benefiting from Scott's vision in achieving quality at reasonable cost, Shanghai Henlius Biotech Co., Ltd. has adopted innovative technologies in mAb development and manufacturing and, in doing so, significantly reduced the manufacturing costs while maintaining the quality of the products. Before founding his own companies, Scott held key technical and/or leadership positions in several biopharmaceutical companies, including

Amgen, Bristol-Myers Squibb, United Biomedicals (UBI), Tanox, and Maxygen. He was the director of Quality Analytical Labs of Amgen Fremont and took responsibility for quality control operations for late-phase development and cGMP commercial manufacturing of Vectibix. Prior to Amgen, Scott held the post of associate director of Biologics QC in BMS Syracuse, where he was in charge of QC operations for late-phase development and commercial manufacturing of Orencia.

James D. Marks Dr. Marks is an internationally recognized pioneer in the field of antibody engineering and an elected member of the Institute of Medicine of the National Academies of Sciences. He has invented broadly applicable methodologies for the generation and optimization of human monoclonal antibodies. The Marks Lab at UCSF has been involved in the use of diversity libraries and display technologies to generate monoclonal antibodies (mAbs) since the earliest days of the technologies. As a graduate student in Greg Winter's Lab, he developed the first universal PCR primers which could amplify and isolate human V-gene repertoires and was the first to show that human mAbs could be directly isolated from nonimmune (naïve) phage antibody libraries. He also was the first to demonstrate the use of phage display for *in vitro* antibody affinity maturation and the direct selection of mAbs to cell surface proteins using cells. The Marks Lab has developed methods capable of generating very large nonimmune phage and phagemid single-chain Fv (scFv) antibody libraries capable of generating panels of antibodies to virtually any antigen. These libraries have been used within the Marks Lab and provided to collaborators where they have been extensively validated as a source of useful mAbs. Several antibodies generated in his lab have been tested in the clinic.

Dr. Marks serves as the chief of medical staff at San Francisco General Hospital and is professor of anesthesia and perioperative care at the University of California, San Francisco (UCSF).

Dr. Leonardo Mirandola graduated in biology and biotechnology in 2006 and received his Ph.D. in Molecular Medicine in 2009 at the University of Milano (Milan, Italy). He has published studies on intracellular signaling pathways, and his work as a Ph.D. student contributed to the molecular mechanisms by which oncogenic signals affects the response to chemokines in hematological malignancies, namely, acute leukemia and multiple myeloma. In 2010, he joined the Division of Hematology and Oncology at the Texas Tech University Health Sciences Center (TX, USA), where he focused on the discovery and validation of new immunologic targets for the development of cancer vaccines and diagnostic tests for multiple myeloma and ovarian, prostate, and breast cancer. Additionally, he leads a research and development project for the generation of new biotech drugs capable to

block tumor-associated lectins, thus increasing cancer cells susceptibility to chemotherapy. His current research focuses are the design of a recombinant adeno-associated virus (rAAV) vector with modified tropism and controllable transgene expression driven by a tissue-specific promoter and the development of a new system to generate clinical-grade dendritic cells *in vitro*.

Jennifer R. Moore Meline is an associate in the Medtronic, Restorative Therapies Group Memphis office. She focuses her practice on intellectual property, with an emphasis on patent litigation, and has experience relating to a wide range of technologies, including medical devices, pharmaceuticals, biology, chemistry, and financial services. She also has experience performing prelitigation investigations, product clearance, and opinion work on behalf of clients.

Jennifer earned a B.S. in Biochemistry and English from the University of California at Los Angeles and her J.D. from Columbia Law School.

K. John Morrow, Jr. A molecular biologist, K. John Morrow, Jr. is president of Newport Biotechnology Consultants. He specializes in writing and consulting in the area of immunology, with a focus on antibody technology. He obtained his Ph.D. from the University of Washington and did postdoctoral studies in Italy at the Università degli Studi di Pavia and at the Fox Chase Cancer Center in Philadelphia. He has been employed at several universities as a faculty member including Texas Tech and Kansas University and has also worked in the private sector. For the last 10 years, he has been engaged in a number of consulting contracts as well as authoring many articles, reviews, books, and commentaries in the area of biotechnology.

Morrow has been employed since 1996 as a consultant at Meridian Bioscience, Inc. in Newtown, OH. He is presently chair of their Institutional Biosafety Committee and has performed contractual work with Meridian in the investigation and development of recombinant antibody technologies. He is also a member of the Institutional Biosafety Committee at Cincinnati Children's Hospital, Cincinnati, OH. He is a member of the Sinclair College Biotechnology Advisory Board in Dayton, OH.

He has written a total of more than 280 peer-reviewed papers, marketing reports, articles in the biotech trade press, commentaries, book chapters, and book reviews. In 2014 he published a book on epigenetics, *Cancer, Autism and Their Epigenetic Roots*.

Reena Nair is currently senior consultant in the Department of Clinical Hematology, Tata Medical Center, Kolkata. She earned her Doctor of Medicine (M.D.), postgraduate degree in internal medicine, from Goa Medical College under Bombay University in 1989. She completed her medical oncology training at Tata Memorial Hospital, Parel, Mumbai, from 1989

to 1994. Her former clinical appointments include Medical Oncology Department lecturer (1996) and professor (2008–2012) at Tata Memorial Hospital, Mumbai, India. She has been primary investigator in 35 investigator-initiated clinical trials and over 25 sponsored trials since 1994 onward. Her publications include over 95 original papers in peer-reviewed journals, both national and international. She has special research interest in lymphomas, leukemia, and breast cancer management and improving outcomes. She is also initiating collaborative clinical trials and data registries for lymphoma in India.

Dr. J. Drew Payne is currently an internal medicine resident at Texas Tech University Health Sciences Center. He completed undergraduate with honors from Texas Tech University receiving a degree in clinical laboratory sciences. He gained experience in clinical blood banking prior to receiving his doctorate of osteopathy from A.T. Still University Kirksville College of Osteopathic Medicine. He was chief resident for his third year of internal medicine training and has coauthored several peer-reviewed publications. His current interests include hematology–oncology training.

Dr. Camilo Pena is an international medical graduate currently training with the Internal Medicine Program at Texas Tech University Health Sciences Center. He graduated from the Universidad El Bosque—Escuela Colombiana de Medicina in Bogota, Colombia. He spent his last year of medical school as a foreign student in Miami, Florida, at the Jackson Memorial Hospital—University of Miami. He also became involved with the Lumen Foundation and Lumen Global, a cardiovascular research organization, where he participated in publishing 8 book chapters (*Textbook of STEMI Interventions Second Edition—2010*) and more than 10 peer-reviewed and published papers, abstracts, and posters. He then became certified by the Educational Commission for Foreign Medical Graduates (ECFMG) and obtained a residency spot with his first choice program at TTUHSC. He is currently working with the hematology and oncology division in different research projects.

Steven J. Projan Prior to joining to his industrial career, Dr. Steven J. Projan was an associate at the Public Health Research Institute, continuing his studies on plasmid replication, antibiotic resistance, and staphylococcal virulence though 1994. In 1987 he also became a senior scientist and then group leader at Applied Microbiology, Inc. (at that time an in-house biotech company at the Public Health Research Institute) working on antimicrobial enzymes and bacteriocins (small antibacterial proteins). There he developed a novel protein-based method for the prevention of bovine mastitis that was marketed to the dairy industry, and he also developed a novel protein expression system for the production of an antistaphylococcal protein that has entered clinical trials.

Dr. Projan attended MIT for his undergraduate education, receiving an S.B. degree (in the life sciences and nutrition and food science) in 1974. He then graduated with a Ph.D. from Columbia University in 1980 (also receiving M.A. and M.Phil. degrees from that institution), spending 1977–1980 at the University of Utah. His graduate work was done with Jim Wechsler and they were successful in developing the first *in vitro* system that initiated chromosomal DNA replication from the *Escherichia coli* origin of replication. He then became a postdoctoral fellow with Richard Novick at the Public Health Research Institute in New York City studying plasmid replication and virulence in *Staphylococcus aureus*.

Dr. Projan joined Wyeth in 1993 as a group leader in anti-infectives research. He became an associate director of Bacterial Genetics in January of 1997 and then director of Antibacterial Research in June of 1998. In May of 2003 he was appointed assistant vice president of Protein Technologies, and in September 2004 he was promoted to vice president and head of the newly created Department of Biological Technologies which was responsible for delivery of novel biologics to development (this group is now part of Pfizer). At Wyeth he was the biology team coleader of the Glycylcycline Discovery Team that produced tigecycline, which had been approved in the United States for the treatment of bacterial infections including those caused by multidrug-resistant strains. In 2008 he became VP and global head of Infectious Diseases at the Novartis Institute of Biomedical Research, and 2010 he assumed his current position of SVP of R&D and iMed Head for Infectious Diseases and Vaccines at MedImmune (the biologics arm of AstraZeneca).

Dr. Projan has authored over one hundred and ten papers and book chapters, several short stories, and one teleplay. He is a past chair of the Gordon Research Conference on Staphylococcal Diseases, makes an award-winning cheesecake, and served (and continues to serve) as a member of several NIH peer-reviewed study sections. He serves on five editorial boards, is a past member of the Program Committee for ECMID and ICAAC, and is a past chair of Division A (Antimicrobial Agents) of the American Society for Microbiology. Of his numerous academic appointments and honors, he has been a visiting professor at the UCLA School of Medicine and is an adjunct professor in pharmacology at Boston University. In 2004 he was elected a fellow of the American Academy of Microbiology. He has been a tireless advocate for the public and private study of microbiology and since the 1990s has sounded the alarm over declining and ineffective antibiotic research.

David Rabuka received a Ph.D. in Chemistry at the UC Berkeley as a Chevron fellow in the lab of Carolyn Bertozzi. His research included developing and applying the aldehyde protein tagging platform technology to cell surface modification. Prior to joining Bertozzi's lab, he worked at the Burnham Institute synthesizing complex glycans followed by Optimer Pharmaceuticals,

which he joined as an early employee, focusing on the development of glycan- and macrolide-based antibiotics. He was CSO, president, and cofounder of Redwood Bioscience where he continued to develop novel protein conjugation methods and biotherapeutic applications such as antibody-drug conjugates. Redwood Bioscience was acquired by Catalent Pharma Solutions in October 2014, where he has continued to apply bioconjugation technologies with various collaborators and partners as a global head of R&D. He graduated with a double honors, B.S. in chemistry and biochemistry from the University of Saskatchewan, where he received the Dean's Science Award, and holds an M.S. in Chemistry from the University of Alberta. He is an author on over 35 major publications, as well as numerous book chapters and patents.

Ronald A. Rader, B.S. (Microbiology), M.L.S. (Library Science), has 35+ years' experience as a biotechnology, pharmaceutical, and chemical information specialist, author, consultant, and publisher. He is the author and publisher of BIOPHARMA: Biopharmaceutical Products in the US and European Markets, a reference source dealing with biopharmaceuticals. He is also the author/publisher of the Biosimilars/Biobetters Pipeline Database, a follow-on biologics-tracking information resource, and Biopharmaceutical Expression Systems and Genetic Engineering Technologies. Since 1988 he is the author and publisher of the *Antiviral Agents Bulletin*, a periodical specializing in antiviral/HIV drug and vaccine development. From 1994 to 2000, he authored and published the Federal Bio-Technology Transfer Directory.

Dr. Rakhshanda Layeequr Rahman is currently director of the TTUHSC Breast Center of Excellence at Amarillo, and the Interdisciplinary Breast Fellowship Program and is associate dean for faculty development at the Amarillo campus. She earned her undergraduate and medical degrees at the Aga Khan University in Pakistan and completed her fellowship in Breast Surgical Oncology at the University of Arkansas for Medical Sciences in 2004. She is board certified in surgery from the Royal College of Physicians and Surgeons of Glasgow and from the Royal College of Surgeons of Edinburgh, United Kingdom. She was also inducted as a fellow at the American College of Surgeons in 2005. She has received Texans Caring for Texans Award and Career Achievement Award by the Amarillo Women's Network. She is the Endowed Chair for Excellence in Women's Health. She specializes in the diagnosis and treatment of breast cancer and novel immunotherapeutic strategies for biologically diverse breast cancers. She is the author of numerous scientific abstracts, peer-reviewed publications, book chapters, and leadership literature.

Adair Reidy is a researcher at Kiromic, LLC, where she examines the expression of novel tumor-associated antigens for the development of

immunotherapeutic treatments. She completed her B.S. in Biology at Texas Tech University and is currently pursuing an MBA with a focus in science, technology, engineering, and mathematics (STEM). While working at Kiromic and under Dr. Chiriva-Internati at the Texas Tech Health Sciences Center, she participated in various peer-reviewed projects, abstracts, posters, and patents.

Jeanene ("Gigi") Robison, MSN, RN, AOCN, works as the oncology clinical nurse specialist at the Christ Hospital, Cincinnati, OH. She earned her B.S.N. from the University of Cincinnati College of Nursing and Health in 1982 and her master's degree in oncology nursing at Case Western Reserve University in 1988. She has maintained her advanced oncology nursing certification since 1997. She has practiced in oncology nursing for 33 years and works with patients receiving chemotherapy and biotherapy drugs. She frequently presents on topics related to oncology nursing and has taught courses on chemotherapy and biotherapy since 1990.

Regis Sodoyer completed Ph.D. in Organic Chemistry at the University of Nice in 1980. Then he joined the Centre d'Immunologie INSERM/CNRS de Marseille-Luminy (CIML) in Marseille, where he directed his research to the polymorphism and structure–function relationships of HLA class I genes of the human major histocompatibility complex. He completed a second Ph.D. in Molecular Immunology in 1986. He then joined the vaccines division of Sanofi Pasteur in 1986. During this period, he was a group leader, head of the Molecular Microbiology Platform, head of the Experimental Design and Modeling Platform, and director for Technology Innovation. He recently moved to Bioaster as a biotechnology specialist, responsible for the company's training program. His fields of expertise include vaccinology, molecular biology, immunology, antibody engineering, and phage display and expression systems for the production of recombinant biotherapeutics.

Samuel D. Stimple received his B.S. degree in chemical and biomolecular engineering from the University of Notre Dame in 2012. At Notre Dame, he was an undergraduate research assistant in the laboratory of Dr. Basar Bilgicer, where his research focused on the development of a novel, small-molecule-based platform technology for the affinity chromatography purification of antibodies without the use of protein A. He is now a university fellow in the third year of his Ph.D. in Chemical and Biomolecular Engineering at the Ohio State University, where he works in the laboratory of Dr. David W. Wood. His graduate research focuses on downstream bioprocess development. Namely, the Wood lab focuses their research in the field of protein engineering, with the aim of developing protein-based biosensors and improving a platform technology for the purification of non-mAb proteins of interest using

self-cleaving intein-purification tag technology. His research also includes the development of synthetic biology tools for applications in RNA and metabolic engineering.

Dr. Natallia Suvorava is currently at the last year of training in the Internal Medicine Residency Program at Texas Tech University Health Sciences Center in Lubbock, Texas, and incoming hematology oncology fellow at the University of Minnesota. She earned her undergraduate and medical degrees at the Gomel State Medical University in Belarus. She coauthored a number scientific abstracts and peer-reviewed publications.

Dr. Rashmi Verma is currently a second-year fellow in hematology and oncology at Texas Tech University, Lubbock, TX. She earned her medical degree with honors at the University College of Medical Sciences, New Delhi, India. Subsequently she did postgraduate studies at the University of Delhi in women's health and ob-gyn. She also worked as senior medical officer for 3 years at Delhi Government Hospital in 2001. She did her internal medicine residency internship at the University of South Dakota in 2009. She finished her internal medicine residency at UMDNJ, Cooper University, New Jersey, in 2012. She is board certified in internal medicine. She worked as a staff at Internal Medicine Hospitalist in 2012–2013. She started her fellowship 2013 and currently is a second-year fellow. Her interests include women's health, breast oncology, ovarian oncology, solid tumors, cancer genetics, and benign hematology. She has participated in various abstract presentations at the national level, case reviews, and peer-reviewed journals.

Adrienne R. Whitlow is a registered nurse previously employed as a critical care nurse in the Medical Intensive Care Unit at UMC Health System and is currently a pediatric nurse through Pediatric Home Health. She also serves as a second Lieutenant part-time in the Army Nurse Corps as a Medical-Surgical Nurse. She earned her B.S. degree in nursing through TTUHSC School of Nursing.

David W. Wood is an associate professor of chemical and biomolecular engineering at the Ohio State University. He received his undergraduate degree from Caltech in 1990 with a double major in chemical engineering and molecular biology. His work experience includes bioprocess development at Kelco, manufacturing at Amgen, and research at Bristol-Myers Squibb. He completed his Ph.D. in 2001 at Rensselaer Polytechnic Institute, where he was coadvised by Georges and Marlene Belfort. His primary field of research is protein engineering, especially the development of self-cleaving affinity tags for applications in recombinant protein purification. He has held faculty positions at Princeton University and the Ohio State University, where he currently resides.

He received the NSF Career Award and has two patents on intein-based technologies.

Dr. Tao Wu is a specialist in antibody research and discovery. Currently working at Boehringer Ingelheim Pharmaceuticals, Inc., he has over 10 years' experience in antibody discovery and pharmaceutical industrial development at Boehringer Ingelheim, Pfizer, and Merck, building and managing different teams and directing therapeutic antibody programs from early discovery stages into development. Currently, he is leading the antibody screening group and the cross-functional assay optimization team at Boehringer. Trained as a physician and molecular biologist, he earned his M.D. at the University of Wuhan in China and his Ph.D. in Microbiology with distinction at the University of Hong Kong. He thereafter completed postdoctoral study at the Centers for Disease Control in Atlanta, GA, and the Winship Cancer Institute at Emory University. He has published a number of peer-reviewed scientific papers in the *Journal of Immunology*, *Blood*, *Journal of Biological Chemistry*, *Molecular Biology of the Cell*, *Cancer Research*, and others.

Florian M. Wurm was trained as a biologist/molecular geneticist in Germany. He worked in industry (Behringwerke AG, Virology Department, Marburg and Genentech, Inc., Process Sciences Department, San Francisco) for 15 years during the earlier parts of his career. His work at Genentech contributed to the generation and manufacturing of several high-value products, such as Herceptin®, an antibreast cancer antibody; Pulmozyme®, a treatment for cystic fibrosis; and TNKase TPA®, a highly potent thrombolytic agent (cumulatively now sold for multibillion dollars/year globally). In 1995 he was appointed professor for biotechnology at Swiss Federal Institute of Technology, Lausanne (EPFL), where he established a research group for the field of process sciences with animal cells in bioreactors and where he gave classes in pharmaceutical biotechnology. As founder and CSO of ExcellGene (2001), he combined academic research and teaching with an entrepreneurial activity. Florian is member and past chairman of the European Society for Animal Cell Technology. He has published more than 200 papers and filed more than 30 patents, covering aspects of expression and manufacture of clinical proteins using mammalian cells in bioreactors. Some of his technology and process inventions have become globally used tools in research and in manufacturing of proteins with animal cells.

Zhinan Xia attended Nanjing College of Pharmacy in Nanjing, China, where he obtained his B.S. and M.S. degrees. He then earned his Ph.D. at the University of Kentucky College of Pharmacy in Lexington, KY. He did postdoctoral study at the Dana-Farber Cancer Institute at Harvard Medical School and subsequently worked in the private sector as principal scientist at Wyeth Discovery Research

and at Pfizer Worldwide Research. He then became director of Protein engineering at Synageva Biopharma and is now head of Biotherapeutics at Moderna Therapeutics. He has worked in the fields of mAb design, Fc fusion, enzyme replacement therapy for lysosomal storage diseases, and other biotherapeutic drug development. He has published more than 35 peer-reviewed journal articles and has obtained multiple patents on therapeutic enzymes and Fc fusion proteins.

Ningning Xu is a Ph.D. candidate in the Department of Chemical and Biological Engineering at the University of Alabama. She is working on cell line development for biotherapeutic proteins and CHO cell engineering using omics technologies for antibody quality regulation. She received her bachelor's degree in the School of Chemical Engineering and Technology at Tianjin University, Tianjin, China.

Dr. Su Yan is head of Human Antibody Discovery at Eureka Therapeutics, a California company dedicated to the development of innovative cancer immunotherapy. She received her degree in medicine from Beijing University College of Medicine and a Ph.D. in Comparative Biochemistry from the University of California, Berkeley. She is coinventor of multiple issued US and international patents, including the technology on antibody ADCC enhancement. She has made major contributions in the discovery of human antibody against WT-1, an intracellular target for cancer immunotherapy, which resulted in half a dozen peer-reviewed scientific publications in the field of therapeutic antibody discovery and engineering.

Jianguo Yang holds a Ph.D. in Biotechnology from the Illinois Institute of Technology. He is an advisor and reviewer for *BioProcess International Journal* and executive director of the Sino-American Pharmaceutical Professionals Association, New England (SAPA-NE), and has several patents and numerous scientific papers.

Yang has over 20 years of experience in cell line and cell culture development and has worked at a number of global 500 pharmaceutical companies in the United States, including positions as principal scientist and senior manager at Genzyme/Sanofi, a group leader scientist at MedImmune/AstraZeneca, and as a biochemist at Abbott Laboratories. In addition he held an academic research position, working in the College of Pharmacy at the University of Illinois. He was vice president and chief scientific officer responsible for R&D in Qilu Pharmaceutical, China, and currently, he is CEO of Abpro China.

Dr. Junming Yie is a molecular biologist working in the field of metabolic diseases and drug discovery. Currently at Amgen, he has over 14-year biotech/pharmaceutical industrial experience at Amgen, Inc. and Pfizer, Inc., building groups and directing R&D projects including delivering one monoclonal

antibody, three small-molecule candidates, and three recombinant protein candidates into clinical development. Trained as biochemist and molecular and cell biologist, he earned his Ph.D. with distinction in Biochemistry and Molecular Biophysics in Columbia University College of Physicians and Surgeons in New York and obtained his postdoctoral training with Dr. Bruce Spiegelman at Dana-Farber Cancer Institute and Harvard Medical School. He also holds an MBA degree from UCLA Anderson School of Management. He has published a number of high impact scientific papers in *Nature*, *Cell*, *Molecular Cell*, *PNAS*, *EMBO J*, Molecular and Cellular Biology and other journals.

Dr. Ziyang Zhong is vice president of Henlius Biotech Co., Ltd., a start-up biotechnology company located in Taiwan, with the mission to develop novel antibody therapeutics. He has more than 20 years of experiences in preclinical research and process development. Prior to joining Henlius, he worked for several start-up/medium-size biotech companies, including Scios (acquired by Johnson and Johnson), Chiron (acquired by Novartis), Abgenix, and Kosan Biosciences (acquired by BMS). He has expertise downstream purification, analytics, and bioanalytics. He earned a Ph.D. degree from Texas A&M University and a master's degree and a bachelor's degree from Sun Yat-sen University (Guangzhou, China).

Yu Zhou Department of Anesthesia and Perioperative Care, University of California San Francisco, San Francisco General Hospital, 1001 Potrero Ave., San Francisco, CA 94110, USA.

Dr. Yu (Eunice) Zhou received her B.A. and Ph.D. Molecular Biology and Biochemistry from Beijing University and is currently is an associate adjunct professor in the Department of Anesthesia and Perioperative Care, University of California, San Francisco. She has developed cancer cell internalizing antibodies and researched novel ways to identify and engineer human monoclonal antibodies to target breast cancer cells, especially cases with poor prognosis. She has also published a report of a novel method to screen phage display antibodies using both cancer cells and yeast-displayed antigens, making it possible to generate disease-associated monoclonal antibodies to virtually any antigen. Her research interests include engineered antibodies as targeted drug delivery system for cancer therapeutics and diagnosis.

Preface

With the expiration of patent protection for a range of antibody therapeutics, there is a rising expectation within the global biopharmaceutical community concerning the construction of knock-offs, so-called biosimilar antibodies, that has huge ramifications to the multibillion-dollar industry and the health of millions of patients.

Biologic agents are increasing economic demand on healthcare systems worldwide, and it is widely recognized that they represent an opportunity to increase access and reduce costs for patients and healthcare systems. A systematic approach to the characterization of the structural and functional similarities of a biosimilar molecule to the originator molecule provides specifications to guide the development of biosimilars. Identical sequence and similar host cell lines are key to development of biosimilars. A well-designed development program establishes biosimilarity based on structural, functional, preclinical, and clinical evaluation. Biosimilar endpoints are defined for each tier of characterization, and evaluation of successive tiers addresses additional relevance of biosimilarity.

Biosimilars are considered to be one of the fastest-growing sectors in the pharmaceutical industry, accelerated by the expiry of patents on multiple brands of biologics. Biosimilars in development include a large number of candidates from established and many new entrants to the biopharmaceutical industry. Nearly 20 biosimilars are already marketed in the EU and some other major market countries having implemented biosimilar approval pathways. A database tracking the biosimilars/biobetters pipeline reports nearly 700 biosimilars and 500 biobetters in development worldwide, with the great majority targeted to enter the US and other major markets where patents and other granted exclusivities are expiring. Many companies view biosimilars (and to a lesser extent, biobetters) as a more affordable, lower-risk way to enter the lucrative US and EU markets and gain credibility as a biopharmaceutical developer. With only comparative testing and trials required for biosimilar approval, this mechanism enables market entry at a cheaper and faster pace, when compared with the pursuit of innovative products receiving traditional full approvals.

Risks are also much lower, generally involving much the same active agent that has been on the market for more than two decades.

Monoclonal antibody biosimilars are expected to dominate the biosimilar field, even though it is a newcomer into the market. The value of the 10 top selling products, the majority of them antibodies and antibody-like molecules, had reached more than $50 billion in 2009, and the value of new antibodies and biosimilars is estimated to rise 7–15% per year. In the last three decades, protein yields from recombinant cell lines in bioreactors have increased 10- to 100-fold, as the result of improvements in media, bioprocess design, and cell culture process control. At the same time, the largest vessels in use for CHO cells have a working volume of about 20,000 l. This improvement of yield efficiency and production scale has lowered the production cost of making biosimilar antibodies. The strong "disposable" trend has emerged, which uses "single-use bioreactors" (SUB) and presterilized plastic bags, and dramatically reduces the capital investment in manufacturing "biosimilar" products. The huge market potential, reduced production cost, and lowering of capital investment in manufacturing facility have made the field of antibody biosimilars an attractive business.

However, there are multiple challenges facing the burgeoning monoclonal antibody biosimilars industry. As detailed by the authors in this book, the complexities and methods of manufacture create an important difference between biosimilars and conventional generic small-molecule drugs: while chemical generics can be fully characterized as identical to the originator product, biosimilars cannot. Full guaranty of similarity is only granted after equivalence parallel trials assessing PK, efficacy, safety, and immunogenicity comparing the biosimilar candidate and the originator. As the first European Medicines Agency (EMA) approval monoclonal biosimilar (infliximab) shows, biosimilars are licensed through a comparability exercise with the reference product and clinical studies to ensure equivalence of efficacy and safety profiles. Guidelines produced by the EMA detail manufacturing process requirements and the range of protein structure, isoform, aggregate, receptor binding, and biological activity assays necessary to demonstrate biological equivalence. It also outlines the required clinical and nonclinical pharmacokinetic (PK), pharmacodynamic (PD), and pharmacotoxicological evaluations necessary to assess safety and efficacy before approval. In the United States, the FDA released draft guidance for the regulatory review of biosimilars in early 2012, which is a significant step toward the first approved monoclonal antibody biosimilar in the US market.

This volume is comprised of a series of chapters dealing with this dynamic and diverse topic. It summarizes work in a number of aspects of the field to bring together the present-day state of the art. Here we review the major points laid out by the authors of this work.

Dr. Regis Sodoyer provides us with an introductory chapter describing the history of therapeutic monoclonal antibody technology. As he explains,

although the power of the immune system to protect the body from disease has long been recognized, murine hybridoma fusion technology, developed in the early 1970s of the last century, has dominated drug development since its inception. Easy access to the production and engineering of murine monoclonal antibodies drove a revolution of drug discovery. The first two decades between 1975 and 1995 were marked by many challenges and the development of the field of molecular engineering. At this critical time, antibodies, still omnipresent in both diagnostic and research domains, have come to dominate the field of immunotherapy.

New technologies, such as phage display, humanized transgenic mice, and repertoire mining, have been perfected and standardized, allowing for the isolation of fully human antibodies. The natural complexity of the antibody molecules and the rapid implementation of engineering methodologies helped make them preferred candidates for the solution of complex immunotherapeutic challenges. Sodoyer updates the different antibody-derived molecules as well as a survey of the latest antibody engineering technologies. In addition, the chapter reviews the critical issue of the development of expression systems suitable for large-scale and cost-effective production of recombinant antibodies.

In Chapter 2, Dr. Zhinan Xia describes the structure, classification, and naming of therapeutic antibodies. He explains the evolution of antibody discovery, moving after the first wave of innovation based on mouse mAbs to the recombinant DNA technology providing the tools for chimeric, humanized, and fully human mAbs as well as recombinant antibody fragments and bispecific therapeutic mAbs. Over the decades, therapeutic mAbs have evolved as innovative pharmaceutical compounds, not only for the treatment of cancer but also of autoimmune and infectious diseases. A new generation of antibodies including anti-PCSK9 mAbs, now in clinical development by Amgen, Pfizer, and Regeneron (in partnership with Sanofi), respectively, may represent a class of blockbuster drugs aimed at cardiovascular disease. We are now entering a third wave of scientific advancement based on antibody architecture modification and engineering, taking advantage of new insights into Fc effector function, glycoengineering, and antibody-drug conjugation.

Among the new wave of antibody technology platforms, antibody-drug conjugates are the most prominent, with their main focus on oncology indications. Because they can offer targeted delivery of chemotherapeutic or radioactive agents, they will increase the performance of mAbs and offer a toolkit for product differentiation and life cycle management.

In Chapter 3, Dr. Yu Zhou and Dr. James Marks cover the mechanisms of action of therapeutic antibodies. Elaborating on the development of multiple antibody-based drugs directed at a single target, in the case of the autoimmune disease targeting TNFa, the differences in Ab–Ag binding properties, the ability to induce effector functions, and other factors resulted in three full-length IgG mAbs, one TNFR–Fc fusion protein, and one pegylated Fab. Meanwhile, a

better understanding of antibody mechanism of action has led to the expansion of antibody drugs with increased potency and greater precision.

Therapeutic monoclonal antibodies and their targets are the topic of the fourth chapter by Dr. Jose Figueroa and his colleagues. These investigators state that neoplastic diseases are currently treated by a variety of mAbs that use diversified mechanisms against cancer-specific targets, including natural cytotoxic mechanisms as well as target-neutralizing functions, in order to produce clinical activity against various cancers. Targets presently used in mAb treatments include CD20 for patients with chronic lymphocytic leukemia and non-Hodgkin's lymphoma; CD30 for HL and ALCL; CTLA-4 and PD-1 for melanoma; HER-2 for HER-expressing breast cancer and gastroesophageal cancer; EGFR-1 for CC and squamous cell carcinoma; VEGF for CC, glioblastoma, non-small cell lung cancer, ovarian cancer, and metastatic renal cell carcinoma; and VEGFR-2 for GE cancer, gastric cancer, and NSCLC. While no specific cancer-related antigens have yet been identified, novel tumor targets, for instance, *c*ancer/*t*estis *a*ntigens (CTAs) such as SP-17, are highly restricted to cancer cells with normal expression limited to testis and placenta. The authors predict a bright future for antibody development and application. They assert that the use of new protein expression platforms and glycoengineering techniques, the design of multispecific binding domains, heterologous fusion constructs, molecular safety switches, and effective linkers will result in an explosion of more effective products with fewer and less serious side effects.

Dr. Roy Jefferis authored the fifth chapter on the topic of antibody posttranslational modifications. He explains in detail that the clinical efficacy of recombinant antibody therapeutics has resulted in their worldwide adoption as drugs of choice, for example, in oncology and inflammation. Antibodies are often referred to as "adapter molecules" as they form a bridge between a specific target and the activation of downstream molecular and cellular effector functions. Immune complexes of each of the four human IgG subclasses may bind and activate multiple host ligands with varying downstream outcomes. Recognition and activation of host ligands are dependent on interactions with the IgG-Fc region and can vary depending on the precise structure of the naturally occurring isoforms, for example, subclasses and glycoforms. Antibody therapeutics must therefore be fully characterized, structurally and functionally, as must a candidate biosimilar. Jefferis enumerates and discusses the multiple posttranslational and chemical heterogeneities that may arise within recombinant antibodies and possible consequences for mechanisms of action *in vivo*.

In Chapter 6, Drs. Ningning Xu, Meimei Liu, and Margaret Liu present the pharmacology, pharmacokinetic, and pharmacodynamic aspects of recombinant antibodies. The authors point out that pharmacological studies of anticancer mAbs focus on the interaction between a patient population and the biopharmaceutical agent. Specifically, these investigations describe the interaction between specific mAbs and deranged cancer cells as opposed to those dynamics

involving normal cells. In sum, the branch of pharmacological antibody studies deals with the uses, effects, and modes of action of mAbs on the body.

Pharmacokinetics (PK) on the other hand is concerned with the biological processes that a drug undergoes in the intact organism, that is, what the body does to a drug.

The third branch of pharmacological studies is referred to as pharmacodynamics. It focuses on the therapeutic action of a drug on an organism, that is, pharmacologic response, as well as the duration and the magnitude of the response associated with the concentration of drug at an effectual site of the organism. The authors lay down a set of general principles describing *in vivo* antibody behavior to serve as a guide for initial drug development leading to future clinical applications.

In Chapter 7, Jeanne Robson outlines the clinical performance of currently approved anticancer antibodies, considering their developmental history, target biology, mechanisms of action, clinical efficacy, and FDA-approved indications.

Dr. Reena Nair discusses the development of biosimilar rituximab and her institution's clinical experience with their biosimilar in Chapter 8. Rituximab was the first monoclonal antibody approved for cancer treatment. Since its launch in 1997, it has been at the forefront of treatment for B-cell non-Hodgkin's lymphoma. Reditux™ (Dr. Reddy's Laboratories Ltd.) is the first in a series of biosimilar products that dramatically expand the affordability of antibody therapy for patients in India and beyond. Regulatory requirements for biosimilars are evolving. Education of physicians and healthcare providers, patients, and payers about biosimilars will assist in informed decision making and promote acceptance of biosimilars into clinical practice. Scientific bodies should formulate practice guidelines and position statements to establish biosimilarity in efficacy, safety, comparability, and interchangeability with the reference originator biologic molecule.

In Chapter 9, Dr. S.J. Projans discusses monoclonal antibodies for infectious and cardiovascular diseases. The advent of the "age of antibiotics" in the 1940s had a major impact on extending lives, but it also brought with it a false sense of security. The overenthusiastic use of antibiotics, sometimes as a substitute for good sanitation and hygiene, has resulted in unintended consequences. We now know that drug resistance developed by these life-threatening bacteria has become a big problem for hospitals treating infectious disease. Finding new ways to prevent and treat infections caused by resistant organisms will be a major challenge in the twenty-first century. Identifying "at-risk" patients (e.g., those colonized with *Staphylococcus aureus*) and taking preemptive measures (e.g., decolonization) or perhaps developing a protective vaccine may well obviate the use of the antibiotics that have made the practice of medicine of the late twentieth and early twenty-first centuries possible. A new term of art has been coined "precision medicine," and we now have the ability to identify an

infecting pathogen within 2 h. This will allow for the use of narrow-spectrum agents that, in and of themselves, cannot obviate either toxicity or the development of resistance but may well prevent cross-resistance to current antibiotics as well as ameliorate, if not prevent, horizontal gene transfer. In addition to the continued hunt for effective vaccines, currently there are numerous monoclonal antibodies in clinical development to prevent and/or treat infections caused by *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Clostridium difficile*, and others. To date, these monoclonal antibodies, mainly of human origin or design, have had an enviable safety record, and these may represent the future of antibacterial treatments.

In Chapter 10, Drs. Yie and Wu discuss monoclonal antibodies directed against markers for musculoskeletal, central nervous system, and other diseases. There are seven approved monoclonal antibodies available for the treatment of various nonmalignant conditions. They are natalizumab for autoimmune disease such as multiple sclerosis and Crohn's disease, eculizumab for rare diseases associated with the dysfunction of the complement system, ranibizumab for macular diseases, denosumab for bone diseases, and daclizumab, basiliximab, and muromonab-CD3 as immunosuppressive agents for solid organ transplantations. The approval of these seven monoclonal antibodies spanned more than two decades, reflecting the evolution of the investigative process, from murine to human/murine chimeric antibody to humanized antibody and finally a fully human antibody. In this chapter, drug development history, target biology, mechanism of action, clinical efficacy, and approved indications of each monoclonal antibody are covered thoroughly. Appropriate tables, factsheets, and sequences of the relevant biologicals round out the discussion.

In Chapter 11, the manufacture of recombinant therapeutic proteins using Chinese hamster ovary (CHO) cells in large-scale bioreactors is presented in detail by Drs. Wűrm and De Jesus. Immortalized mammalian cells in suspension culture have been used in large-scale bioreactors for the production of recombinant protein therapeutics since the mid-1980s. All recombinant antibody products with greater than $1 billion US sales/year on the market today are produced in CHO cells. Clearly they have achieved "superstar" status since more than 50% of all protein pharmaceuticals on the market are produced now in these cells. Eight of the ten top selling biologicals in the market today are derived from CHO cells. When considering products made in animal cells only, CHO cells are probably used in more than 90% of cases. Other immortalized cell lines, such as NS0, BHK, or HEK-293 cells, are used for a few individual products, but they play an insignificant role in the overall sales picture. All processes here considered involve "stable" cell lines, that is, cells that were engineered by inserting the desired gene(s) of interest into the genome (chromosomes) of the host cells.

The contribution of expression vector constructs and host cell engineering is, contrary to what numerous publications and even recent reviews are claiming, relatively minor. The reason for this surprising and counterintuitive

statement is clear. While the academic world has been very active in solving problems in CHO technology by addressing them through cell engineering, the industry was bound by the conservative approaches necessary in pharmaceutical manufacturing and by the benefit of "sticking to approaches that worked." Significant investments (cell banking, testing, established operational handlings) into a relatively solid approach with a proven track record from a nonmodified cell host could not easily be abandoned just because an improving cell cycle or antiapoptotic gene might eventually provide some (minor) benefit. In fact, the backlog of knowledge concerning a given cell host provided ample opportunity to reap the benefits from modifications of media and process, with resultant dramatic improvements.

In general, the same vector components are used today as 20 years ago. Codon optimization of gene sequences coding for the desired protein of interest have improved overall expression of some proteins, but did not result in "breakthrough" yield increases. The identification and selection of the suitable clonal populations from transfected cells are facilitated by the widely accessible and cost-efficient equipment that allows high-throughput screening, including the use of flow cytometry and sorting. Some DNA elements in plasmids and novel vector/gene-transfer approaches have pushed primary expression higher. Currently marketed protein therapeutics are exclusively produced in large-volume steam-sterilizable, stainless steel bioreactors, and the majority of production processes therein can be characterized as "fed-batch" cultures, with processing times of 10–20 days. However, over the last 10 years, a strong "disposable" trend has emerged, particularly not only with innovative products that are entering the clinic but also with "biosimilar" products under development. "Single-use bioreactors" (SUB) and presterilized plastic bags, mounted into or onto containers or platforms, serve as a physical barrier between the nonsterile environment and cell culture process liquids. While stirring with marine and pitched blade impellers is still the main method for mixing of both sterilizable stainless steel and single-use bioreactors, other impeller-free approaches have emerged as well that are now applied in single-use bioreactors.

In Chapter 12, Stimple and Wood consider process development at the downstream end. They summarize several advances in antibody purification at industrial scale, with an emphasis on alternatives to conventional protein A affinity batch processing. At the molecular level, these alternatives include the use of newly developed affinity ligands, as well as reemergence of conventional chromatographic processes. The chapter also covers several novel process configurations, including the use of disposable technologies and the rapidly increasing focus on continuous processing. The general trend of these advances is toward highly flexible manufacturing environment, which will provide rapid process development as well as production on demand of antibody therapeutics in multiproduct facilities. The authors cover each of these developments, with the relative strengths, weaknesses, and potential impacts evaluated.

The impact of biosimilars and biobetters on biopharmaceutical manufacturing and contract manufacturing organizations is covered in Chapter 13 by Ronald A. Rader. He discusses the fact that biosimilars are subjected to an abbreviated but rigorous approval process involving direct comparisons of the biosimilar to its reference product, including analytical profiles and head-to-head comparative pharmacokinetics and clinical trials. Biosimilars will raise the bar for product quality expectations for all biopharmaceuticals, with regulators, healthcare professionals, patients, and the public focusing on the details of manufacturing processes and the analytical, bioprocessing, and related quality differences between products.

Dr. Rader believes that by the end of the decade, we can anticipate 10–12 entries for every successful reference product. While this may seem a chaotically competitive market, there already are many or more sources for some major selling generic drugs. The United States is projected to be the dominant biosimilars market. Many new and established companies will be moving aggressively into this market, even if this results in overcrowding and a market so fractured that it becomes impossible to realize desired profit margins. The biosimilars, biobetters, and biogenerics market will be much like the generic drug market. This includes active pharmaceutical ingredient sources and competing products originating from a range of international companies. As international competition and the available number of biosimilars and other follow-ons increase, buyers will favor the US and EU manufactured products. As a result, unlike the generic sector, development and manufacturing will be located in the industrialized countries.

Dr. Jianguo Yang's Chapter 14 covers cell line production and cell culture development for biosimilar antibody-drug manufacturing. Almost all major pharma companies are heavily committed to biosimilar product development, resulting in many active players within the industry. Likely, pharma companies with well-integrated drug R&D divisions and robust clinical trial and sales networks will come to dominate the field. Due to a wealth of current biosimilar antibody-drug candidates in various development stages, biopharmaceutical companies are increasingly interested in a platform process for mammalian cell line and cell culture development to meet productivity and quality attributes with maximum efficiency.

Cell line and cell culture processing are the most critical steps for biosimilar antibody development since both can determine productivity, quality, and cost/benefits, which are of vital importance to the bottom line. For the last several years, average productivity of mammalian cell lines has reached 3–6 g/l. These gains were achieved using fed-batch process, high-productivity cell lines, and optimized media and bioreactor platforms. These innovations are dramatically cutting costs and speeding drug development. Meanwhile, product quality is the key factor for success as many companies compete against one another in a cutthroat landscape.

Drs. Weidong Jiang, Scott Liu, and Ziyang Zhong review in Chapter 15 the analytical and bioassay methods used to characterize the finished antibody products based on predetermined specifications for biosimilar antibodies. For their identification, it is typical to have the primary sequence confirmed to the reference standard using the LC/M method. There are many options available on the market for this purpose, including quadrupole time of flight (QTOF) and Orbitrap. Both Waters and Agilent make excellent TOF instruments, while Thermo/Finnigan is the undisrupted leader in the Orbitrap technology sphere. All of these instruments offer techniques to provide both primary sequences, as well as information concerning disulfite bonds. Their purity and integrity, on the other hand, are demonstrated by conventional chromatography, such as SDS-PAGE and IEF. Similarly, HILIC is the standard method to characterize the glycan profile, which plays a key role in ADCC.

In Chapter 16, Rafiq Islam reviews the development of bioanalytical tests of biosimilar antibodies, including glycosylation pattern, ADCC/CDC activity, and FcγR binding profile. Critical features of such characterizations are the test results required to demonstrate similarity to the original product, as stated in the regulatory guidelines issued by the FDA and EMEA.

Drs. João Fonseca and João Gonçalves cover preclinical and clinical development of biosimilar antibodies in Chapter 17. They touch on a recurrent theme in this book: that biosimilars offer a highly attractive strategy for reducing medical costs and increasing accessibility to targeted biologic therapies. However, unlike small molecules, the development and production of candidate original biologics and biosimilars can be hampered by unpredictable variability that should be tackled as far as possible during the preclinical development process. Biological therapies are inherently variable, creating unavoidable differences between even subsequent batches of the same product.

Clarinda Islam covers regulatory issues in Chapter 18 providing an overview of the regulatory process with a focus on biosimilar antibody-based therapeutics. Of major concern are the challenges faced by regulatory authorities globally and at the national level as they seek to evaluate biosimilar products for safety and potency. Some of the issues include the question of how similar is similar enough and what are the implications of the observed differences for antibody performance and patient safety. This chapter will also address limitations of the regulatory process and the patients' responsibility to report safety concerns and symptoms.

Legal considerations concerning biosimilar antibodies are subjected to detailed analysis in Chapter 19 by K. Lance Anderson, Jennifer R. Moore, and Jonathan Ball. Follow-on biologics, also known as biosimilars, face unique statutory and regulatory frameworks, the understanding of which is essential for successful commercialization. Modeled after existing schemes for generic drugs, such as the Hatch–Waxman Act, biosimilars are afforded the opportunity for special treatment due to their similarity to an existing innovator

product. However, due to the variable nature of a biosimilar product, whether from its origin, impurities, or method of manufacture, this process has become codified in its own legislation designed to address the additional inherent product variation. The Biologics Price Competition and Innovation Act of 2009 (BPCIA) was signed into law by President Barack Obama on March 23, 2010, as a subchapter of the Patient Protection and Affordable Care Act. The objective of the legislation was to amend the Public Health Service Act (PHS Act) to provide the FDA a framework for approving follow-on biologics. Other regulatory authorities such as the European Medicines Agency (EMA) have developed similar guidelines, although such statutory frameworks do vary.

In essence, the BPCIA provides an abbreviated approval, or licensure, pathway for biological products shown to be biosimilar to, or interchangeable with, an FDA-approved regulatory product. The FDA-licensed biological product, or reference product, may be relied upon so long as similarities can be shown between the biosimilar product and the reference product. In doing so, an abbreviated regulatory pathway is available for the biosimilar product.

In focusing on the BPCIA as it amends the PHS Act, and related FDA guidances issued periodically with regard to the applicable legislation, the basic framework of the licensure process will be appreciated in light of the reference products to which they pertain. Additionally, certain product exclusivity periods may be obtained, or must be permitted, within the biologics licensure process. As discussed further, these aspects will continue to remain of importance in coming years due to known expirations of biological products approved under Section 351(a) of the PHS Act. The exclusivities afforded may vary due to the nature of the biologic products and the sponsors involved. Further, the consideration of patent analysis and procedures critical to the BPCIA process will be discussed, along with recent guidance documents issued by the FDA, submissions of biosimilar applications, and early court cases regarding such submissions.

In Chapter 20, Dr. Cheng Liu, editor of this volume, considers antibody-dependent cell cytotoxicity enhancement technologies for next-generation therapeutic antibodies. ADCC enhancement is a key strategy for improving therapeutic antibody-drug efficacy. Recent clinical studies provided further evidence in support of the technology. It has the potential of lowering effective drug dosage, hereby benefiting patients through lower drug cost. Antibodies with ADCC enhancement are expected to eliminate variations in patient response to antibody treatments caused by genetic polymorphism and improve survival of cancer patients. The commercial value of the technology has been demonstrated by the licensing agreement between Amgen and Kyowa Hakko Kirin on an anti-CCR4 antibody, which includes $100 million up-front fee plus $420 million milestone payment and double-digit royalties. The FDA approval of obinutuzumab and its superior efficacy compared with the first-generation anti-CD20 antibody, rituximab, in leukemia was a milestone success of ADCC

enhancement technology in new mAb drug discovery and development. Meanwhile, ADCC enhancement is also becoming a core technology for developing next-generation therapeutic antibody drugs with favorable clinical outcomes.

Dr. K. John Morrow, Jr., coeditor of this book, is the author of Chapter 21 which considers technologies for engineering improved antibody half-life. Biosimilar antibodies include a subcategory of "biobetters," that is, antibodies that actually represent an improvement over the original product. The loss of patent protection and technological improvements are predicted to generate a wave of biosimilar products in the coming years that will force deep inroads into the market share of the original products. The competition brought about through the introduction of biosimilars may cut costs of antibody-based therapeutics by 50% or more.

Because the IgG framework is able to sustain great variability, it lends itself to a range of engineering possibilities. Advances in cloning and engineering technologies accomplished in recent years have opened the door to improving antibody performance, in particular increasing the half-life of therapeutic mAbs. Fundamental investigations of the mechanism of half-life extension have shown that the complexing of the Fc region of the antibody with the FcRn protein is critical to the prolongation of serum half-life. This receptor protects the IgG molecule from cellular catabolism by way of a pH-dependent recycling and transcytosis pathway.

Antibody half-life can be extended by increasing the affinity of the antibody for the FcRn molecule. Extensive molecular mapping studies have revealed the mechanism of binding of the two molecules and the best mutational substitutions to the Fc region to optimize affinity. Alternatively, in some cases it may be desirable to interfere with the binding in order to shorten the half-life of the antibody. This is the strategy that is being applied in therapeutic application to various autoimmune diseases.

The FcRn molecule complexes with immunoglobulin and also with a totally unrelated molecule, albumin. This property allows for the development of stable albumin fusion molecules with notable therapeutic applications. As new biosimilars are developed, some preclinical and clinical evaluation will be required in order to establish their safety and efficacy. Since preclinical studies involve the use of mice, the recent controversy concerning the accuracy of the mouse as a model for human disease conditions should be mentioned. While there is reason for concern, the weight of the evidence indicates that the mouse is an accurate model of the human immune system, with certain caveats.

One of the most promising approaches to protecting the half-life of engineered antibodies is the use of nanotechnological devices. In its most fundamental state, the design concept is a drug-containing core in a soft sheath covered with polyethylene glycol, which attracts water molecules surrounding the nanoparticle with a liquid halo. Currently there are a number of nanotech

applications that have received FDA approval, although none involving an antibody therapeutic.

Despite the problems associated with the development of biosimilars and biobetters, the future of the industry appears bright. There is an extremely robust market for biologics, now 27% of pharmaceutical sales in Europe. In the United States alone, there are 907 biologics in development, targeting more than 100 diseases. Extension of the half-life of these molecules could constitute a direct and rapid approach to improving their performance, so it is certain that this will be a major part of an R&D biobetters program.

In the final Chapter 22, Patrick G. Holder and David Rabuka discuss technologies for antibody-drug conjugation. As described in the preceding chapters, therapeutic mAbs leverage their specificity to induce cellular cytotoxicity through a variety of mechanisms. Small-molecule chemical pharmacophores can also induce cytotoxicity. An antibody-drug conjugate (ADC) is prepared by chemically joining therapeutic mAbs with these potent chemical cytotoxins. *In vivo*, a circulating ADC binds its target antigen on the cell surface and is internalized, carrying with it the chemical pharmacaphore. This "payload" can then be delivered through a variety of release mechanisms and potentiate cell death. The combination of a mAb that targets a carefully chosen antigen (Chapter 4) with a highly potent toxin can produce ADCs that significantly increase the therapeutic index of targeted treatments.

A "technology" for antibody-drug conjugation describes any of the modular components that are chemically assembled in order to produce a functional ADC. These include the location on the antibody to which drug is attached, the chemical reaction used for conjugation, the components of the linker joining mAb and cytotoxin, and the chemical functional groups that can trigger release of the cytotoxin. The exact choice of cytotoxin will depend upon the target cell and its sensitivity to a specific mode of action.

Researchers have been designing and building ADCs for over 50 years. As the construction of ADCs has changed, so have the underlying technologies. Pertinent reviews of ADC technologies have been prepared along the way; this chapter assembles current knowledge and critiques best practices.

Cheng Liu, Ph.D., and K. John Morrow, Jr. Ph.D.

1

The History of Therapeutic Monoclonal Antibodies

Regis Sodoyer

Expression Systems and Protein Engineering, Technology Research Institute Bioaster, Lyon, France

1.1 Summary

Antibodies, a main component of the immune response, have been recognized, more than a century ago, for their proven therapeutic value. The hybridoma fusion technology, proposed in the early 1970s, for the first time gave easy access to the production and engineering of murine monoclonal antibodies. The potential of these new molecules, as laboratory tools, was largely exploited during the two following decades. At present antibodies, still omnipresent in both diagnostic and research domains, have progressively come to dominate the field of immunotherapy. New technologies, such as phage display, humanized transgenic mice, and repertoire mining, have been proposed, allowing for the isolation of fully human antibodies. The natural complexity of the antibody molecules and the rapid implementation of engineering methodologies helped in making them ideal candidates for new applications and for the solution of complex immunotherapeutic challenges. The first chapter is a current update on the different antibody-derived molecules as well as a survey of the latest antibody engineering technologies. In addition the chapter reviews the critical issue of the development of expression systems suitable for large-scale and cost-effective production of recombinant antibodies.

1.2 Introduction

The historical roots of immunotherapy trace their origins to the end of the nineteenth century. In collaboration with Shibasaburō Kitasato, the first bacteriologist to succeed in cultivation of *Clostridium tetani*, Emil Adolf von Behring demonstrated the efficacy of "so-called" antitoxins to protect animals against

Biosimilars of Monoclonal Antibodies: A Practical Guide to Manufacturing, Preclinical, and Clinical Development, First Edition. Edited by Cheng Liu and K. John Morrow, Jr.

tetanus and diphtheria [1, 2]. Although not immediately recognized, the discovery was adapted to the treatment of diphtheria-stricken children and was found to significantly reduce mortality [3]. For some years serum therapy was widely adopted before being gradually replaced by active immunization. Several decades passed before the composition, physiological behavior, and chemical nature of the antitoxin components were thoroughly characterized.

These components, referred to as gamma globulins according to their electrophoretic mobility and as immunoglobulins and antibodies according to their immunological function, were at the center of multiple investigations involving a steadily increasing number of renowned scientific teams. From the early 1950s until now, the definition of antibody (Ab) became more precise, as well as the genetic aspects behind the creation of their molecular diversity. In parallel, advances in cellular biology were under way around the world, allowing a better understanding of the precise role of B cells in the immune response. Paving the way toward the advent of modern immunotherapy, hybridoma technology described by Köhler and Milstein in 1975 gave, for the first time, easy access to murine monoclonal antibodies (mAbs) [4]. mAbs were recognized as revolutionary laboratory tools from their inception, although the *in vivo* applications and therapeutic potential of these molecules were still controversial, raising some skepticism in the early 1980s. Nevertheless, significant progress in molecular biology techniques allowed mAbs to move from research to diagnostics and applications in therapy.

More recently, monoclonal and recombinant antibodies have become the focus of new technologies, such as bacteriophage or other *in vitro* display techniques, mice or larger animal transgenesis, and other technologies permitting direct access to fully human antibodies. Importantly, the pressing need for large quantities of mAbs was a major driver for the development and optimization of recombinant protein production systems. The contribution and complementarity of these different approaches will be considered in the context of large-scale industrial production of therapeutic mAbs.

1.3 New Markets for Old Antibodies, Old Markets for New Antibodies

To date, more than 40 mAbs have been approved by the US Food and Drug Administration (USFDA) or European Medicines Agency (EMA) for therapeutic applications: 5 are of murine origin, including 2 bispecific constructs, 8 chimeric, 18 humanized, and 13 fully human. Thanks to the novel technologies available [5], fully human antibodies are rapidly taking over the market, and it seems probable that even humanized molecules will be marginalized in the coming years. The composition of the early-stage antibody pipeline is indicative of this trend. In terms of revenue, substantial returns have been realized

both on existing and new markets. A good illustration is provided by recent data from Lawrence and Lahteenmaki: [6] among the top 10 selling biological drugs of 2014, 5 are mAbs. Humira® (adalimumab, AbbVie Inc., North Chicago, IL, the United States), with applications to several conditions (such as rheumatoid arthritis, juvenile rheumatoid arthritis, Crohn's disease, psoriatic arthritis, psoriasis, ankylosing spondylitis, and ulcerative colitis), is number one, with a total revenue exceeding $10.5 billion. Remicade® (infliximab, Janssen Biotech, Inc., Titusville, NJ, the United States) for multiple indications (rheumatoid arthritis, psoriatic arthritis, ulcerative colitis, Crohn's disease, ankylosing spondylitis, and severe or disabling plaque psoriasis) is in second place with a total revenue over $9.2 billion. Rituxan® (rituximab, Biogen Idec Inc., Cambridge, MA, the United States, and Genentech USA, Inc., San Francisco, CA, the United States) for multiple indications (rheumatoid arthritis, chronic lymphocytic leukemia/small-cell lymphocytic lymphoma, non-Hodgkin's lymphoma, antineutrophil cytoplasmic antibody-associated vasculitis, indolent non-Hodgkin's lymphoma, and diffuse large B-cell lymphoma) is found in the sixth position with a total revenue over $7.5 billion. Avastin® (bevacizumab, Genentech USA, Inc., San Francisco, CA, the United States) and Herceptin® (trastuzumab, Genentech USA, Inc., San Francisco, CA, the United States) are, respectively, ranking seventh and ninth positions with individual revenues fluctuating between $6.8 and $7.0 billion.

Beside the success stories of some blockbusters, mAbs are set to play a role in the rapid control of emergent diseases. A striking example is the 2014 Ebola outbreak, for which more than 20 laboratories and research groups around the world, including those from Canada, Japan, Israel, Uganda, and the United States, are working simultaneously to develop therapeutic mAbs against the virus. The dire state of emergency will no doubt facilitate and shorten the approval process. A mixture of three mAbs known as ZMapp (LeafBio, Inc., San Diego, CA, the United States), never tested in humans, was exceptionally accepted despite the fact that very little is known regarding the safety and effectiveness of this treatment.

1.3.1 Intellectual Property

One possible explanation for the great commercial success of therapeutic antibodies is the proper management of intellectual property and accurate designation of strategies for protecting antibodies or antibody-derived products [7]. This is particularly meaningful if we consider the emblematic and well-known Cabilly patent filed by Genentech, one of the most ubiquitous patents in biotechnology, which covers a fundamental method for the production of therapeutic recombinant mAbs that cannot be ignored by anyone planning to commercialize an antibody [8]. Besides the uniqueness of this example, one should not underestimate the inextricably complex patent situation governing

the recombinant antibody world. As a matter of illustration of this complexity, according to Sandercock and Storz [9], it might be possible to patent the precise definition of an epitope with the possibility to claim later-generation antibodies targeting the same epitope.

1.3.2 Biosimilars

A consequence of the astronomical commercial value of mAbs associated with the patent expiry date (or estimated expiry date) is their attractiveness as candidates for biosimilar drugs [10]. The European Union (EU) has been the first to establish a regulatory framework for marketing authorization application (MAA) and has named "copycat" biotherapeutic products with the term biosimilars, a term also recently adopted by the USFDA [11].

Biosimilars offer a highly attractive mechanism for reducing the cost of medical care and thus should be considered positively [12]. Nevertheless, the abbreviated approval pathway associated with their adoption requires reinforced pharmacovigilance after a biosimilar mAb is approved, in order to ensure its long-term safety and efficacy. Unfortunately, it has been observed that the studies carried out to obtain approval of the reference product are not always adequate to ensure comparability of biosimilar mAbs. A similar efficacy does not necessarily imply a similar safety profile between the innovator and biosimilar products [13]. A major challenge to be addressed is the prediction and assessment of immunogenicity of subsequent entry biologicals (SEBs) and, in particular, biosimilar products [14]. It is nevertheless important to keep in mind that the global cost of manufacturing and licensing a biosimilar product remains high, and the reduction in cost may be more limited than for a nonbiological small-molecule drug and its generic version. Vital et al. provide a good illustration for biosimilar versions of the antibody rituximab [15]. In addition to the industrial challenges of development, testing, and marketing, biosimilars and "biobetters" [16] are raising new analytic challenges concerning product characterization and immunogenicity profiling [17a, 17b].

1.3.3 Modified Antibodies, Nontherapeutic Applications

Recent technological innovations will facilitate access to alternative antibody formats including bispecific antibodies (BsAb), conjugated antibodies, and antibody fragments. The demand for these molecules is expected to rise in significance [18]. mAbs are set to play a significant role in the treatment of a wide number of indications in various therapeutic domains, although oncological therapeutics will continue to dominate the majority of applications [19, 20].

The goal of cancer therapy is to cause the direct or indirect destruction of cancer cells, by specifically targeting the tumor (Rituximab®) or the vasculature that nourishes the tumor (Avastatin®). Numerous anti-inflammatory

antibodies already on the market (Remicade, Humira) have also shown the great potential of mAbs. Asthma, transplant rejection, inflammatory conditions, autoimmune disease, cardiovascular conditions [21], atherosclerosis [22], and infectious disease [23] are new domains of treatment. While treatment of infectious disease will increase in the future, full-blown success will require the development of original screening strategies to obtain broadly neutralizing antibodies [24] and/or the combination of several antibodies targeting different epitopes or stages of the pathogen life cycle [25].

1.4 Antibody Engineering: A New Approach to the Treatment of Disease

In 1975, Köhler and Milstein developed and described but did not patent a revolutionary technique for the generation of mAbs [4], based on the immortalization of mouse B cells through fusion with myeloma tumor cells. Mouse hybridomas, generated upon fusion of the two parental cell partners, became the first reliable source of mAbs, facilitating the laboratory-scale production of murine mAb (Mumab) specific for a given antigen. The first Mumab, as therapeutic agent, was unsatisfactory due to their very short half-life in serum, poor or absent activation of human effector functions, and undesirable stimulation of human anti-mouse antibody (HAMA) responses in patients when repeated administration protocols were applied [26].

These issues were addressed using genetic engineering or chemical coupling techniques but have not been entirely resolved. The different strategies chronologically developed to avoid, mask, or redirect this human immune response are "chimerization" by fusion of mouse variable regions to human constant regions [27], "humanization," [28] and "deimmunization" by removal of *in silico* predicted T-cell epitopes [29] or introducing regulatory epitopes to induce tolerance [30].

1.4.1 Chimerization: The Stone Age of Antibody Engineering

In chimeric antibodies, the murine constant regions are replaced with human equivalent regions, since the constant region significantly contributes to the molecule's immunogenicity. In addition, the presence of human constant domains guarantees a more robust interaction with human effector cells and the complement system. This strategy led to therapeutic successes such as basiliximab (Simulect®: IgG1 anti-CD25, developed by Novartis Pharmaceuticals, East Hanover, NJ, the United States) or cetuximab (Erbitux®: IgG1 anti-EGFR, developed by ImClone, LLC, New York, NY, the United States). Nevertheless, chimeric antibodies, even if perceived as less foreign and therefore less immunogenic than mouse mAbs, have been shown to induce

human anti-chimeric antibody (HACA) responses. This has been seen with infliximab/Remicade [31]. The advent and increasing sophistication of humanization technologies and the direct access to fully human antibodies will likely represent the death knell for chimeric antibodies.

1.4.2 Humanization: Improvement Never Ends

In order to move forward to reduce both HAMA and HACA, antibody humanization technologies were developed and made possible by, at the least, transferring all xenogeneic complementary determining regions (CDRs) onto the framework of a human antibody. Different approaches to CDR grafting have been tested according to the human template used as a matrix: sequences with known crystalline structure [32], rearranged somatic sequences, unmodified germ line sequences [33], or consensus sequences [34].

The first humanized antibodies were constructed based on human sequences with known crystalline structures, which permits the identification of residues contributing to antigen binding. In the "best fit" strategy, the closest human sequence, usually rearranged, is used as a framework to receive the murine CDRs. Another approach for humanizing an antibody is to choose the closest human germ line sequence [33]. Indeed, human antibody genes are formed *in vivo* by rearrangements of germ line gene segments. Later in B-cell ontogeny, the hypermutation process takes place, tailoring the initial sequences to improve recognition of the specific target antigen (Ag). The body thus may be theoretically more tolerant to germ line-encoded Abs. It is likely that there will be a reduction in clinical issues if germ line sequences are used for constructing humanized Abs.

The consensus method utilizes variable light (V_L) and variable heavy (V_H) domain frameworks derived from the most common amino acid found at each position within a given human subgroup. Whatever the method, CDR grafting might not result in the complete retention of antigen-binding properties since some framework residues can directly or indirectly interact with the antigen [35] or may affect the conformation of CDR loops [32c]. In this case, the antibody must be re-engineered or back-engineered to fine-tune the structure of the antigen-binding loops and restore its original high affinity. On the other hand, humanization of a xenogeneic Ab does not necessarily abolish the immunogenicity of the molecule, since the humanized Ab can still induce response against its xenogeneic CDRs. Not all residues within the CDRs of an Ab are essential for binding to its Ag. In fact, the Ag-binding site of an Ab usually involves only 20–33% of the residues [36]. These residues have been designated as specificity-determining residues (SDRs) [37]. Therefore, a murine Ab can be humanized through restrictively grafting only its SDRs onto the human template, minimizing the immunogenicity [38].

Another humanization strategy, termed resurfacing, was proposed by Padlan and involves the replacement of solvent exposed murine framework residues in

the variable regions with human residues [39]. More recently, Hanf et al. [40] have proposed humanization by redesign of CDR residues in close proximity to the acceptor framework, yielding an antibody with better binding capacity than simple CDR graft with reduced immunogenicity compared to framework redesign.

Almagro and Fransson [41] have divided the field of humanization into two main trends: rational methods based on design cycle and precise knowledge of the antibody structure and sequence information and empirical methods using large combinatorial libraries and selecting the desired variants, a so-called "rational" strategy.

In contrast, a prime example of an empirical approach is "guided selection," a process that transfers the specificity of a Mumab to novel human mAb (Humab) by creating a hybrid library of the murine heavy chain and random human light chains, subjecting them to a selection process of binding antibodies and repeating the protocol with the human light chains previously isolated and a library of human heavy chains. Adalimumab (Humira) is the first phage display-derived human antibody and was generated by "guided selection" starting from a Mumab [42].

Humaneering™ is a KaloBios Pharmaceuticals' proprietary method for converting nonhuman antibodies into engineered human antibodies. The resulting antibodies are as close to the human germ line sequences as the products of fully human antibody generation techniques. The authors assert this platform maintains epitope specificity and increases affinity.

Finally, humanization technology has taken advantage of the recent advances in bioinformatics and *in silico* modeling. Zhang et al. [43] proposed a novel antibody humanization method based on this strategy, including an epitope-scanning algorithm designed to identify antigenic residues in the framework regions (FRs) that are mutated to their human counterpart in the humanization process.

The process of humanization is not restricted to murine antibodies and can similarly be applied to antibodies from other species. Nishibori et al. [44] have described a simple method for humanizing chicken mAb by CDR grafting followed by framework fine-tuning using a chicken phage-displayed mAb, phAb4-31, as a model antibody.

Despite the reduction in the proportion of exogenous sequences, the level of immunogenicity of humanized Abs ranges from negligible to highly detrimental. The humanized anti-HER2/neu Ab Herceptin gave as low as 0.1% of human anti-human antibody (HAHA) in breast cancer patients [45], while the humanized A33 Ab elicited 49% of HAHA among colon cancer patients treated with this Ab [46]. In this case, immunogenicity might result from HAHA responses, in particular to the paratope of the antibody (anti-idiotype antibody response) [47].

To date, it is difficult to determine whether "fully human" mAbs are less immunogenic than humanized mAbs as full immunogenicity data are available

only for a limited number of mAbs in each category. The answer is complex and most probably will be resolved through a case-by-case consideration and detailed analysis.

Finally, anti-allotype reactions are predicted to occur during therapy of a genetically diverse population with a single antibody reagent. Moreover, allotypic variation is not equally distributed to all antibody isotypes and might be taken into consideration before designing a therapeutic strategy and effector antibody [48].

1.4.3 Deimmunization

Biovation (the United Kingdom, www.biovation.co.uk) has developed the DeImmunization™ technology consisting of identification and removal of T helper (Th) cell epitopes from antibodies [29]. An example of a product of DeImmunization actually under clinical trials, J591 is a modified antibody binding to prostate-specific membrane antigen (PSMA) [49].

Antitope, a subsidiary of Abzema, is conducting the same kind of approach to limit the development of antidrug antibodies (ADAs), which can reduce efficacy through rapid clearance. The associated EpiScreen™ immunogenicity assessment technology is used to confirm that T-cell epitopes have been removed [50].

The company Epivax is sharing the same field and proposes *in silico* epitope discovery, immunogenicity assessment, and protein deimmunization. They include "Tregitope Analysis" based on the identification within each submitted sequence of putative regulatory T-cell epitopes. These are subregions contained within the submitted sequences, which may relate to natural regulatory T cells and which may help to dampen the immune potential of the submitted antibody sequence [51, 52].

1.5 Fully Human Antibodies, What Else?

mAbs of human origin may have greater therapeutic value; thus several methods have been developed to generate Humab: selection from human hybridomas, selection from "humanized" transgenic mice, construction of *in vitro* combinatorial libraries, or direct cloning from immunized individuals.

1.5.1 Human B-Cell Hybridoma and Immortalized B-Cell Lines

The recovery of stable human B-cell hybridoma producing high-affinity IgG mAbs has rarely been achieved, due to the lack of a suitable human myeloma cell line. The most satisfactory results were obtained using heteromyelomas (mouse–human hybrid myelomas) as fusion partners. However, the mouse–human heteromyelomas that have been used for fusion with human lymphocytes are often

unstable. The situation was ameliorated by the recent development of the novel SPYMEG cell line, a human lymphocyte fusion partner, codeveloped by Naomasa Yamamoto, of Ohu University and MBL. SPYMEG was established by the cell fusion of MEG-01 with a murine myeloma cell line. The SPYMEG cell line overcomes the problem usually encountered by hybridoma cells of human origin that are prone to chromosome deletions.

As an alternative, human antibody-secreting cells (ASCs) can be immortalized by Epstein–Barr virus (EBV) infection. However, EBV-infected cells are somewhat difficult to clone and usually produce only very low yields of immunoglobulin [53]. An improved method of B-cell immortalization by EBV involving the addition of a polyclonal B-cell activator (CpG/TLR-9 agonists) has been described [54, 55]. In contrast to plasma cells, which are terminally differentiated, memory B cells retain substantial growth potential and can be immortalized by EBV [56].

The final disadvantage restricting the human B-cell hybridoma approach (and, more generally, all the technologies giving access to human antibodies) is the fact that the human circulating antibody repertoire does not generally retain specificity to "self" proteins, which represent a significant number of targets for human antibody therapeutics.

1.5.2 *Ex Vivo* Stimulation

A recent technology using a process of *ex vivo* stimulation has been proposed by Duvall et al. [57]. Naive human B cells, isolated from tonsil tissue, are immortalized. The *ex vivo* stimulation of these cells induces class switching and somatic hypermutation. The resultant human library of different IgG antibodies can then be screened against any antigen using a standard limiting dilution protocol. By eliminating immunization and humanization steps, this platform should reduce both cost and time in producing a therapeutic mAb of interest.

1.5.3 Mice and Other Animal Species Producing Human Antibodies

1.5.3.1 SCID and Other Immune-Deficient Mice

A potential source of Humab can be obtained upon transplanting a functional human immune system into immunocompromised mouse strains, such as severe combined immunodeficient (SCID), SCID-bg, Trimera, or $\gamma c^{-/-}$/RAG2$^{-/-}$ mice.

SCID mice, lacking mature T and B cells and virtually devoid of endogenous serum immunoglobulins, can be successfully reconstituted with human peripheral blood lymphocytes (PBLs). Such mice reconstituted with a competent human immune system would represent an invaluable tool for producing human immunoglobulin, after immunization with antigen. However, the use of SCID mouse can be limited by shortened life spans, spontaneous production of functional lymphocytes with aging, and residual innate immunity leading to

variable levels of engraftment. Mouse natural killer (NK) cells in particular would be detrimental to engraftment of human lymphoid cells [58]. For example, the natural depletion of NK activity in SCID-bg mice facilitated engraftment of human PBL from anthrax-vaccinated (AVA) donors. Stable recombinant cell lines producing Humab were generated by hybridoma formation, and human anti-protective antigen (PA) neutralizing mAbs of high affinity were obtained [59]. Other immunodeficient strains of mice were developed, for instance, the $\gamma c^{-/-}$/$RAG2^{-/-}$ mice. In addition to T- and B-lymphocyte deficiency, $\gamma c^{-/-}$/$RAG2^{-/-}$ mice are completely deficient in NK activity [58]. In recent years, the passing fad for such technologies has been completely masked by the gain in maturity of transgenic mice expressing human antibodies.

1.5.3.2 Humanized Transgenic Mice

The availability of transgenic strains of mice expressing human Ig genes (XenoMouse®, HuMab™ Mouse, TransChromo Mouse) provides a breakthrough for isolating Humab [60]. The occurrence of rearrangements and hypermutations confirmed that the endogenous cell signaling machinery of the mouse is compatible with human immunoglobulin sequence elements [61] and would constitute a marked advantage.

XenoMouse strains from Abgenix (Fremont, CA), were engineered by functionally inactivating the murine heavy-chain and k light-chain Ig loci and incorporating megabase-sized inserts of human DNA yeast artificial chromosome (YAC) carrying Ig heavy-chain and k light-chain loci that express the vast majority of the human Ab repertoire [62]. Three different strains of XenoMouse mice have been produced, constrained to class switch from IgM to IgG1, IgG2, or IgG4. The immune repertoire of XenoMouse strains was further increased by the introduction of the entire human Igλ locus [61]. To date, results obtained in preclinical and early clinical trials with human antibody from XenoMouse mice confirm their relative lack of immunogenicity [63]. However, human antibodies from mice can be distinguished from human antibodies produced in human cells by their state of glycosylation, particularly with respect to their Galα1-3Gal residue [64]. This carbohydrate residue is widely distributed among nonprimate mammals. Anti-Gal antibodies are produced in humans throughout life, as approximately 1% of circulating Ig [65]. Thus, antibodies bearing that residue would probably be subject to an accelerated immune clearance [66].

In a parallel strategy, human minichromosomes (derived from human chromosome 2 and 14) containing the complete germ line clusters for heavy and k light chains were introduced into TransChromo mice [67]. These mice, developed by the Kirin Brewery Company (Japan), are capable of producing every subtype of fully human Ig, including IgA and IgM. However, the instability of the transchromosome carrying the Igk locus was particularly detrimental, as hybridoma production was less than 1% of that seen in wild-type mice. An

instant solution to this problem was to crossbreed the Kirin TC mouse carrying the human chromosome fragment 14 (locus IgH), with the Medarex YAC transgenic mouse carrying about 50% of the Igk locus. The resulting mouse (KM) performed as well as normal mice with regard to immune responsiveness [67b].

In 2006, panitumumab, the first fully human antibody generated from transgenic mice, was approved for clinical use by the USFDA. In 2012, seven of such antibodies were approved, which clearly indicates the important contribution of humanized transgenic mice in the pipeline of novel therapeutic mAbs [68].

Over the years, models of transgenic mice have risen in sophistication. According to Murphy et al. [69] from Regeneron Pharmaceuticals, a common weakness of humanized transgenic mice is a lack of fully functional immune systems intrinsically due to the strategy used for their genetic humanization. The VelocImmune mice model efficiently produces human–mouse hybrid antibodies in which the mouse constant regions (which are rapidly convertible to fully human antibodies) are kept intact and have fully functional humoral immune systems. The rationale was that the introduced human variable region gene segments would function indistinguishably in their new genetic location, whereas the retained mouse constant regions would allow for optimal interactions and selection of the resulting antibodies within the mouse environment.

The strategy adopted by Kymab is quite similar; Kymouse™ transgenic mice have been generated upon insertion of the genetic regions corresponding to the variable genes from all three human immunoglobulin loci, including λ genes, into precise locations in the corresponding loci of mouse embryonic stem (ES) cells using a proprietary technology called sequential recombinase-mediated cassette exchange (S-RMCE) [70].

Besides the aforementioned transgenic mice, MeMo® mouse developed by Merus produces, upon immunization, human antibodies composed of a single common light chain and diversified immunoglobulin heavy chains. These antibodies serve as building blocks for the easy and direct generation of BsAb for therapy (Biclonics™). Simultaneous transfection of two cLC V_H genes gives greater than 99% pure BsAb from a single cell and might additionally provide an innovative solution to the complex problem of coexpressing a mixture of several antibodies.

1.5.3.3 Humanized Transgenic Chicken

Transgenic chickens expressing human antibodies are designed to access human targets and epitopes that have been intractable in mammalian hosts because of tolerance to conserved proteins. The major difficulty lays in the mechanism for the generation of diversity, which in the chicken is accomplished through gene conversion, a very different system from that occurring in the human and murine immune response. To resolve this issue it was necessary to confirm that a knockout chicken B-cell line lacking V_H and V_L loci was able to undergo gene conversion with inserted human V_H and V_K

regions. Gene targeting in avian primordial germ cells may provide a unique approach to antibody engineering, particularly in the field of antibody discovery [71, 72].

1.5.3.4 Humanized Transgenic Bovine: Polyclonal Revival?

Antigen-specific human polyclonal antibodies (hpAbs), produced by hyperimmunization, have proven to be useful for treating many human diseases or infections. However, yields from available transgenic mice carrying human immunoglobulin loci are too low for direct therapeutic applications. Kuroiwa et al. [73] have produced transgenic bovine antibodies after transferring a human artificial chromosome bearing the entire unrearranged human immunoglobulin heavy (hIGH) and kappa light (hIGK) chain loci to bovine fibroblasts in which two endogenous bovine IgH chain loci were inactivated. Up to 2 g/l of hIgG, either associated with human kappa light chain or with bovine kappa or lambda light chain (chimeric), can be obtained from these animals. Polyclonal sera from cattle immunized with anthrax PA proved to be highly active in an *in vitro* toxin neutralization assay and protective in an *in vivo* mouse challenge assay. The transgenic bovine platform was recently fine-tuned to further increase the proportion of fully hpAbs produced after triple genetic knockout eliminating the bovine lambda cluster [74]. A first clinical trial for pathogen-specific fully human antibodies derived from Tc bovine (currently in preparation) will assess the value of the approach. The possibility of a yearly production, of approximately 450 l of plasma per transgenic animal, makes this strategy especially appealing.

1.5.4 Antibody Display

A major step forward in the production of fully human antibodies after selection from antibody gene repertoires expressed either *in vivo* on the surface of cells or filamentous bacteriophages [75, 76] or *in vitro* took place during the early nineties. Display technologies made possible the selection of antibodies from large repertoires, and the physical linkage between genotype and phenotype enabled the recovery of the DNA encoding the selected antibody fragment.

1.5.4.1 Phage Display

The bacteriophage platform is still the most widely used and well-established technique for antibody display and library screening [77]. Large repertoires of single-chain Fv fragment (scFv) or antigen-binding fragment (Fab) antibody genes are cloned into phage or phagemid vectors as a fusion product to one of the phage coat protein genes. Expression of the fusion product and its subsequent incorporation into the mature phage coat result in the antibody display on the phage surface. During the screening procedure generally termed

"biopanning," phages that display a relevant antibody will be retained on a given surface coated with antigen, while nonspecific phages will be washed away. Multiple rounds of panning are made possible and necessary to select high-affinity binders.

1.5.4.2 Naive, Immune, and Synthetic Repertoires

The different types of antibody libraries are distinguished by the source of their repertoires: naive, immune, or synthetic. Naive libraries are constructed by cloning the antibody variable domain genes from pools of nonimmunized donors [78]. Since the description of the first antibody libraries, substantial effort has been directed toward the assembly of universal large-sized repertoires. The theoretical justification for large repertoires was the expectation that they would serve to isolate antibodies with nanomolar affinities against any antigen [79].

However, the primary limiting factor is the bacterial transformation step, inherent to the process of library construction. Even if techniques based on the so-called combinatorial infection [80] have provided an elegant mean of breaking this technological barrier, the vast majority of described "naive" repertoires, with a complexity over 10^{10} clones, have been assembled through the massive and tedious accumulation of multiple small-sized subrepertoires [78b].

By definition, the complexity of a given library is the overall number of different $V_H - V_L$ combinations obtained at the DNA level. However, experience has shown a significant difference between the encoded diversity and the displayed diversity for different reasons, including toxicity upon bacterial expression, nonproper folding or assembly, competition between wild-type and protein III molecules linked to antibody fragments, and proteolysis of the displayed moiety.

Lastly, it became obvious that the major issue was not the assembly of a large repertoire but rather its maintenance over time and the prevention of the drift of its content. This reality has necessitated a move to focus on smaller or biased repertoires obtained from immunized subjects [78b]. Nevertheless, some examples of universal large-sized synthetic [79b, 81] and semisynthetic [82] repertoires are available. They are based on the use of a limited number of universal frameworks selected for their capacity to be overexpressed in *Escherichia coli*. According to the technique employed, CDRs are totally synthetic or derived from a pool of naturally expressed ones [83, 84].

1.5.4.3 Display Formats and Optimization

From their inception, display methods have been based on fusion of the C-terminus of the phage λ tail protein pV or both the N- and C-termini of the capsid D protein, which is part of the phage head. Alternative phage display systems have been described using bacteriophages T4 [85] and T7.

Because of its numerous positive features, the filamentous bacteriophage M13 became the platform of choice for antibody display. Although M13 coat protein pIII and pVIII [86] display formats have been the most instrumental in the construction of antibody libraries, other display systems have been reported, such as those based on the minor coat protein pIX [87] or pVI [88]. The value of phage display depends not only on the diversity of the library at the DNA level but also on the efficiency with which the encoded proteins are displayed on the phage surface. Extensive studies of M13 assembly and structure have enabled improvements in phage display technology [89]. Indeed, phage selections often suffer from the amplification of nonspecific binding molecules. In order to alleviate this problem, three groups have developed a system coupling the binding of a displayed peptide or protein to its target with the amplification of the displaying phage: selectively infective phage (SIP) [90], selection and amplification of phage (SAP) [91], and direct interaction rescue (DIRE) [92]. The SIP technology exploits the modular structure of the phage protein pIII, which consists of three domains: N1, N2, and CT. The N-terminal N1 domain is absolutely essential for *E. coli* infection, while the CT domain is absolutely essential for phage morphogenesis [93]. Thus, SIP consists of two components:

1) A phage particle made noninfective by replacing its N-terminal domains of pIII with an antibody fragment.
2) An "adapter" molecule in which the antigen is linked to N-terminal domains. Infectivity is restored when the displayed protein binds to the ligand. Consequently, phage propagation becomes strictly dependent on the protein–ligand interaction [94].

A vector system allowing for the display of bivalent Fabs fused to leucine zippers on phagemid virions has been reported by Lee et al. [95]. The "bivalent display" format is a way to effectively mimic the binding avidity of natural antibodies and greatly reduce the off rate for phage bound to the immobilized antigen.

1.5.4.4 Yeast Display, Bacterial Display, Anchored Periplasmic Expression (APEX)

A complementary approach is based on the display of antibody libraries on the surface of bacteria [96] or yeast cells [97]. The display of Ab on the surface of bacteria is not only an alternative expression system for the screening of binders from libraries but also opens new potential applications, such as the delivery of passive immunity to mucosal body surfaces [98] or whole-cell catalyst [99]. Unlike phage, the relatively large size of bacteria and yeast allows screening by flow cytometry [100].

A protein library-screening technology based on anchored periplasmic expression (APEX) has been developed [101]. In this method, proteins are anchored on

the periplasmic face of the inner membrane of *E. coli*. After disruption of the outer membrane by tris-EDTA-lysozyme, inner membrane-anchored proteins bind fluorescent ligands, allowing screening by flow cytometry.

Baculovirus displaying antibody moieties such as scFvs has been constructed for targeting specific cell types for gene therapy protocols. But at this time the baculovirus system is not considered as an attractive choice for the construction of antibody libraries [102], and this situation will not change in the foreseeable future.

1.5.4.5 *In Vitro* Display Technologies

One limitation of the *in vivo* selection systems is the library size that could be generated and handled. The efficiency of transfer of DNA into cells often limits the library size to 10^9–10^{10} members. In addition, selection in the context of the host environment (*E. coli*) could lead to the loss of potential candidates due to their growth disadvantage or even host toxicity. The most popular *in vitro* display technologies are ribosome display [103] and mRNA display [104] based on an original idea from G. Kawasaki (US patent no. 5,643,768 and 5,658,754). These *in vitro*-based antibody selection methods have proven to be successful in the construction and selection of libraries with a high diversity and complexities (potentially up to 10^{14} members). Inherent characteristics of the *in vitro* systems could obviously be turned into advantages:

- Easily amenable to an automated process.
- The RT-PCR step between screening rounds can be performed according to error-prone conditions, thus generating an amplification of diversity [105].
- An *in vitro* process is more likely to tolerate screening steps under nonphysiological conditions such as elevated temperature or highly denaturant environment [105b].

Other potential *in vitro* systems for antibody display are covalent antibody display (CAD) and polysome display [106]. In the covalent display technology, a protein is fused to P2A, a bacteriophage DNA-nicking protein that covalently binds its own DNA and thereby is subjected to selection regimes similar to those for phage display [107].

The polysome display is a modified ribosome display method, exploiting the interaction between a tandemly fused MS2 coat protein (MSp) dimer and the RNA sequence of the corresponding specific binding motif, C-variant [106].

Finally, among the nonconventional display systems, the strategy proposed by Sepp and Griffiths [108] based on *in vitro* compartmentalization through packaging in microdroplets should be mentioned. Briefly, domain antibodies (dAbs) are *in vitro* expressed in fusion to the N-terminus of single-chain variant of phage P22 Arc repressor DNA-binding domain that links the compartmentally expressed protein molecules to their encoding PCR fragment-based genes via cognate operator sites present on the DNA.

1.5.5 Next-Generation Technologies for a Direct Access to Fully Human Monoclonal Antibodies

1.5.5.1 Single-Cell Isolation

Recent progresses in high-throughput technologies and associated platforms allow one to consider rapid and large-scale automated screening of multiple antibody-expressing cells. In combination with cloning technologies based on single-cell RT-PCR, the direct access to human antibodies is now possible. The isolated antibody sequences are then directly inserted into expression vectors for further investigation.

Trellis Bioscience, employing a 10-day human antibody platform, has screened 10 million B cells from 25 RSV-infected donors with their CellSpot technology. This approach allows the discovery of rare antibodies that neutralized both the A and B subtypes of G protein [109]. More recently, a variation of this approach was described that reveals suites of cross-clade antibodies directed to discontinuous epitopes. It takes advantage of an iterative feedback between antigen probe designs based on structure and function information. Its high-throughput and multiplexed screening method is a generally applicable strategy for efficient identification of safe, native, finely tuned antibodies with the advantage of high genetic barriers to viral escape [110].

Valneva's VIVA|Screen® technology (www.valneva.com) is a microarray-based single-cell screening proprietary technology that allows rapid high-throughput analysis and discovery of fully human therapeutic antibodies obtained directly from human donors. The development of this technology was made possible through the following:

1) Access to a very large population of healthy or diseased human donors
2) The optimization of culture and expansion of human primary memory B cells
3) The use of specifically designed microarray chips that contain 62,500 wells with size and shape optimized for a single human B lymphocyte per well

In a work by Zwick et al. [111], human neutralizing mAbs are generated from vaccinated individuals who received a booster immunization against influenza virus. Blood cells, collected at day seven post immunization, are then specifically sorted by flow cytometry for an early population of ASCs. Neutralizing mAbs against a panel of influenza subtypes were subsequently isolated from single cells and expressed as recombinant proteins.

1.5.5.2 High-Throughput Sequencing and Repertoire Mining

The rise of next-generation sequencing (NGS) technologies allowed Reddy et al. [112] to bypass the tedious screening step inherent in the isolation of antigen-specific mAbs. The strategy employs high-throughput DNA sequencing and bioinformatic analysis to mine antibody variable region (V)-gene

repertoires from bone marrow plasma cells (BMPCs) of immunized mice. V-gene repertoire of BMPCs, indeed, becomes highly polarized after immunization, with the most abundant sequences represented at frequencies high enough to be sorted out of the total repertoire. The most represented variable heavy (V_H) and variable light (V_L) genes based on their relative frequencies are paired expressed as recombinant antibodies in *E. coli* or mammalian cells.

Even though extremely attractive, repertoire mining is unlikely to totally replace current screening technologies. Saggy et al. [113] have shown that phage display and repertoire mining of immune repertoires are complementary technologies that can yield different antigen-specific antibody clones.

The functional antibody repertoire of the rabbit has been investigated through NGS by Kodangattil et al. [114] as a first step toward engineering rabbit V regions to enhance their potential as therapeutic agents.

We do not have, so far, examples of repertoire mining, through NGS, from human samples leading to the isolation of a candidate therapeutic antibody, but this gap will probably be rapidly fulfilled. In addition to the discovery of novel antibodies, information gained from high-throughput sequencing (HTS) of immunoglobulin genes can be applied to detect B-cell malignancies to guide vaccine development and to understand autoimmunity. A wider application of NGS will nevertheless require the development of a standardized experimental framework that will enable the sharing and meta-analysis of sequencing data generated by different laboratories [115].

1.6 Antibody Design

1.6.1 Antibody Isotype: The Specific Case of IgG4

In the process of designing a therapeutic antibody, the rational choice for a given isotype is determined by different factors such as *in vivo* half-life, need for recruitment of effector functions, or the extent of allotypic variation. In such a context IgG4, the least abundant of the four subclasses of IgG in serum, displays unique biological properties. It can undergo heavy-chain exchange, also known as Fab-arm exchange, ending with the formation of half antibody molecules that can randomly reassociate and generate BsAb [116–118]. Its weak interaction with FcγRII and FcγRIII, and lack of complement activation, makes it relatively "noninflammatory" [119].

1.6.2 Antibody Fragments

For many therapeutic applications, antibody functions (such as cytokine inactivation, receptor blocking, or viral neutralization) do not require the recruitment of effector functions through the crystallizable fragment (Fc) portion. An antibody's therapeutic performance is to a large extent dependent on factors

such as size, tissue penetration, distribution, half-life, effector functions, affinity, stability, and immunogenicity. This can explain why smaller Ab (scFv or Fab) fragments may be preferred. Consequently, one of the goals of antibody engineering has been to reduce the size to a minimum antibody fragment while still retaining both binding affinity and specificity. Another advantage of small antibody fragments is that they can be expressed in *E. coli* and yeast, dramatically reducing the cost associated with large-scale mammalian cell culture.

The Fv fragment, formed by the heterodimeric association of the two variable domains V_L and V_H of the light (L) and heavy (H) chain, respectively, can be genetically engineered into an scFv with a flexible polypeptide linker. The most commonly used linker is a flexible 15-mer peptide $(Gly_4Ser)_3$. Changing the linker length between V-domains induced oligomerization into "diabodies" or even into higher order valency antibody fragments (tribodies, tetrabodies), potentially increasing their avidity [120]. Diabodies, the best characterized of these molecules, have shown high functional affinity, greater tumor retention, and slower systemic clearance than their monovalent counterparts in preclinical studies [121]. To stabilize the association of the V_H and V_L domains, different linkage strategies have been proposed, for instance, through disulfide bridges [122] and "knob into holes" mutations [123]. An alternative format to scFv, named MoaFv, has been proposed to stabilize the Fv fragment and restore the bivalency of the antibody [124]. This MoaFv was constructed by replacing the C_{H1} and C_L domains of the Fab with heterotetrameric molybdopterin synthase (MPTS).

The fragment containing the antigen-binding (Fab) component is a heterodimer of $V_H - C_{H1}$ and $V_L - C_L$ linked together through a disulfide bond. In comparison with whole antibodies, small antibody fragments such as Fab or scFv exhibit better tissue penetration pharmacokinetics. However, Fab and scFv are monovalent and often exhibit fast off rates and poor retention time on the target.

The half-life of circulating antibody fragments (Fab, scFv) can be improved by site-specific coupling of polyethylene glycol (PEG) to the fragment, the so-called PEGylation [125]. For example, site-specific PEGylation into the hinge region has prolonged the circulating half-life of an anti-TNF, a human fragment (CDP 870, Celltech), to 14 days. Covalent attachment of PEG increases the circulating *in vivo* half-life of the antibody fragment, augmenting its apparent size above the glomerular filtration limit. PEG may have the additional benefit of increasing solubility and resistance to proteolysis and reducing immunogenicity [126]. Kitamura et al. have shown that PEG attachment to an intact Mumab reduced the HAMA response significantly when compared to the parent intact antibody [127]. The linkage of PEG to antibody fragments, done by chemical coupling, has some disadvantage in terms of global cost and the complexity of the associated downstream processing. In order to partly overcome this problem, PASylation has been proposed as an alternative. The