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Contributors

Christopher E. Andoniou
Virology Program, Centre for Ophthalmology and Visual Science, The University of Western Australia, Western Australia, Australia and Centre for Experimental Immunology, Lions Eye Institute, Nedlands, Western Australia, Australia.

Anna Balato
Department of Dermatology, University of Naples Federico II, Naples, Italy.

Per H. Basse
Faculty of Immunology, Hillman Cancer Center, University of Pittsburgh, Pittsburgh, Pennsylvania, United States.

Vasileios Bekiaris
Division of Molecular Immunology, La Jolla Institute for Allergy & Immunology, La Jolla, California, United States.

Giovanni Bernardini
Department of Experimental Medicine and Pathology, Instituto Pasteur-Fondazione Cenci Bolognetti, University of Rome, Rome, Italy.

Edward L. Briercheck
Integrated Biomedical Graduate Program, Medical Scientist Program, Ohio State University College of Medicine, Columbus, Ohio, United States.

Maryanne A. Bryan
Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, United States.

Lisa H. Butterfield
University of Pittsburgh Cancer Institute, Departments of Medicine and Immunology, University of Pittsburgh, Pittsburgh, Pennsylvania, United States.

Michael D. Cahalan
Department of Physiology and Biophysics, Center for Immunology, University of California, Irvine, California, United States.

Michael A. Caligiuri
Department of Microbiology, Virology, Immunology and Medical Genetics, James Cancer Hospital and Solove Research Institute, Ohio State University College of Medicine, Columbus, Ohio, United States.

William L. Camp
Department of Dermatology, University of Pittsburgh, Pittsburgh, Pennsylvania, United States.

Claudia Carlino
Department of Experimental Medicine and Pathology, Instituto Pasteur-Fondazione Cenci Bolognetti, University of Rome, Rome, Italy.

James R. Carlyle
Department of Immunology, Sunnybrook Research Institute, University of Toronto, Toronto, Canada.

Paolo Carrega
Istituto Scientifico Giannina Gaslini, Genoa, Italy.

Benedict J. Chambers
Department of Medicine Huddinge, Center for Infectious Medicine, Karolinska Institutet, Stockholm, Sweden.

Kenji Chamoto
Division of Immunoregulation, Hokkaido University, Hokkaido, Japan.

Sarah Cooley
Division of Hematology, Oncology, and Transplantation, University of Minnesota, Minneapolis, United States.

Régis Costello
Université de la Méditerranée, Institut de Cancérologie et d'Immunologie de Marseille, Service d'Hématologie, Hôpital Nord, Marseille, France.

Jerome D. Coudert
Virology Program, Centre for Ophthalmology and Visual Science, The University of Western Australia, Western Australia, Australia and Centre for Experimental Immunology, Lions Eye Institute, Nedlands, Western Australia, Australia.

Heike Daldrup-Link
Department of Radiology & Biomedical Imaging, University of California, San Francisco, San Francisco, California, United States.

Lesley R. de Armas
Department of Microbiology and Immunology, University of Miami, Miller School of Medicine, Miami, Florida, United States.

Mariapia Degli-Esposti
Virology Program, Centre for Ophthalmology and Visual Science, The University of Western Australia, Western Australia, Australia and Centre for Experimental Immunology, Lions Eye Institute, Nedlands, Western Australia, Australia.

Julie Y. Djeu
H. Lee Moffitt Cancer Center and Research Institute, Immunology Program, Tampa, Florida, United States.

P.K. Epling-Burnette
H. Lee Moffitt Cancer Center and Research Institute, Immunology Program, Tampa, Florida, United States.

Jia Fan
Department of Surgery, Liver Cancer Institute, Zhongshan Hospital, Fudan University Shanghai Medical School, Shanghai, China.

Cyril Fauriat
Université de la Méditerranée, Institut de Cancérologie et d'Immunologie de Marseille, Marseille, France.

Guido Ferlazzo
Department of Immunology, Laboratory of Immunology and Biotherapy, University of Messina, Policlinico Universitario, Messina, Italy.

Aharon G. Freud
Department of Pathology, Stanford University of Medicine, Palo Alto, California, United States.

Kym R. Garrod
Department of Physiology and Biophysics, University of California, Irvine, California, United States.

Anthony A. Gaspari
Department of Dermatology, University of Maryland School of Medicine, Baltimore, Maryland, United States.

Godfrey S. Getz
Department of Pathology, University of Chicago, Chicago, Illinois, United States.

Angela Gismondi
Department of Experimental Medicine and Pathology, Instituto Pasteur-Fondazione Cenci Bolognetti, University of Rome, Rome, Italy.

Stephen R. Goding
Oncology Department, Johns Hopkins Medical Institution, Baltimore, Maryland, United States.

Segundo González
Department of Functional Biology, University of Oviedo, Oviedo, Spain.

Martin R. Goodier
Department of Immunology, Imperial College London, Chelsea and Westminster Hospital, London, United Kingdom.

Bartosz Grzywacz
University of Minnesota, Department of Pediatrics, Division of Blood and Marrow Transplantation, Minneapolis, Minnesota, United States.

Petter Höglund
Department of Microbiology and Cell Biology, Karolinska Institute, Stockholm, Sweden.

Anne Hosmalin
Département d'Immunologie, Institut Cochin, Université Paris-Descartes, Paris, France.

Franck Housseau
Oncology Department, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States.

Takayuki Ikeda
Department of Food Science and Human Nutrition, Fuji Women's University, Hokkaido, Japan.

Taisuke Ito
Department of Dermatology, Hamamatsu University School of Medicine Handayama, Higashiku, Hamamatsu, Japan.

Priyanka Jha
Department of Radiology & Biomedical Imaging, University of California, San Francisco, San Francisco, California.

Veli-Matti Kähäri
Department of Dermatology, University of Turku, Turku, Finland.

Tatsuya Kanto
Department of Gastroenterology and Hepatology, Department of Dendritic Cell Biology and Clinical Applications, Osaka University Graduate School of Medicine, Osaka, Japan.

Alex Karlsson-Parra
Department of Microbiology and Immunology, Gothenburg University, Gothenburg, Sweden.

Rolf Kiessling
Experimental Oncology, IGT (Immune & Gene Therapy Laboratory), CCK (Cancer Center Karolinska), Stockholm, Sweden.

Hans Klingemann
Tufts Medical Center, Boston, Massachusetts, United States.

Christiane Knopp
Chemotherapeutisches Forschungsinstitut, Georg-Speyer-Haus, Frankfurt am Main, Germany.

Shinichi Koizumi
Division of Immunoregulation, Hokkaido University, Hokkaido, Japan.

Akira Komoriya
OncoImmunin. Inc., Gaithersburg, Maryland, United States.

Ming-Ling Kuo
Department of Microbiology and Immunology, Chang Gung University, Taoyuan, Taiwan.

Peter J.L. Lane
Medical Research Council for Immune Regulation, University of Birmingham Medical School, Birmingham, United Kingdom.

Yen-Chang Lee
Department of Pediatrics, Chang Gung Children's Hospital, Chang Gung University College of Medicine, Taoyuan, Taiwan.

Jussi Liippo
Department of Dermatology, University of Turku, Turku, Finland.

Syh-Jae Lin
Department of Pediatrics, Chang Gung Children's Hospital, Chang Gung University College of Medicine, Taoyuan, Taiwan.

Siyuan Liu
Department of Radiology & Biomedical Imaging, University of California, San Francisco, San Francisco, California, United States.

Hans-Gustaf Ljunggren
Center for Infectious Medicine, Department of Medicine, Karolinska University Hospital, Huddinge, Sweden.

Carlos López-Larrea
Department of Immunology, Hospital Universitario Central de Asturias, Oviedo, Spain, and Fundación Renal "Iñigo Álvarez de Toledo," Spain.

Alejandro López-Soto
Department of Functional Biology, University of Oviedo, Oviedo, Spain.

Michael T. Lotze
Departments of Surgery and Bioengineering, University of Pittsburgh Cancer Institute, University of Pittsburgh Schools of the Health Sciences, Pittsburgh, Pennsylvania, United States.

Tara J. Loux
Department of Surgery, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania, United States.

Lina Lu
Department of Immunology and General Surgery, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio, United States.

Michael Magee
University of Pittsburgh Cancer Institute, Department of Pathology, University of Pittsburgh, Pittsburgh, Pennsylvania, United States.

Robbie B. Mailliard
Celsense, Inc., Pittsburgh, Pennsylvania, United States.

Victoria H. Male
Department of Pathology, University of Cambridge, Cambridge, United Kingdom.

Ofer Mandelboim
Lautenberg Center for General and Tumor Immunology, Hebrew University, Biomedical Research Institute Israel Canada, Hadassah Medical School, Jerusalem, Israel.

Kazutaka Masuko
Division of Immunoregulation, Hokkaido University, Hokkaido, Japan.

Benjamin M. Matta
Department of Surgery and Immunology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, United States.

Domenico Mavilio
Laboratory of Experimental Immunology, IRCCS Instituto Clinico Humanitas, Rozzano, Milan, Italy.

Borna Mehrad
Division of Pulmonary and Critical Care Medicine, Departments of Medicine and Microbiology, University of Virginia, Charlottesville, Virginia, United States.

Jeffery S. Miller
Division of Hematology, Oncology, and Transplantation, University of Minnesota, Minneapolis, Minnesota, United States.

Rieko Mitamura
Department of Food Science and Human Nutrition, Fuji Women's University, Hokkaido, Japan.

Ashley Moffett
Department of Pathology, University of Cambridge, Cambridge, United Kingdom.

Erika Montalto
Laboratory of Immunology and Biotherapy, University of Messina, Messina, Italy.

Alessandro Moretta
Department of Experimental Medicine, and Centre of Excellence for Biomedical Research, University of Genova, Genova, Italy.

Lorenzo Moretta
Istituto Giannina Gaslini, Genova, Department of Experimental Medicine, University of Genova, and Centre of Excellence for Biomedical Research, University of Genova, Genova, Italy.

William G. Morice
Division of Hematopathology, Mayo Clinic, Rochester, Minnesota, United States.

Christian Münz
Institute of Experimental Immunology, University Hospital of Zürich, Zürich, Switzerland.

Michael A. Nalesnik
Division of Transplantation and Hepatic Pathology, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania, United States.

Jerry Y. Niederkorn
Department of Ophthalmology, University of Texas Southwestern Medical Center, Dallas, Texas, United States.

Takashi Nishimura
Division of Immunoregulation, ROYCE Health Bioscience Institute for Genetic Medicine, Hokkaido University, Hokkaido, Japan.

Karen A. Norris
Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, United States.

Daniel Olive
Université de la Méditerranée, Institut de Cancérologie et d'Immunologie de Marseille, Marseille, France.

John R. Ortaldo
Laboratory of Experimental Immunology, Cancer and Inflammation Program, National Cancer Institute, National Institutes of Health, Frederick, Maryland, United States.

Beverly Z. Packard
OncoImmunin, Inc., Gaithersburg, Maryland, United States.

Ralf Paus
Department of Dermatology, University Hospital Schleswig-Holstein, University of Lübeck, Lübeck, Germany.

Eckhard R. Podack
Department of Microbiology and Immunology, University of Miami, Miller School of Medicine, Miami, Florida, United States.

Shiguang Qian
Department of Immunology and General Surgery, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio, United States.

Shuang-Jian Qiu
Department of Surgery, Liver Cancer Institute, Zhongshan Hospital, Fudan University Shanghai Medical School, Shanghai, China.

Richard M. Ransohoff
Department of Neurosciences, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio, United States.

Catherine A. Reardon
Department of Pathology, University of Chicago, Chicago, Illinois, United States.

Verena Reinhart
Department of Radiology & Biomedical Imaging, University of California, San Francisco, San Francisco, California, United States.

Jérôme Rey
Université de la Méditerranée, Institut de Cancérologie et d'Immunologie de Marseille, Marseille, France.

Anna Rubartelli
Cell Biology Unit, National Cancer Research Institute, Genova, Italy.

Angela Santoni
Department of Experimental Medicine and Pathology, Instituto Pasteur-Fondazione Cenci Bolognetti, University of Rome, Rome, Italy.

Daniel Scott-Algara
Unité de Régulation des Infections Rétrovirales, Institut Pasteur, Paris, France.

Claudia Semino
Cell Biology Unit, National Cancer Research Institute, Genova, Italy.

Fu-Dong Shi
Center for Neurologic Diseases, Tranjin Medical University General Hospital, Tranjin 3000071, China and Department of Nuerology Institute, Barrow Neurological Institute, St. Joseph's Hospital and Medical Center, Phoenix, AZ 85013.

Helena Stabile
Department of Experimental Medicine and Pathology, Instituto Pasteur-Fondazione Cenci Bolognetti, University of Rome, Rome, Italy.

Noam Stern-Ginossar
Lautenberg Center for General and Tumor Immunology, Hebrew University, Biomedical Research Institute Israel Canada, Hadassah Medical School, Jerusalem, Israel.

Jeff Subleski
Laboratory of Experimental Immunology, Cancer and Inflammation Program, National Cancer Institute, National Institutes of Health, Frederick, Maryland, United States.

Angus W. Thomson
Departments of Surgery and Immunology, Thomas E. Starzl Transplantation Institute, University of Pittsburgh Schools of the Health Sciences, Pittsburgh, Pennsylvania, United States.

Mervi Toriseva
Department of Dermatology, University of Turku, Turku, Finland.

David S. Ucker
Department of Microbiology and Immunology, University of Illinois College of Medicine, Chicago, Illinois, United States.

Andrea Velardi
Division of Hematology and Clinical Immunology, University of Perugia, Perugia, Italy.

Michael R. Verneris
Pediatric Hematology/Oncology and Blood and Marrow Transplant, Minneapolis, Minnesota, United States.

Lazar N. Vujanovic
University of Pittsburgh Cancer Institute, Department of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, United States.

Nikola L. Vujanovic
University of Pittsburgh Cancer Institute, Department of Pathology, University of Pittsburgh, Pittsburgh, Pennsylvania, United States.

Daiko Wakita
Division of Immunoregulation, Hokkaido University, Hokkaido, Japan.

Sheng Wei
H. Lee Moffitt Cancer Center and Research Institute, Immunology Program, Tampa, Florida, United States.

Jonathan M. Weiss
Laboratory of Experimental Immunology, Cancer and Inflammation Program, National Cancer Institute, National Institutes of Health, Frederick, Maryland, United States.

Winfried S. Wels
Chemotherapeutisches Forschungsinstitut, Georg-Speyer-Haus, Frankfurt am Main, Germany.

Michael F. Wendland
Department of Radiology & Biomedical Imaging, University of California, San Francisco, San Francisco, California, United States.

Amy K. Wesa
Departments of Dermatology and Immunology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, United States.

Robert H. Wiltrout
Laboratory of Experimental Immunology, Cancer and Inflammation Program, National Cancer Institute, National Institutes of Health, Frederick, Maryland, United States.

Yong-Sheng Xiao
Department of Surgery, Liver Cancer Institute, Zhongshan Hospital, Fudan University Shanghai Medical School, Shanghai, China.

Dah-Chin Yan
Department of Pediatrics, Chang Gung Children's Hospital, Chang Gung University College of Medicine, Taoyuan, Taiwan.

Makato Yawata
Institute for Stem Biology and Regenerative Medicine, Stanford University, Palo Alto, California, United States.

Nobuyo Yawata
Institute for Stem Biology and Regenerative Medicine, Stanford University, Palo Alto, California, United States.

Alexandra Y. Zhang
Department of Dermatology, University of Pittsburgh, Pittsburgh, Pennsylvania, United States.

Juan Carlos Zúñiga-Pflücker
Department of Immunology, Sunnybrook Research Institute, University of Toronto, Toronto, Canada.

Foreword

High background reactivity in a laboratory assay was the bane of my existence!

It was the early 1970s and I had joined the Department of Tumour Biology at the Karolinksa Institute with Eva Klein and Hans Wigzell as my Ph.D. supervisors. In the early 1960s, Hans did a postdoctoral stint at the Queen Victoria Hospital in Sussex and had established the ^{51}Cr release assay (Wigzell, 1965). It was subsequently used by Drs. Cerottini and Brunner (Brunner et al., 1968) to measure cellular lysis of tumour targets. My job as the new lab rat was to standardize this assay with YAC-1 cells to investigate the T cell reactivity against murine Moloney Virus induced leukemia. Much to my dismay, the background lysis of YAC-1 cells in the presence of mouse splenocytes remained high and persisted despite my best efforts, even with cells from non-immunized, control mice. By hindsight, it was through sheer serendipity that we chose YAC-1 cells but the choice was fortuitous indeed since even today, I cannot think of any cell that is more exquisitely sensitive to natural killer cell activity.

Gradually this "high background" activity evoked our interest and delineating the cell responsible for this phenomenon became the principal focus of my doctoral research. Our group and that of Ron Herberman at the National Institute of Health were the first to systematically characterize the effector cell responsible for the activity (Herberman et al., 1975a; Herberman et al., 1975b; Kiessling et al., 1975a; Kiessling et al., 1975b). The name "natural killer cells" came effortlessly as it was paraphrased on "natural antibodies", a field which was very active in the early 1970s. Nowadays, it is obvious that the cell we defined had several functions other than being a killer cell, such as secreting cytokines and recruiting other immune cells to the site of inflammation. Following its discovery and for several years later, the field of immunology continued to be dominated by research on T and B cells. NK cells were virtually relegated to the role of an interesting artifact or T cell type of unknown significance. Between 1975 and 1979 there were fewer than a hundred publications on NK cells. It was inconceivable that 30 years later NK cells would be investigated and found to have a role in all aspects of immunology including immunity to bacteria and viruses, transplantation, autoimmune disorders and hypersensitivity, as is obvious from the contributions to this volume.

The subject of target recognition by NK cells and their specificity arose very early on after their discovery and continues to be a subject of research even today. Three chapters in this book delve into the complex mechanisms and processes by which multiple ligands and signaling molecules regulate the activation and inhibition of NK target recognition and effector activity. At the onset, NK cell activity was defined by their ability to kill tumor targets and we looked upon NK cells as an alternative to T cell mediated immune surveillance (Kiessling and Haller, 1978). It became apparent soon, however, that the biological relevance of NK cells was much more complex and went beyond that of antitumor surveillance. As early as 1961, Snell and Stevens noticed that F1 hybrid mice derived from two inbred strains of mice often were relatively resistant to small tumor grafts of parental strain origin, compared to syngeneic recipients (Snell and Stevens, 1961). The late Gustavo Cudkowicz, then at University of Buffalo, had for many years pioneered studies on a similar type of "hybrid resistance" controlling rejection of hematopoietic grafts (Cudkowicz and Rossi, 1972). The resistance phenomenon had some rather distinctive characteristics that differed from the tenets of "conventional immunity" held at that time. In the summer of 1976, with America celebrating the bicentennial anniversary of its independence, I visited Gustavo's laboratory for a short and intense visit. As I worked with him and his colleagues it became apparent that there was a striking similarity between the mechanism of resistance to hematopoietic grafts and NK cell rejection (Kiessling et al., 1977).

Klas Kärre joined my laboratory as a doctoral student in the late 1970s. A soft-spoken, eloquent and slightly absent-minded guy, he made a succession of seminal discoveries which were summarized in 1981 in his doctoral thesis titled "On the immunobiology of Natural Killer Cells; studies of murine NK-cells and their interactions with T-cells and T-lymphomas". I delivered the galley proofs to him while he was at the hospital awaiting the birth of his first son. His doctoral defense was rather lively since a member of the advisory committee found his findings too speculative and not adequately substantiated with experimental evidence. However, the evidence for his "alternate immune defense" hypothesis accumulated rapidly in subsequent years. One of the key observations was that the RMA-S lymphoma selected to lack MHC class I expression (due to a mutation in the TAP2 gene (Yang et al., 1992)), was rejected in a T cell- independent, NK-cell dependent manner (Karre et al., 1986). This and other lines of verifications

pointed to an inverse correlation between the expression of surface MHC class I molecules and susceptibility to NK-cell-mediated lysis of target cells. Later, Klas and his first doctoral student, Hans-Gustaf Ljunggren formulated the "missing self" hypothesis (Ljunggren and Karre, 1990). The hypothesis initially stirred quite a bit of controversy since it challenged the prevailing concept of how the immune system discriminated self and non self. To my mind, it was not until then and later following the molecular definition of NK receptors (Ciccone et al., 1992; Karlhofer et al., 1992), that NK biology truly became recognized and established as a bonafide domain of immunology.

The "missing self"-theory predicted the existence of inhibitory receptors that bind MHC class I. These receptors, now termed Ly49A, were identified on murine NK cells (Karlhofer et al., 1992). In parallel, antibodies against the human receptors were made (Ciccone et al., 1992). These discoveries provided a strong impetus to research on NK cells. During subsequent years it became clear that NK cells have a multitude of inhibitory and activating receptors that engage MHC class I as well as molecules similar to or entirely disparate from MHC class I. It is now common knowledge that the balance between these inhibitory and activating receptors ultimately regulates the cytotoxic function of NK cells, as will become apparent from several of the chapters in this book and continues to be the focus of research in several laboratories worldwide. The intricacy of this interaction is particularly perceptible in patients with MHC class I deficiencies. One would expect that these patients with "bare lymphocyte syndrome" would demonstrate high incidence of NK-mediated immunopathologic disease, but surprisingly they do not (Zimmer et al., 1998). One potential reason is that the NK receptors, never having encountered the MHC class I ligand, persists in an "uneducated" state, and is therefore unable to recognize MHC class I low target cells. The understanding of how NK cells are being "educated" is one of the more important aspects of NK research, closely associated with the question of how NK cells are maintained in a "tolerant" state to self, as will be discussed in several chapters of this volume.

Why have NK cells developed and why do we have them? Our early view was that NK cells were a vestigial remain of a primordial immune system which was a forerunner to the more refined adaptive immune system (Kiessling and Wigzell, 1981). However this theory is not compatible with several observations, including the fact that orthologs of most NK cell receptor families cannot be found earlier in evolution than mammals (Walzer et al., 2007). NK and T cells have complementary roles in host defense as well as have commonality in mechanisms of cytotoxicity, which rather suggests a common ancestral cell for NK cells and T cells. The most plausible explanation for why NK cells evolved is that they developed as a complementary system to adaptive T cell immunity for defense against viruses and transformed cells. The "virus activated killer cell" was studied by a handful of virologists in the seventies, which then merged with NK research when it proved that this killer cell was identical to the NK cell (Oldham et al., 1977; Welsh and Zinkernagel, 1977). The antiviral role of NK cells however is not universal and only extends to certain viruses like Herpes Viruses and influenza virus. Direct evidence for the protective effect comes from NK depletion or adoptive transfer experiments in mouse models of the herpesvirus MCMV (Bukowski et al., 1985). NK cell deficient mice infected with coxsackie B3 virus have higher titers of virus and more severe myocarditis compared to NK-replete control mice (Fairweather et al., 2001), demonstrating the importance of NK cells also in controlling immunopathology. There also exists a notable case report of severe Herpesvirus infection in a patient with selective NK cell defect (Biron et al., 1989).

The capacity of NK cells to react also with non-malignant activated or immature myelomonocytic cells was described in the early 1980s (Hansson et al., 1982). NK cells inhibited the development of granulocytic progenitor cells in colony forming assays performed in semisolid agar. This immunoregulatory function of NK cells and their interaction with cells of the myelomonocytic lineage has now been extensively verified, specifically by their ability to influence DC function. NK cells can kill both human and mouse DC, which may influence DC homeostasis and potentially also limit dendritic cell vaccination efficacy (Hayakawa et al., 2004). Paradoxically, however, NK cells can also facilitate antigen presentation by DC since antigens released by target cells following lysis by NK cells can be endocytosed and presented by DC.

Primary immunodeficiencies have frequently offered opportunities to study the input of distinct effector mechanisms towards resistance against microorganisms, but NK cell research has suffered from a paucity of animal models which selectively lack NK cells. Furthermore, patients with selective defects in NK functions are very rare. One may in fact dispute whether any truly selective NK immunodeficiency really exists in mouse or man (Fischer, 2007). Although several studies described deficiencies in NK cells primarily in conjunction with viral infections, a specific molecular defect leading to selective loss of NK function has never been identified. For example, the "beige" mouse, which is the murine equivalent of Chediak Higashi syndrome of man, also displays numerous defects in the monocyte and T cell compartment (Barak and Nir, 1987; Roder et al., 1979).

Regardless of the true biological role of NK cells, there is now much optimism in the NK field that the

coming decades will see the development of NK cell based therapies in the clinical management of diverse diseases caused by infectious pathogens and cancer (Ljunggren and Malmberg, 2007). T cell based therapy of cancer and chronic viral infections has so far met with only limited success in the clinic. The efficacy of T cell therapies is restricted largely due to the strong tendency of tumors and viruses to develop "stealth" strategies based on loss of MHC class I expression. If we could harness the complementary role that NK cells have in eliminating MHC class I low tumor cells and utilize our rapidly increasing understanding of the NK receptors and their tumor ligands, it would have a significant impact on future immunotherapy. Pioneering studies were done with peripheral blood lymphocytes activated with IL-2 into "lymphokine-activated killer" (LAK) cells whose function can principally be attributed to activated NK cells. LAK cells combined with IL-2 can achieve very significant and long lasting responses in melanoma patients as well as other solid tumors (Rosenberg, 2000). Initially it was thought that the 15–20% response rate typically noted in patients could be improved by increasing the dose of IL-2 or LAK cells which was unfortunately not realized. It is presently known that several reasons including dose-limiting toxicity may curb the clinical response rate of IL-2 based therapies and it has become apparent that IL-2 also induces apoptosis in NK cells or expands the regulatory T cell subset which can directly inhibit NK function (Ghiringhelli et al., 2005; Rodella et al., 2001) . Additionally, tumor targets in most of the treated patients may lack the appropriate combination of activating and inhibitory receptors, and therefore cannot simply be eliminated by the IL-2 activated NK cells. While most NK assays are performed with long term in vitro cultured tumor lines, freshly explanted human tumors are relatively resistant to NK mediated cytotoxicity, although some non-cultured tumors such as ovarian carcinomas which frequently display various defects in MHC class I presentation (Norell et al., 2006) can be recognized and killed even with non-activated NK cells (Carlsten et al., 2007).

A high priority will be the discovery of methods to manipulate the activity of NK cells e.g. approaches tilting the balance in favor of activating versus inhibitory receptors or administration of the right combination of growth factors. Recently, there has been a major breakthrough in the treatment of leukemias which is directly related to the findings of NK cell mediated killing of lymphomas, the "missing self" hypothesis and the definition in molecular terms of HLA class I inhibitory receptors (Ruggeri et al., 2005). It is an attractive possibility to also utilize similar treatment modalities for solid tumors, as will be discussed in this volume in several chapters.

Clinical therapy with antibodies has been a real success story for modern biotherapy. Approximately 12 antibodies are currently approved for therapeutic use. ADCC by NK cells is known to be a major factor mediating the clinical effect of mAbs such as Rituximab reactive to CD20 and used for treatment of lymphoma and Herceptin specific for the oncogene Her2/neu expressed in a proportion of breast-cancers (Cartron et al., 2002). The ADCC is largely mediated by the CD56 dim NK cell subset, which has a high expression of the low-affinity Fc γ receptor IIIA, CD16. The conclusion that ADCC plays a major role in the efficacy of Rituximab and Herceptin is established from experiments with Fc receptor gamma deficient mice and more recently from the correlation between improved clinical efficacy and an Fc gamma IIIa gene polymorphism which results in a higher affinity for these antibodies (Cartron et al., 2002; Clynes et al., 2000). This knowledge has stimulated interest in combinatorial therapies with mAb administered together with therapies known to increase NK activity.

There is also an increasing awareness of the potential synergistic effects of combinatorial cancer therapies; not only focused on merging T cell and NK cell modalities but also those combining immunotherapies with conventional chemo-radiotherapy (Zitvogel et al., 2008). The "preconditioning" of patients with chemotherapy may have several effects which may increase NK- mediated tumor killing by boosting NK activity or increasing the target sensitivity to NK lysis. A non-myeloablative regimen with low dose cyclophosphamide and 5-fluorouracil was shown to preferentially eliminate regulatory T cells. Objective clinical responses were observed in 50% of advanced melanoma patients who received this regimen prior to adoptive transfer of tumor infiltrating lymphocytes (TIL). Since NK cells are also suppressed by regulatory T cells by a TGF-β-dependent mechanism (Ghiringhelli et al., 2005), the combination of the chemotherapy regimen with NK-based immunotherapy is particularly promising. Other combinatorial possibilities involve up-regulating ligands for activating NK receptors, such as the NKG2D, through low doses of chemotherapy or ionizing irradiation (Gasser et al., 2005). These regimens act through the DNA-damage response pathway, which may upregulate NKG2D, thereby "sensitizing" tumor cells to recognition by NK cells. Another example of combinatorial treatments based on NK cells and drugs is the recently approved combination of IL-2 and histamine in the treatment of patients with acute myelogenous leukemia (AML) (Brune et al., 2006). AML Patients in remission were shown to have a prolonged relapse free survival as a result of IL-2 and histamine treatment which activates both NK cells and CD8+ T cells. Histamine was shown to protect NK cells from oxidative stress-induced apoptosis, particularly the CD56 subpopulation which is of major importance for cytotoxicity against tumor targets.

In conclusion, there has been a tremendous increase in our knowledge of NK biology and function. We are beginning to develop approaches for utilizing NK cells for clinical therapy of malignancies, or in contrast neutralizing them to protect organ transplants or abrogate autoimmune disorders. These approaches are still in their infancy but will be greatly facilitated by the ever-expanding knowledge of the activation and inhibition pathways in NK cells. It has been a long and astonishing journey for a cell that started its life as a background noise in a laboratory assay.

Rolf Kiessling

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Preface

> ***Nature**, in the broadest sense, is equivalent to the **natural world**, **physical world** or **material world**. 'Nature' refers to the* phenomena *of the physical world, and also to* life *in general. Manufactured objects and human interaction generally are not considered part of nature, and are referred to as* artificial *or man-made. Nature is generally distinguished from the* supernatural. *It ranges in scale from the* subatomic *to the* galactic. *The word* nature *is derived from the Latin word* natura, *or 'essential qualities, innate disposition', but literally meaning 'birth'. Original sense is in 'human nature' (Harper, 2001a).* Natura *was a Latin translation of the Greek word* physis (φύσις), *which originally related to the intrinsic characteristics that plants, animals, and other features of the world develop of their own accord.*[1] *This is shown in the first written use of the word* φύσις, *in connection with a plant.*[2] *The concept of nature as a whole, the physical* universe, *is one of several expansions of the original notion; it began with certain core applications of the word* φύσις *by pre-Socratic philosophers, and has steadily gained currency ever since. This usage was confirmed during the advent of modern* scientific method *in the last several centuries.*[3,4]

Indeed, there have been many changes in the source of information and quotes both ancient and more modern, decorate the beginnings of each of our chapters within this 'First Edition of Natural Killer Cells'. Given the changes that now occur in the means by which we derive information, it seemed particularly appropriate to use a rather democratic source of information available to all on the Internet, 'Wikipedia', to launch our preface. The scientific method, distilled as repeated observations attempting to nullify a central hypothesis, was applied in all of our efforts to launch this effort with many of our colleagues and mentors. They demeaned the process of writing books, now possibly considered dinosaurs of erudition, in favour of writing grants, giving talks, or crafting reviews for high-impact factor journals. Most of these are more remunerative directly or indirectly but we felt the democracy of a set of firm, experienced highly selected hands laying siege to individual topics, a steady editorial assistant in the person of Kristi Anderson, to whom we are indebted, and the iterative events of review and resubmission in a single volume was well-deserved labour for this most singular of cells, the so-called NK cell. Together, we have introduced other changes here: electronic availability through libraries for each chapter, the development of an abstract/construct to introduce each chapter comparable to what is available for journal articles and available through ScienceDirect for ready access and citation management systems. We thank our authors for their industry and willingness to commit to this volume and suffer our reminders and pursuit of them in their labours. For the value of this edition, we owe them everything; any defects remain with us.

Natural. It is now over 40 years since the first cytolytic assays were performed with dye exclusion and then ^{51}Cr release assays revealing in fine detail, the ability of lymphoid cells to mediate lytic activity against cultured tumour targets (Brunner et al., 1968). Flying in the face of conventional notions of immune specificity, it was subsequently found that some cells, so-called natural killer cells (Herberman, 1975a,b; Kiessling et al., 1975a,b) could kill tumour cells without prior sensitization and without MHC restriction. Since then the emphasis has rather been on the cytolytic capability of these cells, more than on their nature and their natural role. With this volume we explore the many other natural traits

[1]A useful though somewhat erratically presented account of the pre-Socratic use of the concept of φύσις may be found in Naddaf (2006). The word φύσις, while first used in connection with a plant in Homer, occurs very early in Greek philosophy, and in several senses. Generally, these senses match rather well the current senses in which the English word *nature* is used, as confirmed by Guthrie (1965).

[2]The first known use of *physis* was by Homer in reference to the intrinsic qualities of a plant:
ὣς ἄρα φωνήσας πόρε φάρμακον ἀργεϊφόντης ἐκ γαίης ἐρύσας, καί μοι φύσιν αὐτοῦ ἔδειξε. (So saying, Argeiphontes [=Hermes] gave me the herb, drawing it from the ground, and showed me its **nature**.) (The word is dealt with thoroughly in Liddell and Scott's *Greek Lexicon*.) For later but still very early Greek uses of the term, see earlier note.

[3]Isaac Newton's *Philosophiae Naturalis Principia Mathematica* (Newton, 1687), for example, is translated 'Mathematical Principles of Natural Philosophy', and reflects the then-current use of the words 'natural philosophy', akin to 'systematic study of nature'.

[4]The etymology of the word 'physical' shows its use as a synonym for 'natural' in about the mid-fifteenth century (Harper, 2001b). From Wikipedia, downloaded on Sunday, 29 March 2009.

of these cells, well expanding beyond simple killing of tumours, suggesting that in a less-innocent world we should consider these rather N cells, comparable to B and T cells, reflecting now on their ability to regulate TH1 responses, promote DC maturation, promote autophagy, and support the vascularization of the placenta, putting it in a critical role in perpetuation of our own species. Most recently the notions of NK cell progenitors being critical for the development of lymph nodes and all adaptive immune cells make them indeed the most natural of cells, critical for their emergent role throughout modern immunology. Although perhaps misplaced here, we should connote this important cell with a single letter like its brethren the T, the B, and the dendritic but perhaps we will have to wait for the next edition. Indeed, as abundantly revealed throughout this text, NK cells do far more than just kill and they have been revealed in a degree of complexity and importance rather unimagined in their debut almost 35 years ago. Thus 'N Cells' awaits your approbation and consideration.

Stress. Life is stressful. Nutrient loss, hypoxia, genomic stress, ER stress, infection, damage. Writing new textbooks in a difficult time with all scientists and clinicians oversubscribed with the promise and threat of instant information and manuscripts managed online and without the buffeting of the postal service is also stressful. Thus the tempo and temperament of the writing process has assumed the same hurried moment as the recruitment of inflammatory cells, including the NK cell to sites of stress. Perhaps following in the 'cell-steps' of the macrophage with which they interact early during inflammatory processes, on an evolutionary scale, NK cells are likely in our estimation the primordial adaptive cell. NK cells similarly have an ability to expand, contract and respond with, at the very least, short-term memory to microbial stress and, we suspect, tissue damage or injury (Sun et al., 2009). At a fundamental level, NK cells script and focus their myeloid brethren on a dangerous world full of pathogens and tissue damage. As such they define the quality and nature of the immune response in the setting of stress, serving as mobile paracrine agents releasing cytokines or inducing their production dependant on their integration of multiple signals, multi-tasking within the lymph node and peripheral tissues. The first section of our volume (Chapters 1–9) focuses on the development of NK cells from progenitors in rodents and humans, how they signal, how they identify stress in tissues and cancer, and how they interact coordinately with other cells within these tissues.

Recognition of stress. There are many ways for cells to communicate in multicellular organisms, through cytokines that indeed 'move' cells to change their shape or biology, through chemokines directing the to and fro of cells within tissues and secondary and tertiary lymphoid sites, and through direct cell–cell interactions. This goes beyond just their interaction with each other as inflammatory cells and is defined as the integrant of interactions with cells in the tissues including the endothelial cells, epithelial cells, stromal cells, and sessile inflammatory cells. NK cells are sophisticated communicators, sensing signals from all of these cells and providing necessary feedback, eliciting programmed cell death (apoptosis] when necessary or programmed cell survival under dire threats (autophagy). The rough and tumble of life makes NK cells exquisitely sensitive to signals emanating from the postcapillary venules to allow their rapid emigration into these sites and within lymph nodes across high endothelial venules, coordinately enhancing their response to stress. These important interactions are captured in Chapters 10–24.

Integration of signals within organs. As different are the NK cells serving as helper cells steering new blood vessels to the implanted embryo, are those pow-wowing with their dendritic cell intimates within the eye, the brain, the gut, and the liver. Thus, a nuanced and balanced role for their function is required. It is one thing to eliminate an hepatocyte which is stressed and readily regenerated as it is to over-react to the microbial flora within the gut. Indeed NK cells are necessary diplomats, cajoling when necessary, creating outright warfare when discussion is beyond reason, and defining the nature and shape of the negotiating table. This they do with remarkable insights into the various tissues within which they find themselves. Thus to understand NK cells in aggregate, more importantly, one should consider them within various tissues within which they share some central properties but not all. Focusing on their identification and varied roles at these sites are exemplified in Chapters 25–33.

Roles of NK cells in disease. With acute injury, there is a requisite need to mobilize resources to limit damage and repair, and in an informed way, prepare for similar insults with an enhanced response, what immunologists refer to as memory (Sun et al., 2009). NK cells are charged to (1) recognize damage; (2) limit further damage; (3) regenerate and vascularize damaged tissues; and (4) learn from the experience and in particular be prepared for further encounters with the same or similar pathogens (basically, the role of adaptive immunity). This characteristic originally relegated to a perceived more 'noble' T cell and B cell has now been recognized in NK cells. They too can commit sins of omission, with failure to recognize either cryptic microbial or neoplastic antigens or commission with the undesired aspects of autoimmunity and graft-versus-host or host-versus-graft disease. These can be readily defined as issues for both NK cells and T and B cells. It should at least be contemplated that NK cells' exuberant or deficient response to stress in the setting of disease may allow their promotion of a damaging TH1 response or a permissive and emergent role as NK regulatory cells, squelching

immune reactivity where necessary and promoting programmed cell survival or autophagy. Their role in various disease states is considered in detail in Chapters 34–45.

Special issues in NK cells and 'Wicked Problems'. The role of new technologies in genetically modifying NK cells, assessing their cytolytic activities, or imaging them in vivo deserved special consideration, here assembled in Chapters 46–50. The 'dilemma' of understanding NK cells requires a deeper understanding of the problems of all 'social scientists' considering the emergent properties of complex organisms as presciently outlined in the journal *Policy Sciences*, published in 1973 by Elsevier, a Dutch company in Amsterdam and our publisher for this volume but, interesting to us, printed in Scotland (Rittel and Webber, 1973). Here Rittel and Webber wrestled with how to plan when neither the problems were well-understood nor suitable solutions with any well agreed upon and indisputable public good could be identified. This indeed, 'Dilemmas in a General Theory of Planning' deserves some comment for the social nature of NK cells in the organisms within which they evolved. Thus, ending with a quotation from their treatise seems only appropriate:

> *A great many barriers keep us from perfecting such a planning/governing system: theory is inadequate for decent forecasting; our intelligence is insufficient to our tasks; plurality of objectives held by pluralities of politics makes it impossible to pursue unitary aims; and so on. The difficulties attached to rationality are tenacious, and we have so far been unable to get untangled from their web. This is partly because the classical paradigm of science and engineering—the paradigm that has underlain modern professionalism—is not applicable to the problems of open societal systems. …The error has been a serious one. The kinds of problems that planners deal with—societal problems—are inherently different from the problems that scientists and perhaps some classes of engineers deal with. Planning problems are inherently wicked. As distinguished from problems in the natural sciences, which are definable and separable and may have solutions that are findable, the problems of governmental planning—and especially those of social or policy planning—are ill-defined; and they rely upon elusive political judgment for resolution. (Not 'solution'. Social problems are never solved. At best they are only re-solved—over and over again.)*

And thus NK cells, thrust into their societal role within the organism, have this difficult problem of resolving the problems of complex biology over and over again. The relevance of the body politic and political bodies seem indeed congruent in this instance. And thus, undoubtedly, we will need to revisit this cell in a second edition, finding their solution impossible, but in their understanding, something wicked.

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Dedication to our Institute Directors, Fadi Lakkis and Nancy Davidson. It is appropriate in this era of increasingly complex science, administration and clinical activities to dedicate this volume to our new directors. Both have dedicated themselves to the art and science of Transplantation Immunology and Cancer Biology, respectively. Our ability to construct this first volume has been dependant on their emergent support for the academic mission, creating and organizing knowledge during a period of extraordinary expansion of that knowledge, now during a period of unprecedented economic travail, and concerns about how this knowledge will be applied and expanded. Indeed, 'natural' progression of our innate understanding of immunity in the context of the problems of both acute and chronic inflammatory diseases mediated and modulated by NK cells will require their continued gentle ministrations.

Chapter **One**

1

Developmental stages and pathways of NK cell maturation

Bartosz Grzywacz, Jeffery S. Miller, Michael R. Verneris

CHAPTER CONTENTS

Yes, there are two paths you can go by, but in the long run, there is still time to change the road you are on….

Robert Plant of Led Zeppelin (1970)

ABSTRACT

Hematopoietic stem cells (HSCs) by definition can differentiate into all types of blood cells. There are several factors and events that promote HSC differentiation into the natural killer (NK) cell lineage. These include soluble factors, with a prominent role for interleukin 15, as well as contact- or gradient-dependent signals, such as Gas6/Tyro family of ligands and factors activating Wnt pathway. A complete understanding of the factors that control NK cell differentiation may allow for manipulation of NK cell reconstitution following hematopoietic cell transplantation and efficient ex vivo generation of NK cells for adoptive immune therapy.

KEY WORDS

NK cells, Development, Hematopoiesis, Transcription factors, Differentiation, Lineage CHOICE, Cytokines and receptors, Morphogens, Innate immunity

The early events in hematopoiesis

Hematopoiesis sustains the production of blood cells throughout life and is the best understood model of stem cell differentiation. By way of asymmetric cell division, hematopoietic stem cells (HSCs) give rise to two daughter cells. One of these daughter cells maintains HSC characteristics, while the other goes on to generate progeny that ultimately develop into mature blood cells (Orkin and Zon, 2008). During stem cell differentiation two parallel processes are imposed upon the cell: (1) the gradual acquisition of features of a given cell lineage, and (2) the loss of the ability to give rise to

other cell lineages. Differentiation entails the selective expression of proteins that determine the identity of a particular lineage. This is accompanied by the repression of genes that direct differentiation towards other lineages.

Hematopoiesis has been schematically depicted as a series of binary choices faced by a multipotent HSC. The most widely accepted model, according to Weissman and colleagues, describes the initial developmental choice between the myelo-erythroid vs. lymphoid fates. Accordingly, common lymphoid precursors (CLPs) have no myeloid potential (Kondo et al., 1997), and common myeloid progenitors (CMPs) lack lymphoid generating capacity (Akashi et al., 2000). This model is based on the prospective isolation of CLPs that give rise to lymphocytes but not myeloid cells upon transplantation. Similarly, CMPs can replenish the myeloid and erythrocyte compartments but do not generate lymphocytes after adoptive transfer. The simplicity of this model has been called into question by the demonstration of progenitors that lack erythroid potential but retain myeloid and lymphoid capacity (Adolfsson et al., 2005; Katsura, 2002). Thus, there is still some ambiguity regarding the order of choices during early hematopoiesis.

NK cells as a distinct cell type

The observations of natural killing (cytotoxicity without prior antigen priming) by a population of non-T lymphocytes and non-B lymphocytes have led to the identification of the natural killer (NK) cell lineage (Herberman et al., 1975; Kiessling et al., 1975). The advent of monoclonal antibodies and flow cytometry allowed for a phenotypic definition of NK cells (i.e. $CD56^{+}CD3^{-}$) (Lanier et al., 1986). Although a lymphoid vs. myeloid origin of NK cell development had been debated early in their discovery based on early application of monoclonal antibody typing (Li et al., 1994; Ortaldo and Herberman, 1984), the demonstration of a common T/NK precursor in human (Sanchez et al., 1994) and murine thymic tissue (Carlyle et al., 1997) placed NK cells developmentally close to T cells. The lymphoid origin of NK cells was more formally demonstrated by Akashi who isolated murine CLPs and demonstrated their ability to give rise to NK cells (in addition to T cells and B cells) upon transplantation into congenic animals (Kondo et al., 1997). Thus, NK cells can be derived from lymphoid progenitors.

Unlike the other progeny of CLPs (i.e. T cells and B cells), NK cells do not mediate conventional adaptive immunity. This is due to the lack of rearranged antigen-specific receptors, such as the T cell and B cell receptors that are generated following somatic recombination. Instead, NK cells express diverse sets of germline-encoded receptors responsible for antigen recognition (Lanier, 2005). This strategy (expression of multiple nonrearranged receptors) is commonly employed by the innate immune system. Recently however, NK cells have been shown to be closer to the adaptive immune system than previously appreciated. In this regard, NK cells can mediate recall or secondary immune responses, including contact dependent hypersensitivity to secondary challenge by chemical irritants (O'Leary et al., 2006). Recall responses by murine NK cells expressing Ly49H have also been observed after infection with mCMV (Sun et al., 2009). In human NK cells studies, expansion of NK cell clones expressing inhibitory receptors specific for ligands missing in the host have also been observed following HSC transplantation (Ruggeri et al., 1999, 2002). Despite this evidence for the expansion of reactive NK cell clones mediating secondary immune responses, NK cells lack the fine and exclusive specificity for the challenging antigen that is conferred by the B cell and T cell receptors. Likewise, questions still remain as to how long NK cell 'memory' persists.

While NK cells do not fully conform to the definition of adaptive immunity, they also differ from members of the innate immune system. For instance, NK cells do not mediate phagocytosis and lack bactericidal enzymatic systems. Rather, they express intracellular proteins associated with effector functions also used by cytotoxic T cells, including granzymes and perforin. As well, NK cells rapidly release a wide array of cytokines upon activation, including IFN-γ and TNF-α, which serve to shape adaptive immune responses. Consequently, owing to their CLP derivation, NK cells are developmentally close to the adaptive immune system, while functionally they retain features more in line with the innate immune system, perhaps suggesting a more ancient origin compared to T cells and B cells.

Lineage specific growth factors

A significant breakthrough in the understanding of hematopoiesis came with the demonstration of lineage-specific growth factors (reviewed by Kaushansky, 2006). Examples of such factors include G-CSF, which promotes the granulocytic lineage; M-CSF, which leads to monocytic development, or erythropoietin resulting in erythrocyte generation. Acquisition of a particular receptor renders precursors responsive to a particular lineage-specific growth factor, thereby marking an important event in lineage determination. Thus, precursors of distinct lineages can be identified by the presence of specific growth factor receptors. For example, the erythropoietin receptor marks erythroid precursors.

In the case of lymphoid progenitors, CD127 (IL-7R) has been used to define CLPs (Kondo et al., 1997), and IL-7 is necessary for murine T cell and B cell development (Akashi et al., 1999; Peschon et al., 1994). This supports the notion that IL-7 is a growth factor specific for CLPs. However, murine NK cells develop normally in the absence of IL-7 (He and Malek, 1996). Several cases of human severe combined immune deficiency (SCID), caused by a mutation in the IL-7R (CD127), have also been reported. These patients lack T cells, however, NK cells are present and functional (Giliani et al., 2005; Puel et al., 1998). Thus IL-7, a growth factor specific for the development of CLPs into T cells, is not required for NK cell development. In contrast, deficiency of the cytokine receptor common γ-chain (CD132) in both mice (Cao et al., 1995) and humans (Buckley et al., 1997) results in the lack of T cells, B cells, and NK cells. These observations led to the conclusion that some cytokines that signal through the common γ-chain (including IL-2, -4, -7, -9, -15 and -21) are required for NK cell development.

Early studies of human NK cell differentiation from hematopoietic precursors used IL-2 (Miller et al., 1992). Paradoxically, this cytokine is not abundant in the bone marrow (BM) microenvironment. This suggested that another growth factor present in the BM milieu is responsible for NK progenitor development and expansion. IL-15 is expressed by BM stroma and was a possible candidate (Mrozek et al., 1996; Puzanov et al., 1996). The receptor for IL-15 shares β and γ subunits with IL-2 receptor, explaining the redundancy between IL-2 and IL-15 in vitro. Murine studies identified IL-15 as an NK-specific growth factor since IL-15$^{-/-}$ mice show a near absence of NK cells (Kennedy et al., 2000) Similarly, the deficiency of the IL-15 receptor β-subunit (CD122, shared with IL-2R) also results in a profound decrease in NK cells (Gilmour et al., 2001; Lodolce et al., 1998) As well, mice lacking the α subunit of the IL-15 receptor (15Rα$^{-/-}$) have a reduction in NK cells due to the lack of IL-15 transpresentation (Kawamura et al., 2003; Lodolce et al., 1998), and IL-15 transpresentation (via IL-15Rα) supports human NK cell differentiation in a xenogeneic mouse model (Huntington et al., 2009). In summary, IL-15 has emerged as a requisite NK specific growth factor, although it is not entirely NK specific, as it also acts on CD8^{+} T cells.

In line with this, CD122 (IL-15Rβ) has been used to isolate NK precursors (Rosmaraki et al., 2001). Primitive, nonlineage specific growth factors, including stem cell factor (SCF), FLT-3L and IL-3, also influence NK cell development (Williams et al., 1997). These growth factors act upon the early hematopoietic precursors, inducing IL-15Rβ (CD122) expression, thereby conferring IL-15 responsiveness (Yu et al., 1998). In line with this, IL-15$^{-/-}$ or 15Rα$^{-/-}$ mice are nearly devoid of mature NK cells (above), but they do have NK precursors (Vosshenrich et al., 2005). These findings suggest that at least one function of IL-15 is to provide survival signals to the developing NK cell. This has been confirmed by the demonstration that NK cell development in CD122 (IL-15Rβ)$^{-/-}$ mice can be restored by enforced, constitutive expression of the anti-apoptotic survival factor Bcl-2 (Minagawa et al., 2002). The downstream signalling through the IL-15/IL-2 receptor involves activation of JAK1/JAK3 and STAT3/STAT5b (Imada et al., 1998; Waldmann and Tagaya, 1999). Deficiencies of JAK3 or STAT5b also result in severe impairment in NK cell development (Imada et al., 1998; Park et al., 1995).

Sites of NK development: the importance of the developmental environment

BM HSCs give rise to lymphoid precursors and, at certain stages of development, these cells migrate to sites that facilitate terminal differentiation. Migration is an important factor in determining lineage fate. The environmental cues present at a particular site are required for the initiation of developmental programs. While it is well established that the thymus is the site for T cell development and B cells develop in the BM, we are just beginning to unravel the sites of NK cell development.

Initially, NK cell development was believed to occur exclusively in the BM (Kim et al., 2002). The critical role of BM for NK cell maturation in mice has been shown by using bone-seeking radioactive isotopes that injure the BM stroma, inducing a profound block in NK cell maturation (Mellen et al., 1982). However, recent studies show that NK cells also develop in human secondary lymphoid tissue (Freud et al., 2005). Freud and coworkers have identified consecutive stages of NK cell development starting from CD34^{+} precursors, resulting in functional CD56^{+} NK cells in lymph nodes (Freud et al., 2006). The relative importance of lymph nodes in NK cell development has not been completely established. Other tissues, such as intestinal epithelial layer (villous and crypt regions), also contain NK precursors (Chinen et al., 2007; Lynch et al., 2006) and are likely sites of NK cell development. The CD34^{+} hematopoietic precursors isolated from gut tissue frequently co-express CD56 and differentiated into NK cells upon short-term culture with IL-15.

The thymus also appears to be a potential site of NK cell development. Thymocytes up to the double negative 2 stage retain the capacity to give rise to NK cells (Schmitt et al., 2004; Spits et al., 1998). Vosshenrich and colleagues (2006) have defined a thymus-dependent NK cell developmental pathway in mice. These thymic-derived

NK cells expressed high levels of CD127 (IL-7R), the transcription factor GATA-3 and differed functionally from the majority of murine (splenic) NK cells. This example (thymic NK cell development) underscores the importance of the particular environment (i.e. niche) in guiding the maturation of NK progenitors. Distinct niches likely provide unique combinations of developmental cues (discussed later) that shape NK cell function.

A peculiar population of NK cells is abundant in the decidua of the pregnant uterus, and the properties of these NK cells distinguish them from peripheral blood NK cells (Koopman et al., 2003; Yadi et al., 2008). These cells are characterized by a $CD56^{bright}$ phenotype and a lack of cytotoxicity. It has not been clearly resolved whether uterine NK cells migrate from peripheral blood or are derived from precursors that differentiate locally within the uterine environment (Ashkar et al., 2003; Keskin et al., 2007; van den Heuvel et al., 2005). The latter possibility would explain their unique characteristics. Thus, one of the emerging views is that NK cells can complete differentiation in various organs outside of the BM, and depending upon the site, these cells differ functionally. Elucidation of the factors present in a particular site and mechanistic explanations for how they impact NK development requires further study. Furthermore, how various sites of differentiation contribute to the heterogeneity of NK cell subsets is not well established.

Fate determining interactions with stroma

Receptor-ligand systems that direct cellular development and differentiation have been collectively referred to as 'morphogens'. They are the means by which the environment shapes the fate of developing progenitors (Moore, 2004). Signalling systems, including Notch, Wnt and others, are highly conserved throughout evolution (Pires-daSilva and Sommer, 2003). These systems are important in many aspects of embryogenesis and, thus, in the differentiation of multiple organ systems. Importantly, the actions of morphogens are developmental stage- and context-dependent. For example, triggering of the Notch receptor represses B cell differentiation and skews lymphoid precursors to T-cell lineage (Schmitt et al., 2004). However at later stages of B-cell differentiation, Notch signalling is required for terminal B-cell maturation (Santos et al., 2007).

The difficulty in studying the importance of a particular morphogen in hematopoiesis is related to their vast redundancy, due to multiple homologues that mediate similar (or overlapping) functions. Thus, the elimination of a single factor may be compensated for by other homologues. Furthermore, morphogens have major roles in early embryogenesis and therefore genetic manipulations often result in lethal defects, prohibiting the study of their role in NK development. Methods to circumvent this include the creation of BM chimeras by transplantation of foetal liver hematopoietic precursors into irradiated wild type recipients. This method can be used if the mouse embryo with deleted genes survives beyond the initiation of hematopoiesis (approximately day 9 post conception). Alternative approaches include conditional knock-out strategies using tissue specific or drug-inducible promoters driving the CRE recombinase. In this way, a given gene can be eliminated from a selected tissue (or lineage) and/or at the desired time by administration of the promoter-inducing drug.

Notch signalling plays a critical role in directing hematopoiesis and lymphocyte development (MacDonald et al., 2001; Maillard et al., 2003). In particular, thymic stroma supports T cell differentiation by the expression of the Notch ligand, delta like ligand-1 (DLL1). Accordingly the murine BM stromal cell line OP9, engineered to express DLL1, efficiently promotes in vitro T-cell differentiation by providing continuous Notch engagement (Schmitt and Zuniga-Pflucker, 2002). Early Notch signalling induces the acquisition of CD7 (La Motte-Mohs et al., 2005) and CD161 by hematopoietic precursors. Both also mark NK precursors (Bennett et al., 1996; Miller et al., 1994) , perhaps suggesting a role for Notch in NK commitment. Additional evidence for Notch involvement in NK differentiation comes from studies on murine early progenitors with lymphoid and myeloid developmental potential (EPMLs), where Notch signalling favoured NK development (Rolink et al., 2006). While DLL1 is the most extensively investigated ligand, Notch engagement by a different ligand, Jagged 2, also promotes in vitro NK differentiation from hematopoietic precursors (DeHart et al., 2005). We interpret these (and our unpublished studies) to show that signalling through Notch induces the development of common T-NK precursors. Upon continuous Notch engagement progenitors advance towards the T-cell lineage (Schmitt et al., 2004), whereas early Notch signalling appears to be sufficient for NK cell development. Importantly, Notch signalling is not absolutely required for NK cell differentiation (Radtke et al., 2000). Hematopoietic precursors cultured with, but not without Notch triggering (De Smedt et al., 2007) expressed cytoplasmic CD3ε. Subsequently, NK cells resembling Notch-dependent in vitro derivatives could be found in human cord blood but not in adult blood. While Notch signalling is not absolutely required for NK cell differentiation, it is mandatory for T-cell development (Radtke et al., 2000). Collectively, the key factors required for T-cell differentiation, IL-7 and Notch signalling, are both dispensable for NK development, questioning whether the

common T/NK precursor is the only pathway for NK cell development.

The Wnt signalling system represents another family of morphogens that influences NK cell development. Numerous Wnt proteins exist (18 identified members in mammals) and can interact with a receptor complex made up of the frizzled receptor and the LDL receptor-related protein (Staal et al., 2008). There are at least two intracellular Wnt signalling pathways, known as canonical and noncanonical. Canonical Wnt signalling leads to stabilization of β-catenin, which activates the LEF (lymphocyte enhancer factor) and TCF (T-cell factor, gene name tcf7) family of transcription factors. In the absence of LEF/TCF, neither T cells nor NK cells develop (Held et al., 2003). Gain-of-function and loss-of-function variants also demonstrate the importance of Wnt signalling in lymphopoiesis. In particular, a nondegradable, constitutively active β-catenin imposed lymphoid potential onto myeloid precursors (Baba et al., 2005). Despite the role of β-catenin in Wnt signalling, conditional deletion of β-catenin did not abolish lymphocyte development. Such cells showed sustained LEF/TCF expression, suggesting the contribution of an alternative, β-catenin independent pathway leading to LEF/TCF upregulation (Staal and Sen, 2008). Overall, these studies show that canonical WNT signalling via TCF/LEF is important in lymphopoiesis, including NK cell generation. In support of this, TCF expression was detected in NK cells, specifically in the $CD56^{bright}$ subset (Toor et al., 2001).

Another interaction recently shown to be involved in stroma-dependent NK cell differentiation is between GAS6 and protein S. These two highly homologous ligands, expressed on stroma, trigger the Tyro3/Axl/Mer protein tyrosine kinase receptors present on NK precursors. In animals lacking all three receptors, NK cells were phenotypically and functionally impaired (Caraux et al., 2006). Lack of only one receptor of this family (i.e. Axl) had a modest effect on NK cell development. Subsequently, fibroblasts expressing recombinant Gas6 could be shown to support in vitro NK cell differentiation (Caraux et al., 2006). Interestingly, the downstream signalling of Axl is reciprocally associated with IL-15 signalling (Hafizi and Dahlback, 2006) since Axl and IL-15Rα can heterodimerize. As a result, the IL-15 and GAS6-Axl pathways transactivate one another (Budagian et al., 2005). Indeed this association between IL-15 and Axl is operational in NK cell differentiation from human HPCs in vitro (Park et al., 2008). Perhaps the notion that GAS6–Axl interactions can transactivate the IL-15 pathway might provide the clue as to why IL-15-deficient mice have a residual population of NK cells (Vosshenrich et al., 2005).

Other receptor-ligand pairs with morphogenic functions in the differentiation of organ systems may also be involved in hematopoiesis. Members of the TNF superfamily of surface receptors are important in the development of lymphoid tissues (Mebius, 2003). Reciprocal interactions between hematopoietic progenitors and the nonhematopoietic stroma involve the expression of lymphotoxin-α on progenitors and lymphotoxin-β-receptor on stroma. This interaction (between LTα and LTβ-R) triggers IL-15 production by stroma, which in turn, supports NK cell development (Iizuka et al., 1999; Lian et al., 2004). Still other morphogenic receptors, including hedgehog, TGF—Smad, may also be involved in NK cell development; however, their roles have not been fully investigated.

Transcription factors involved in NK cell differentiation

Signalling through cell surface receptors results in a cascade of events that may ultimately lead to the activation of transcription factors. These DNA-binding proteins recognize consensus sequences in the promoter regions of target genes and influence gene transcription. The balance of multiple transcription factors, often with opposing functions, ultimately dictates whether initiation or repression of gene transcription occurs. Thus, depending upon the stimuli, certain genes are transcribed, while others are repressed. In this way, differentiation is directed towards a particular lineage. Consequently, several branching points in hematopoiesis are regulated by the balance of opposing transcription factors, such as Id proteins vs. E proteins or PU.1 vs. GATA-1 (see Figure 1.1). These opposing transcription factors regulate the expression of a number of genes, including receptors for lineage specific growth factors (discussed earlier). In this respect, PU.1 and C/EBPα drive expression of receptors for myeloid growth factors (G-, M- and GM-CSF), whereas GATA-1 induces erythropoietin receptor expression (Zhang et al., 1996). This opposition controls myeloid vs. erythroid lineage choice at a molecular level.

Transcription factors that play a dominant role in guiding differentiation towards a particular lineage have been referred to as master regulators. Expression of a master regulator is absolutely required for progression past a defined stage of differentiation. Precursors deficient in that factor are therefore unable to advance past a given checkpoint. PAX5 is an example of a master regulator of B-cell development. Cells deficient in PAX5 are unable to differentiate into mature B cells but retain myeloid, T cell and NK cell potential (Schaniel et al., 2002). Numerous transcription factors are involved in NK development and functional maturation. Mice lacking these factors have impaired NK cell generation. However, in most cases, the defect is not confined to

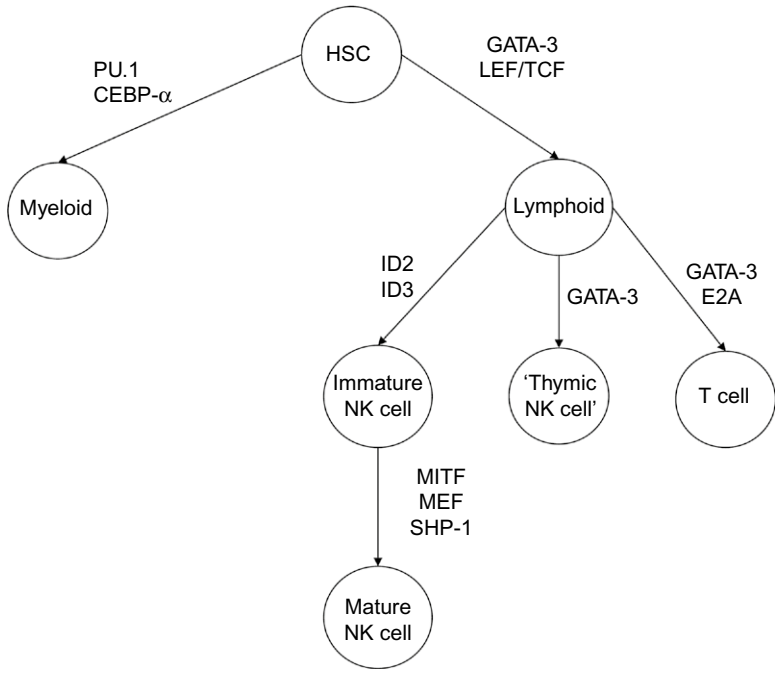

Figure 1.1 • Transcription factors important for lineage choice at pertinent branching points in differentiation of HSCs towards the NK lineage. Key transcription factors control the myeloid vs. lymphoid choice, such that PU.1 (dose dependent) and CEBPα favour myeloid lineage, whereas GATA-3 and LEF/TCF family members (downstream of Wnt) promote the lymphoid lineage. ID2 expression is required for NK cell development and antagonizes T cell fate promoted by E proteins (E2A, which is downstream of Notch). GATA-3 is necessary for both T cell and 'thymic NK cell' development. Other transcription factors, including MITF, MEF and SHP-1, are required for functional NK cell maturation.

the NK lineage. Along these lines, no master regulator (equivalent to PAX5 for B cells) has been identified or agreed upon for the NK lineage.

Consistent with the dominant role for IL-15 in NK cell development, transcription factors that are either down-stream of the IL-15R or are involved in IL-15 production by stroma are important for NK development. Accordingly, transcription factor defects influencing IL-15 signalling can be either intrinsic or extrinsic to the progenitor cell. Stat5a and Stat5b are transcription factors involved in signalling through the IL-2 and IL-15 receptor common β chain (i.e. CD122). Deficiency in Stat5b resulted in a ~50% reduction in splenic NK cells. These mice also showed a profound attenuation in NK cytotoxicity, which was not rescued by exogenous IL-2 or IL-15 (Imada et al., 1998). In parallel studies, Stat5a deficiency was associated with a marginal influence on either the number of NK cells or their function. As an example of hematopoietic progenitor cell-extrinsic factors, IRF-1 (interferon-regulatory factor-1) is required for IL-15 expression. Accordingly, irf-$1^{-/-}$ mice show an NK cell deficiency resembling IL-$15^{-/-}$ animals (Ogasawara et al., 1998). Hematopoietic progenitors from these irf-$1^{-/-}$ mice differentiate into NK cells with exogenous IL-15. Thus, the IRF-1 deficiency results in the impairment of the environmental support required for NK cell development. In contrast, irf-$2^{-/-}$ mice appear to have a defect that is intrinsic to NK precursors, resulting in a selective loss of mature NK cells in periphery, while BM NK cells are relatively unimpaired (Taki et al., 2005).

As described previously, Notch signalling has been implicated in NK cell development. Downstream effectors of Notch include the helix loop helix (HLH) proteins from the E protein family, including E2A and E47. These transcription factors are involved in the development towards the T-cell lineage (Ikawa et al., 2006). Similar to Notch deficiency (MacDonald et al., 2001), E47 deletion abrogates T-cell development, but NK cells and myeloid cells develop normally (Ikawa et al., 2006). A different set of transcription factors, which negatively regulates E proteins, is the set of dominant negative helix-loop-helix proteins, Id2 and Id3 (inhibitor of DNA binding 2 and 3). Both Id2 and Id3 are highly expressed by NK precursors. Upon further development, Id3 expression falls dramatically, while Id2 is sustained. These results support the involvement of Id2 in NK cell maturation. In fact, in the absence of Id2, NK cells fail to expand and mature, even though $CD122^{+}DX5^{-}$ NK precursors are present in BM (Yokota et al., 1999). The activity of Id proteins in NK cell development involves the dominant negative regulation of T-cell differentiation, in favour of NK cell development (Heemskerk

et al., 1997; Ikawa et al., 2001). The balance between Id2 and E2A dictates the ability of precursors to expand and terminally differentiate into NK cells. Moreover, the maturational defects seen in the absence of Id2 can be corrected by the deletion of E2A (Boos et al., 2007). Thus, Id transcription factors antagonize the Notch induced E proteins, diverting progenitors from the T-cell lineage towards the NK lineage (Fujimoto et al., 2007).

As described earlier, Wnt signalling results in activation of the transcription factors TCF-1 and LEF-1. In the absence of both, mice displayed a profound reduction in NK cells and T cells but not B cells (Held et al., 2003). The reduction in NK cells was seen both in the BM and spleen. Further studies showed that compared to LEF-1, TCF-1 played a more substantial role in NK cell differentiation and phenotypic maturation. Human studies confirmed that TCF-1 is acquired by HPCs as they differentiate into NK cells in vitro and that TCF-1 transcript and protein could be found in the $CD56^{bright}$, fraction of PB NK cells (Toor et al., 2001).

The transcription factor PU.1, a member of the Ets family, is strongly associated with myeloid differentiation (Dahl and Simon, 2003). PU.1 functions as a dose- and stage-dependent regulator of lineage fate in hematopoiesis. At an early branching point in hematopoiesis, PU.1 and GATA-1 antagonize one another, facilitating myeloid or erythroid differentiation, respectively (Stopka et al., 2005). At later developmental stages, PU.1 represses NK cell and T cell specific genes, favouring alternative lineages, including myeloid and B cells (Kamath et al., 2008). In $PU.1^{-/-}$ mice, NK cell differentiation is impaired, although not as severely as the T-cell and B-cell lineages (Colucci et al., 2001). Thus, the defect conferred by PU.1 deficiency is not NK specific. In contrast, a related transcription factor, Ets-1, is specifically required for NK development (Barton et al., 1998). In $Ets\text{-}1^{-/-}$ mice, the NK cell numbers were reduced (~threefold), and cytotoxicity was virtually absent, whereas T cells, B cells and other blood lineages were quantitatively normal. As a result, affected mice were more susceptible to lymphoid tumours upon challenge with the RMA-S cell line. MEF (myeloid Elf-1 like), another member of the Ets family, is also important for NK cell development. MEF appears to be involved at the latter stages when NK cells gain cytotoxicity. Deletion of MEF resulted in greatly impaired NK cell killing, as well as IFN-γ production. Mechanistically, the lack of cytotoxicity reveals a role for MEF in regulating perforin gene transcription (Lacorazza et al., 2002).

Similar effects on NK cell differentiation have been observed with C/EBPγ, a member of the leucine zipper family of transcription factors. These CCAAT/enhancer binding protein (C/EBP) transcription factors play a critical role in myeloid vs. lymphoid lineage determination. C/EBPα antagonizes the Tribbles homolog 2 (TRIB2) protein, one of the downstream effectors of Notch signalling (Wouters et al., 2007). Thus, C/EBPα antagonizes Notch signalling, favouring myeloid development. Another member of the C/EBP family, C/EBPγ plays a specific role in NK development. $C/EBP\gamma^{-/-}$ mice showed nearly normal numbers of NK cells, albeit with diminished cytotoxicity and cytokine production capacity, suggesting a maturational defect resembling MEF-deficient animals (described earlier). Likewise, perforin expression was greatly diminished in splenocytes from $C/EBP\gamma^{-/-}$ mice (Kaisho et al., 1999). Another transcription factor involved in NK cell functional maturation is MITF (microphthalmia transcription factor [MITF]), which similar to MEF, regulates perforin gene expression (Ito et al., 2001).

The family of distal-less (Dlx) homeobox proteins have been implicated in the development of multiple organ systems. This family contains multiple members, including Dlx 1 through Dlx 6. Dlx 3 is expressed preferentially at an immature stage of NK development (Sunwoo et al., 2008). The requirement for Dlx 3 in NK cell development has not been addressed since $Dlx\ 3^{-/-}$ mice die at an early embryonic stage, prior to the onset of hematopoiesis. However, over-expression of either Dlx 1 or Dlx 2 resulted in an arrest of NK development at an immature stage. Quite interestingly, over-expression of Dlx 1 also leads to a profound defect in the development of T lymphocytes and B lymphocytes, in addition to NK cells. These findings may suggest that this factor is important at the CLP stage.

Another TF involved in functional NK cell maturation is T-bet (Townsend et al., 2004), a factor known to control Th-1 lineage commitment and IFN-γ production by T cells (Szabo et al., 2000). $T\text{-}bet^{-/-}$ mice show reduced numbers of mature NK cells in the periphery and an increase in phenotypically and functionally immature NK cells (Townsend et al., 2004). This defect results from a higher rate of NK cell apoptosis in the periphery (i.e. spleen). T-bet is upregulated through STAT1 signalling, which, in turn, is triggered by IFN-γ. However, the requirement for STAT1 in NK cell maturation is less stringent than the requirement for T-bet, indicating that T-bet induced NK maturation can be STAT-1 independent (Townsend et al., 2004).

In contrast to T-bet, which drives Th1 polarization, the transcription factor GATA-3 is critically involved in Th-2 polarization of T cells. T-bet and GATA-3 are thus, expressed in a mutually exclusive fashion by Th1 and Th2 polarized $CD4^+$ T cells, respectively (Ouyang et al., 1998). In the absence of GATA-3, T-cell differentiation is abrogated (Ting et al., 1996), whereas NK cells do develop. NK cells from $GATA\text{-}3^{-/-}$ animals produce less IFN-γ and appear phenotypically immature (Samson et al., 2003). Thus, it appears that GATA-3, a factor critical for Th2 polarized $CD4^+$ T cells, is somewhat paradoxically

required for IFN-γ production by NK cells. The impairment of NK cells from GATA-3$^{-/-}$ mice likely reflects the importance of GATA-3 in the thymic pathway of NK cell maturation, discussed later (Vosshenrich et al., 2006).

Second messenger signalling in NK cell development

NK cells are regulated by surface receptors with activating or inhibitory functions. The majority of activating receptors use adapter proteins (including DAP10, DAP12, FcεRIγ and CD3ζ) to link surface receptors with intracellular signalling pathways, resulting in effector functions. These signalling pathways involve second messengers, including Syk, ZAP70, Lck, PI3Kinases (phosphoinositide 3-kinases), SAP, Fyn, Vav, Grb-2, Phospholipase-Cγ-1 and -2 (reviewed in Lanier, 2008).

The importance of the individual elements of activating receptor signalling pathways on NK cell development has been partially investigated. Animals deficient in DAP10 have normal numbers of NK cells. Since DAP10 serves as adapter protein for NKG2D, these NK cells had reduced NKG2D expression and function. Otherwise, NK cell activity was not diminished. Unexpectedly, DAP10$^{-/-}$ mice showed enhanced resistance to skin carcinoma and did not have increased spontaneous tumour formation (Hyka-Nouspikel and Phillips, 2006). DAP10$^{-/-}$ mice were also more resistant to melanoma challenge, thorough studies indicated that cell subsets other than NK cells (including NK-T cells and Tregs) were also involved in this effect. Similar to DAP10, deficiency of ZAP70 did not result in the impairment of NK cell development (Negishi et al., 1995). To the contrary, NK1.1^{+}CD3^{-} cells were more numerous in ZAP70$^{-/-}$ animals (Iwabuchi et al., 2001). The constitutional lack of other kinases, including Syk, or SAP/Fyn in mice did not block NK cell development either (Colucci et al., 2002; Turner et al., 2000).

PI3Ks are a family of kinases composed of multiple isoforms that encode both regulatory and catalytic subunits. The p110δ isoform of the catalytic subunit of PI3K is required for NK cytokine secretion (IFN-γ, TNF-α and GM-CSF), while the other tested isoform p110γ was not. Neither of these individual isoforms were absolutely required for terminal NK maturation and/or acquisition of cytotoxicity (Kim et al., 2007; Tassi et al., 2007). However, in the absence of both isoforms, NK cell numbers and cytotoxicity were greatly reduced. Studying a related protein, BCAP (B-cell adapter for phosphatidylinositol 3-kinase), it was noted that in BCAP$^{-/-}$ mice the NK cells are overtly long-lived, mature and functionally active (MacFarlane et al., 2008). NK cells from these animals were more resistant to apoptosis, suggesting that BCAP mediated ITAM signalling (and activation of the Akt pathway) negatively impacts NK cell maturation and survival.

The reductionist approach of studying individual proteins in NK cell development is feasible in rodents; however, the nonfully overlapping functions of individual molecules may be a confounding factor when translating these results to humans. Several genetic defects in humans are informative as to the role of these individual proteins in NK development. For instance, DAP12 deficiency results in a rare syndrome known as Nasu–Hakola disease in which presents with impaired osteoclast activity (bone cysts) and dementia. NK cells are normal and functional in these patients (Paloneva et al., 2000). Deficiency of another adapter molecule, CD3ζ, was reported in a single human with T^{-}B^{+}NK^{+} severe combined immune deficiency (SCID) (Roberts et al., 2007). Curiously, the NK cell repertoire of this patient consisted of a peculiar population of CD56^{-}CD16^{+} NK cells. Expansion of CD56^{-}CD16^{+} cells has been previously observed in other situations (following allogeneic hematopoietic cell transplantation, HIV infection and in cord blood). Such cells are thought to be either dysfunctional or immature. In this patient, virtually all NK cells were, CD56^{-} and cytotoxic activity was diminished compared to healthy controls (Roberts et al., 2007). It is difficult to dissect whether this is due to a direct effect of CD3ζ deficiency on NK development or an indirect effect via severe impairment of T cells. T cells are the main source of IL-2, which next to IL-15, is the dominant survival factor for NK cells. The other clinical states characterized by an abundance of CD56^{-}CD16^{+} cells are also associated with functional T cell deficiency (i.e. after HCT, in HIV infections or in newborns).

While activating receptor triggering leads to kinase activation, NK inhibitory receptors signal through phosphatases. These phosphatases oppose kinase function. Phosphatases involved in NK cell signalling include SHP-1 (src homology region 2 domain-containing phosphatase 1; PTPN6), SHP-2 (src homology region 2 domain-containing phosphatase 1; PTPN11) and SHIP (SH2-containing inositol phosphatase; INPP5D) (Vely et al., 1997). SHP-1 deficient mice (motheaten-viable [*me-v*]) show impaired terminal NK cell maturation, characterized by reduced cytotoxicity and IFN-γ production (Clark et al., 1981; Lowin-Kropf et al., 2000). However, competitive transplantation into wild type hosts did not reveal intrinsic defects in NK cells from *me-v* mice (Kim et al., 2005). This was unexpected since inhibitory receptor signalling was important for NK licensing in this study. Perhaps in the absence of SHP-1, other phosphatases (i.e. SHP-2 and/or SHIP) may substitute, as the reverse occurs in SHIP deficient animals (Yusa and Campbell, 2003). The role of SHP-1 in the acquisition of NK function was also studied using a dominant negative variant of this molecule. NK cells expressing the dominant negative SHP-1 were defective at rejecting MHC deficient BM transplants but showed otherwise normal responsiveness

(Lowin-Kropf et al., 2000). SHP-2 deficient murine embryos did not survive to permit analysis of their NK cells. The role of SHIP in NK cell development and function was also tested in two independent models, which showed that in the absence of SHIP, NK cells were more numerous with improved survival (Wang et al., 2002) and higher IFN-γ production (Parihar et al., 2005). Interestingly, $SHIP^{-/-}$ NK cells also had limited expression of MHC-specific inhibitory receptors, resulting in an inability to reject allogeneic BM grafts. Inhibitory receptor skewing may be related to SHP-1 substituting for SHIP (Wahle et al., 2007). The increased IFN-γ production by the $SHIP^{-/-}$ NK cells provides insight into the functional dichotomy between human $CD56^{bright}$ and $CD56^{dim}$ NK cells, since the latter population expressed higher levels of SHIP when compared to $CD56^{bright}$ cells (Trotta et al., 2005). To further prove the point, that differential SHIP expression underlies the observed functional differences between $CD56^{bright}$ and $CD56^{dim}$ subsets, SHIP was overexpressed in $CD56^{bright}$ NK cells, resulting in a significant reduction in IFN-γ production.

The impact of activating and inhibitory surface receptor triggering on NK cell development has also been studied. Collectively, it appears that NK cells with competent inhibitory receptors have superior functionality. In contrast, a rare subset of NK cells that lack competent MHC-specific inhibitory receptors was found to be nonfunctional. This has led to the concept of NK cell licensing—a requirement for inhibitory receptor signalling to attain full functionality (Kim et al., 2005; Lowin-Kropf and Held, 2000; Raulet et al., 2001). In contrast, exposure to ubiquitously expressed ligands triggering activating receptors resulted in a reduced capacity of mature NK cells (Fauriat et al., 2007; Sun and Lanier, 2008; Tripathy et al., 2008). This has fundamental importance for our understanding of the process of NK cell development and functional maturation. While the exact roles of particular inhibitory and activating receptors have not been dissected, the emerging picture is that this mechanism is in place to assure tolerance. Inhibitory receptor signalling capacitates NK cells, while activating signals appears to incapacitate them (Lowin-Kropf and Held, 2000; Raulet et al., 2001). This contrasts with the developmental requirement of T cells and B cells, which principally require activating signalling to successfully progress in development and maturation.

The NK cell ontogeny—lessons from evolution

Evolutionarily primitive species, up to the jawless vertebrates, rely solely on the innate immune system and lack both MHC and RAG gene families. The adaptive immune system, based on the activity of the RAG genes, first appeared in jawed vertebrates. Thus, the hallmark of adaptive immunity is the expression of unique antigen recognition receptors generated by somatic recombination. These receptors, expressed by B cells (BCR) and T cells (TCR) confer specificity and memory. NK cells lack these highly variable receptors, and their activation is controlled by the integrated signalling from numerous germline encoded receptors. Such a strategy is characteristic for cells of the nonadaptive or innate immune system.

It is difficult to establish whether NK cells predated the development of an adaptive immune system. For obvious reasons, phenotypic markers used to define mammalian (human) NK cells cannot be applied to invertebrates. One method of NK cell identification in invertebrates would be to search for cells with functional characteristics of NK cells (i.e. perforin mediated cytotoxicity and IFN-γ production). It is interesting to note that immunocytes (i.e. macrophages) from a molluscan slug (*Incilaria fruhstorferi*) express perforin and can reject skin allografts. In line with this notion, the rejected tissue showed features of perforin-induced cell death (Furuta et al., 2006). Therefore, lack of lymphocytes in this invertebrate is potentially compensated for by immunocytes that mediate both perforin-dependent cytotoxicity and phagocytosis. In higher species (i.e. vertebrates), these functional characteristics are performed by separate types of cells. However, it has been recently documented that human dendritic cells (DCs) (which have phagocytic properties) can also acquire perforin and kill tumours (Stary et al., 2007).

NK cells use the 'missing self' strategy of immune recognition to identify targets for elimination. This approach is reminiscent of the rules governing mating by fusion of sea sponge colonies of the *Botryllus* species (De Tomaso et al., 2005). Individual colonies select fusion-partners on the basis of sharing of histocompatability antigens. Fusion with another colony that is missing a 'self' allele is prevented by a mechanism of rejection, likely immune in nature. Coincidentally, receptors with high homology to the mammalian NK receptors (CD94 and/or CD161) have been identified on hemocytes (i.e. blood cells) of *Botryllus* as well as a related sea sponge, *Ciona intestinalis* (Khalturin et al., 2003; Zucchetti et al., 2008). The CD94 homologues in *Ciona intestinalis* have a similar function to the mammalian receptors since they inhibit hemocyte activation, thereby reducing phagocytosis (Zucchetti et al., 2008). Hence, inhibitory receptors with homology to mammalian NK-related lectin-like receptors are expressed on phagocytic cells in jawless vertebrates. Therefore, the missing-self strategy could have evolved primarily as a selection criterion for mating. Obviously, the use of this same strategy by NK cells could be an independent phenomenon, but it is difficult to overlook the role of NK cells in the process of

foetal implantation in mammals, which resembles the role of missing self recognition in implantation of marine invertebrate *Botryllus schlosseri* (Lightner et al., 2008). Moreover MHC, the target of NK recognition, has been implicated in partner selection in rodents and humans (Yamazaki and Beauchamp, 2007).

The evolution of NK cells as a cell type can also be considered from the perspective of NK-specific receptors. There are two main genomic clusters of receptors that encode proteins that regulate NK cell activation and inhibition. These are the leukocyte receptor cluster (LRC, chromosome 19q13.4) and NK gene complex (NKC, chromosome 12p13.1-2 in humans) (Trowsdale et al., 2001; Yokoyama and Plougastel, 2003). Encoded within the LRC are several gene families that share immunoglobulin-like structure and immune function. Included are the killer immunoglobulin-like receptors (KIR), as well as leukocyte Ig-like receptors or immunoglobin-like transcripts (LILR or ILT) (Cella et al., 2000). Both KIR and ILT clusters are believed to be related, perhaps derived from a common ancestor (Volz et al., 2001). Interestingly, KIR are expressed by NK cells and rare subsets of T cells (Uhrberg et al., 2001). In contrast, ILT receptors are found predominantly on NK cells, monocytes and DCs (Cella et al., 2000).

The NKC encodes a number of lectin-like receptors. Similar to the LRC, the NKC contains NK specific genes that are intermingled with receptors found on other cell types. Some of these lectin-like receptors are found mainly on dendritic and myeloid cells (Dectin1, CLEC4A/DCIR, Lox1), while several are distributed predominantly on NK cells and a subset of T cells (such as CD69, CD94, NKG2D), and still others are expressed by both myeloid/DC and NK cells (LLT1, MICL). The phylogenetic relationships between different lectin-like receptors within the NKC delineated 28 lineages of orthologous genes. The phylogenetic and physical clustering of NKC genes points to their origin by duplications, likely from a common precursor (Hao et al., 2006). As mentioned earlier, receptors encoded by LRC and NKC genes define functional activity of NK cells and are the means by which we classify these cells. The distribution of NK receptors argues that they can be placed evolutionarily between the myeloid and T cell lineages.

Lessons from embryogenesis

During foetal development, the first site of primitive hematopoiesis is the yolk sac. At a later time, it shifts to the AGM region (aorta–gonad–mesonephros) and continues in the foetal liver. Eventually, hematopoiesis is established in the BM. The contribution of the yolk sac to the intra-embryonic and definitive hematopoiesis is a matter of debate (Dzierzak and Speck, 2008; Tavian and Peault, 2005). More likely, these two regions (AGM and the yolk sac) are two independent sites of blood stem cell generation (Tavian and Peault, 2005; Yokota et al., 2006). Hematopoietic precursors from these two sites have been compared for their potential to generate distinct blood lineages (Tavian et al., 2001). This was done using in vitro culture assays for B cell (MS-5 stromal cell line) and T cell (foetal thymus organ culture [FTOC] assay) differentiation. Yolk sac precursors (i.e. extra-embryonic) could generate primitive nucleated erythrocytes, myeloid cells and NK cells, but lacked T cell and B cell generation potential. In contrast, progenitors from the AGM region (i.e. intra-embryonic hematopoietic cells) readily generated both B cells and T cells, as well as other lineages.

Thus NK cells, along with myeloid and erythroid cells, can be derived from yolk sac hematopoietic precursors, whereas T cells and B cells could not. Interestingly, the human embryonic stem cell line, H9, shows the same pattern of generating myeloid and NK cells but not T cells or B cells (Martin et al., 2008). In line with these findings, NK cells have been identified in human foetal liver as early as week 6 of gestation, whereas T cells are first observed in the foetal liver at 15–16 weeks (Phillips et al., 1992). These foetal NK cells had unconventional features, including the cytoplasmic expression of CD3ε and CD3δ subunits. However, they lacked membrane CD3 expression or TCR gene rearrangements, clearly distinguishing them from T cells. These findings demonstrate that NK development precedes that of T cells and B cells during embryogenesis. The presence of CD3ε and CD3δ subunits in the cytoplasm, may point to a common pathway of development for T cells and NK cells. Interestingly a proportion of cord blood NK cells also show cytoplasmic CD3ε, while this trait is not seen in adult PB NK cells. Studies of NK differentiation in vitro (De Smedt et al., 2007) show that cytoplasmic CD3ε expression is induced by DLL1-Notch triggering, perhaps implicating Notch signalling in the ontogeny of foetal liver NK cells but not adult NK cells.

Lessons from NK cell immune reconstitution after hematopoietic cell transplantation

Immune reconstitution is a process of rebuilding the immune system from transplanted HSCs. The reappearance of hematopoietic lineages follows a reproducible order, with monocytoid cells emerging first in the peripheral blood, followed by granulocytes and then NK cells. The recovery of NK cells significantly precedes T cells and B cells, with respect to both cell number and functional maturation (Storek et al., 2008). The timing of NK cell reconstitution coincides with myeloid

recovery, similar to the pattern observed during embryonic development (discussed earlier). This supports the notion that posttransplant immune reconstitution recapitulates ontogeny.

The first wave of NK cells found in peripheral blood, after transplant have distinct features (Jacobs et al., 1992). They are $CD56^{+}$ but are predominantly $CD16^{-}$ and KIR^{-}. The vast majority of these, early recovering NK cells express the CD94/NKG2A inhibitory receptor heterodimer as their sole MHC-specific inhibitory receptor (Cooley et al., 2005; Nguyen et al., 2005; Shilling et al., 2003). In many respects, the early recovering NK cells closely resemble the $CD56^{bright}$ subset of peripheral blood NK cells, whereas the fraction of cells corresponding to the $CD56^{dim}CD16^{+}$ NK cell fraction increase and predominate at later times after transplant (Shilling et al., 2003). This orderly appearance of $CD56^{bright}$ and $CD56^{dim}CD16^{+}$ subsets supports the model that $CD56^{bright}$ cells are recently differentiated and upon further maturation they assume the characteristics of $CD56^{dim}CD16^{+}$ cells. However the confounding role of immunosuppressive agents (Cyclosporin A) cannot be ruled out, as the predominance of $CD56^{bright}$ NK cells could also reflect their relative resistance to Cyclosporin A (Wang et al., 2007). An important factor in the early posttransplant reconstitution of NK cells are high levels of IL-15 elicited by the pretransplant conditioning chemotherapy and irradiation (Miller et al., 2005).

Stages of NK cell development

The differentiation of every type of cell can be seen as a network of phenotypic and epigenetic changes that ultimately leads to the mature cell type. Stages of development represent semistable nodes in this network, at which developing precursors accumulate before traversing to the next juncture (Warren and Rothenberg, 2003). Since developmental stages are mostly defined by surface phenotype, the scheme of NK development elucidated in mice is not easily applicable to humans. This is related to the fact that key phenotypic markers of humans NK cells do not apply to mice (i.e. CD56, CD16, and KIR).

Systematic analysis of phenotypic and functional characteristics of NK cells has led the Yokoyama laboratory to propose five stages of murine NK development (Kim et al., 2002). The first stage is the NK precursor, marked by a $CD122^{+}NK1.1^{-}$ phenotype (Rosmaraki et al., 2001). These cells go on to acquire NK1.1 (NKRp1, CD161) and CD94/NKG2A at stage II, followed by Ly49 at stage III. Notably the acquisition of the two major types of MHC-specific inhibitory receptors (Veinotte et al., 2003) (CD94/NKG2A followed by Ly49) marks important steps in maturation, a pattern also observed in humans (Grzywacz et al., 2006) (discussed later). At the fourth stage, termed the 'expansion stage', NK cells undergo significant proliferation before reaching the final maturational stage, stage V, characterized by full cytotoxic ability and IFN-γ production. Stage V NK cells also acquire Mac-1 (CD11b) and CD43. More recently, cells at this final stage of maturation have been further subdivided according to the level of CD27 expression (Hayakawa and Smyth, 2006). Murine $CD27^{high}$ NK cells progress to a $CD27^{low}$ stage. In contrast to the $CD27^{low}$ subset, $CD27^{high}$ NK cells had lymph node migratory capacity and the ability to interact with DCs. These $CD27^{high}$ NK cells also had higher cytotoxicity and produced more IFN-γ in response to IL-12 and/or IL-18 stimulation compared to the $CD27^{low}$ counterparts. In support of their relative immaturity, the $CD27^{high}$ NK cells uniquely expressed c-kit receptor (CD117) and IL-7R (CD127) and showed a lower proportion of Ly49 receptor expressing cells. How these $CD27^{high}$ NK cells are related to the thymic pathway of NK maturation (Vosshenrich et al., 2006), also marked by CD127 expression, is not known.

The stages of human NK differentiation did not emerge as a direct correlate of murine studies. Beginning with the NK precursor subset, as defined in mice (Rosmaraki et al., 2001), the available reagents for staining of human CD122 (IL-2/IL-15Rβ) do not allow for a clear discrimination of a distinct $CD34^{+}CD122^{+}$ subset of human hematopoietic precursor cells (Grzywacz, unpublished). Instead, CD7 has been used to distinguish cells committed to the NK/T lineage (Miller et al., 1994). NK1.1 is an early marker of murine NK cells, which corresponds to human CD161. Human NK precursors with a $CD34^{-}CD161^{+}CD56^{-}$ phenotype have been characterized in vitro and are also found in peripheral blood and cord blood (Bennett et al., 1996). The loss of CD34 suggests that they have progressed in differentiation; however, these cells have not yet acquired cytotoxic ability. Culture with IL-2 promoted further NK differentiation, with the acquisition of CD56 and other NK receptors, cytotoxicity and IFN-γ production. Other investigators have also found that CD161 marks an early common NK/T precursor found in murine foetal thymus (Michie et al., 2000).

More recently the expression of CD45RA, along with integrin α4β7 has been used to identify a subset of human peripheral blood $CD34^{+dim}$ hematopoietic precursors that preferentially differentiate into NK cells in vitro upon IL-15 stimulation (Freud et al., 2005). The presence of integrin α4β7 has previously been used to distinguish progenitor cells with gut and lymph node homing capacity (Yoshida et al., 2001). The Caligiuri group proposed that $CD34^{+}CD45RA^{+}Integrin\beta7^{+}$ cells home to lymph nodes and differentiate into NK cells at this site. This was based on the identification of lymph node resident cells at four

consecutive stages of NK development (Freud et al., 2006). These include the pro-NK cell ($CD34^+CD45RA^+$ $Integrin\beta7^+$, stage 1), pre-NK cells ($CD34^+CD117^+$, stage 2), iNK ($CD34^-CD117^+CD161^+CD94^-$, stage 3) and $CD117^{low}CD94^+$ $CD56^{bright}$ NK cells (stage 4). The authors go on to hypothesize that $CD56^{dim}$ NK cells constitute the final, fifth stage of NK differentiation, that is completed outside of the LNs (Freud and Caligiuri, 2006). Notably, CD56 acquisition is not an essential criterion in this model; however, consecutive NK developmental stages are marked by increasing CD56 expression. Our own studies on NK cell differentiation in vitro show that $CD34^+$ cells cultured on a monolayer of murine foetal liver cells and in the presence of cytokines (IL-3, IL-7, IL-15, FLT-3L and SCF) robustly develop into NK cells. Using CD56 as a marker of NK cells, we distinguished two $CD56^+$ subsets: immature NK cells ($CD56^+CD117^{high}CD94^-$) that could give rise to a more mature, functional NK cells ($CD56^+CD117^{low}CD94^+$) (Grzywacz et al., 2006). The transition from the $CD56^+CD117^{high}CD94^-$ to the $CD56^+CD117^{low}CD94^+$ stage was associated with the acquisition of activating receptors (NKp30, NKp46 and NKG2D), inhibitory receptors (CD94/NKG2A) and functionality (cytotoxicity and IFN-γ production). The immature NK cells did not express perforin or granzyme B but were $CD161^{+,}$ which, as mentioned earlier, is an early marker of murine and human NK cells. Immature $CD56^+CD117^{high}CD94^-$ cells could be found in UCB and thus, represent physiological relevant intermediates of NK maturation. Moreover, the $CD56^+CD117^{high}CD94^-\rightarrow CD56^+CD117^{low}CD94^+$ transition is equivalent to the stage 3→stage 4 progression in lymph nodes (Freud et al., 2006).

The NK cells derived from in vitro culture rarely expressed KIR or CD16. Moreover, the acquisition of these two receptors was not coordinated at a single cell level, meaning that some developing NK cells acquired KIR but not CD16 (and vice versa) (Grzywacz, unpublished). This is somewhat unexpected considering that in vivo KIR expression is almost exclusive to the $CD16^+$ NK subset. Overall, in vitro derived NK cells as well as the NK cells developing in LN have features reminiscent of the $CD56^{bright}$ subset. The work by Ferlazzo et al. (2004) documented the abundance of $CD56^{bright}$ NK cells in secondary lymphoid tissue. Isolation of these cells and further in vitro culture with IL-2 lead to perforin, KIR and CD16 acquisition. Thus far, the conditions required to advance the in vitro derived NK cells to a $CD56^{dim}CD16^+$ stage are difficult to reproduce. Chan et al. (2007) had the interesting observation that $CD56^{bright}$ NK cells from the peripheral blood can develop into $CD56^{dim}$ cells upon interaction with synovial fibroblasts through a CD56:FGF-R1 interaction. It would be interesting to determine whether in vitro derived NK cells will follow a similar pattern. In another study transpresentation of IL-15 promoted acquisition of CD16 and KIR by $CD56^{bright}CD16^-$ NK cells in mice engrafted with human hematopoietic system (Huntington et al., 2009).

As with murine NK cells, human peripheral blood NK cells can be divided on the basis of CD27 staining intensity. $CD56^{bright}$ and $CD56^{dim}$ human NK cells show $CD27^{high}$ and $CD27^{low}$ expression, respectively. This has led the Smyth group to propose that murine $CD27^{high}$ NK cells correspond to $CD56^{bright}$ cells in humans (Silva et al., 2008). In fact, the LN homing, DC interaction, and IFN-γ production of the murine $CD27^{high}$ NK cells are also properties of $CD56^{bright}$ NK cells (Fehniger et al., 2003). Moreover, murine $CD27^{high}$ cells reconstitute early after BM transplantation, followed by the emergence of $CD27^{low}$ NK cells, reminiscent of the predominance of $CD56^{bright}$ cells early after transplant in humans. However, the $CD27^{high}$ subset in mice is more cytotoxic, as opposed to the relatively low cytotoxicity of human $CD56^{bright}$ ($CD27^{high}$) NK cells. Also, the expression of CD94/NKG2A, universally high on human $CD56^{bright}$ NK cells, does not distinguish murine $CD27^{high}$ and $CD27^{low}$ subsets. Despite these discrepancies, the dichotomy of NK cells subsets distinguished by CD27 expression can be observed in both mice and humans and appears to correspond reasonably well with a linear model of NK cell maturation.

Acquisition of inhibitory receptors during NK cell development

NK cells are restrained from auto-aggression by inhibitory receptors that are specific for MHC class I (HLA). Human MHC-specific inhibitory receptors belong to two structurally distinct families: (1) the lectin-like, CD94/NKG2A complex and (2) the immunoglobulin-like, KIR. Acquisition of CD94/NKG2A by NK progenitors marks an important step in NK development. This is because CD94/NKG2A expression is coordinated with attainment of functionality (activating receptor expression, cytotoxicity and IFN-γ production) (Grzywacz et al., 2006). Thus, the linking of inhibitory receptor expression with effector mechanisms appears to be a form of tolerance during NK development.

The order of inhibitory receptor acquisition during NK cell development appears not to be circumstantial. The ligand for CD94/NKG2A is HLA-E (Braud et al., 1998; Lee et al., 1998). This nonclassical HLA molecule has limited polymorphism. The conserved sequence of HLA-E and its ubiquitous expression, assures that CD94/NKG2A will find its ligand on all healthy cells and tissues (Kaiser et al., 2005). Similarly, CD94 and NKG2A both have strikingly conserved sequences in the human