Natural Killer Activity: Early Days, Advances, and Seminal Observations

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ABSTRACT: This manuscript describes the early history of NK cell discovery, with emphasis on the events in the first decade of NK cell studies, 1972–1982. The authors highlight some of the earliest and most important observations that would later prove to be milestones in the study of NK cells and their activity.

KEY WORDS: NK cells, discovery, characterization

ABBREVIATIONS: FcR: Fc-receptor; **IFN:** interferon; **Ig:** Immunoglobulin; **IL-2:** interleukin-2; **LGL:** large granular lymphocytes; **NK:** natural killer

I. INTRODUCTION

What were the earliest observations of natural killer (NK) activity or associated NK cells? Most important findings have their origins in the work of many people, and the description of NK activity and the associated cells was no different.

The earliest studies on NK function can be linked to several papers that described cellular assays performed in an attempt to elucidate immune reactivity to viral pathogens or tumor cells. In this review, we identify some of the seminal early reports that contributed significantly to the identification of a new immunological process that has become known as NK activity. Although this is not a complete review of all early NK cell studies, we highlight some of the earliest and most important observations that later proved to be milestones in the study of NK cells. We focus on the events in the first decade of NK studies, 1972–1982.

II. EARLY REPORTS

During the study of T-cell immune reactivity to tumor antigens in patients with cancer, several reports emerged of "non-specific" reactivity observed *in vitro*. One early example was a family study by E.B. Rosenberg, R.B. Herberman, P.H. Levine, R. Halterman, J. McCoy, and J.R. Wunderlich,[1] which provided one of the first reports of unexplained "natural" cytotoxic reactivity. Even more importantly, this report was one of the first to suggest that this cytotoxicity was a real phenomenon, not just an *in vitro* artifact. Although the biological importance of the cytotoxicity was not clear at this point, the authors did state in their conclusions: "Positive cellular cytotoxicity reactions to leukemia-associated antigens by lymphocytes of family members and normal unrelated individuals are of great interest. Tumor surveillance mechanisms have been postulated which could explain why lymphocytes from normal individuals would destroy cells bearing tumor antigens."

By early 1972, a number of research efforts were focused on attempting to define immune reactivity to cancer cells and determining the best methodology with which to evaluate this reactivity. In June of that year, the US National Cancer Institute held a "Conference & Workshop on Cellular Immune Reactions to Human Tumor-Associated Antigens" in Bethesda, Maryland.[2] The program committee

11040-8401/14/$35.00

was composed of many noted researchers: Conference Chairman: Ronald B. Herberman; Program Committee: Ronald B. Herberman, Paul H. Levine, Clarice E. Gaylord, and Cathleen L. Baughman; with the monograph of the meeting being edited by Drs. Herberman and Gaylord. This conference focused on (1) cytotoxicity assays, (2) migration inhibition assays, (3) lymphocyte stimulation, and (4) skin tests. In this conference, the recognition of spontaneous cellular activity to tumors of various origins became evident. The participants included a variety of scientists from noted research institutions, including the Karolinska Institute (G. and E. Klein); the US National Institutes of Health (R. Herberman and R. Oldham); UCLA (M. Takasugi and S. Golub); and the MD Anderson Institute (J.G. Sinkovics). A brief summary of the presentations, assays, and key findings is provided in Table 1.

Many key questions were proposed to be addressed at the conference: **(**1) What types of antigens are being detected in the various assays? (2) What are the phases of the immune response that these assays measure? (3) What is the nature of the reactive lymphocytes—are they T-lymphocytes or B-lymphocytes? (4) What role do lymphocyte-dependent antibodies play in the observed responses? (5) What are the relationships of these various assays to each other? (6) How reliable and reproducible are the results of these various assays? (7) Importantly, can the assays be used to differentiate patients with neoplastic diseases from those with benign disease or from normal individuals?

A large portion of the conference examined different *in vitro* assay systems, including; cytotoxicity, migration inhibition, and lymphocyte stimulation. Cytotoxicity assays discussed included microcytotoxicity, 3H-proline release, 51-chromium release, and 125-iodine release assays. Using these assay systems many participants at this conference reported *in vitro* reactivity of "control" or "normal" lymphocytes in their assays. This activity was not well understood at the time but was consistently observed by many participants. Sinkovics et al. concluded, "Cultured lymphocytes were nonspecifically cytotoxic to a battery of target tumor cells. Purified lymphocytes were less cytotoxic." Oldham et al. concluded, "Wide variations in the ability of lymphocytes from normal individual to lyse tissue-culture lines has been evident." McCoy et al. from Litton Bionetics Research Laboratories concluded, "Normal human lymphocytes … directly lysed human tumor cell lines."

Two general conclusions were drawn at the conclusion of this conference: (1) It is certainly possible that some or all normal individuals have immune reactivity against tumor cells or cell lines derived from tumors. (2) This could be activity against some cross-reactive antigens, e.g., bacterial or histocompatibility antigens.

Additional questions were raised in the summary of the meeting: (1) Does the activity seen with leukocytes from normal individuals represent real immunologic activity against tumor-associated antigens? (2) Is this activity just noise or problems with setting the baseline in the assays?

This important conference led to a critical increase in awareness regarding the "spontaneous" *in vitro* antitumor activity of normal leukocytes and the recognition that further studies were necessary to characterize this activity associated with unstimulated leukocytes. Thus began the first major push into the study of the "natural" or "non-specific" reactivity associated with normal, i.e., unstimulated, leukocytes.

III. EARLY DISCOVERIES AND CONTRIBUTIONS

By 1975, a series of key papers has been published that set the stage for important discoveries and the characterization of tumor cell killing by normal leukocytes. While previous immunologists had limited this *in vitro* function solely to sensitized T lymphocytes, two papers by Herberman et al. provided insight into the phenotype(s) responsible for natural cytotoxic activity of leukocytes in the mouse.[3,4] The first paper demonstrated that the antitumor effector cells from non–tumor-bearing mice was mediated by a unique subpopulation of non-adherent lymphoid cells with no known T- or B-lymphocyte cell-surface markers. These cells were termed N cells.[3] The second paper described the broad specificity associated with this lytic activity from normal mice, possibly associated

TABLE 1: Summary of select reports from 1973 NCI Monograph

Author	Test employed	Target(s)	Method of analysis	Major conclusion
J.S. Sinkovics, K. Tebbi, and J.R. Cabiness MD Anderson	Microcytotoxicity	Rhabdomyosarcoma, Renal carcinoma, Breast carcinoma	Visual analysis	Established cultures of lymphocytes were nonspecifically cytotoxic to a battery of target tumor cells.
Charles F. McKhann, MD. Patrick H. Cleveland, Ph.D. and Martyn W. Burk, M.D Univ. Minnesota	Cytotoxicity	Sarcoma	51-Chromium 3H-Thymidinerelease	Nonspecific lysis of target cells by normal lymphocytes is variable and should be considered when using this combination as a control for target cell killing by immune lymphoid cells.
Robert K. Oldham, David Siwarski, James L McCoy, Ernest J. Plata, and Ronald B. Herberman NCI & Litton Bionetics Research	Cytotoxicity	Panel of breast carcinomas, melanomas, colon carcinomas, melanomas sarcomas	125-IUDr release	Wide variation in the ability of lymphocytes from normal individuals to lyse these tissue-culture lines has been evident. Some, but not all, of this variability may be related to the method of lymphocyte purification.
James L. McCoy, Ronald B. Herberman, E.B. Rosenberg, F.C. Donnelly, Paul H. Levine, and C. Alford Litton Bionetics & NCI	Cytotoxicity	Human lymphoid lines: NC-37 and F265	51-Chromium release	The results suggest that true cell-mediated immunologic reactions cause the lysis of human tissue-culture lymphoid cells by normal donor lymphocytes. The relevant antigen(s) appears neither to be "blast" associated nor to be related to antigens of fetal bovine serum.
M. Takasugi, M.R. Mickey, and P.I. Terasaki Univ. California	Micro-cytotoxicity	Cultured human primary tumor cells	Electronic Image Analysis	The choice of a correct control in the test also became a problem when some normal individuals were found to react strongly and feeder effects were observed. A score was developed based on percent reduction from either medium controls or from negative lymphocyte tests with the maximum number of target cells.
Henryk Skurzak, Ladislaus Steiner, Eva Klein, and Ed Lamon Karolinska Institute	Micro-cytotoxicity	Glioma lines; 118 & 158 MG; Glia lines: 587 & 622 CG, Osteosarcoma 2T	Visual Analysis	The pattern of cytotoxicity appeared to reflect target cell sensitivity rather than tumor specificity. Lymphocytes from the nontumor patients often produced cytotoxic effect against the target cells that was most pronounced.

Selected summary from NCI *Conference & Workshop on Cellular Immune Reactions to Human Tumor-Associated Antigens*.[2]

with murine endogenous type-C viruses. This activity was not observed against normal cells or some tumor cell lines.[4]

At about the same time, Karolinska et al. reported similar results in the mouse.[5] They concluded that the spontaneous cytotoxic activity of normal mouse spleen cells against Moloney leukemia cells was exerted by small "undefined" lymphocytes; they termed them natural killer (NK) cells. This name defined the cells based on their function, and the name has been preserved to this day.

In addition, Karolinska et al. also observed strong spontaneous lymphocyte-mediated cytotoxicity against a mouse lymphoma cell line (Yac-1), thus defining the prototype mouse NK target.[6] Sendo et al. reported similar natural cytotoxicity using a Balb/c target, RLmale 1.[7] Soon thereafter, Pross et al. reported the use of K562 to evaluate human spontaneous lymphocyte-mediated cytotoxicity, thus defining the prototype human NK target.[8]

All of these reports from the earliest studies of NK cells defined critical aspects of NK activity and set the stage for a later series of seminal discoveries and conferences (Fig. 1) regarding the nature and characteristics of these cells. The first NK Workshop was held in 1982 in North Carolina and was organized by Hillel Koren. The format of this meeting was very informal due the small attendance (~50 people). As can be seen in Fig. 1, many of the discussions occurred in small discussion groups. Formal presentations were few with many presenters merely stating a scientific observation followed by informal discussions. This initial meeting has been followed by a series of NK workshops that are still held today under the oversight of the Society of Natural Immunity starting in 1992.

IV. NATURE OF NK EFFECTOR CELLS

In spite of the relatively crude research tools available at the time, a number of early studies were able to begin to define the phenotype, specificity, and regulation of NK cells in both mouse and human. During the mid to late 1970s, NK cells were mostly defined by their lack of markers.[9–16] NK cells lacked T- and B-lymphocyte cell-surface markers; most were Fc receptor (FcR) positive. But unlike monocytes they were non-adherent to plastic. Bolhuis et al. observed that human NK cells, which possessed a FcR for IgG, did not utilize this receptor in its NK killing function and concluded that NK cells must utilize an (as yet) undefined receptor(s) to mediate their function.[16]

However, the lack of unique markers on NK cells made their acceptance by the larger immunologic community difficult, and many investigators simply considered the spontaneous *in vitro* cytotoxicity seen with normal leukocytes to bean artifact.

This all changed in 1979 when the uniqueness of NK cells first became evident with the observations that human NK cell activity was highly associated with a relatively minor population of unique leukocytes called large granular lymphocytes (LGLs).[17,18] This observation led to the identification of unique human and rodent markers on these cells,[19–28] which allowed the enumeration, isolation, and functional analysis of purified NK cells. This discovery was a major milestone in the understanding of NK cells because most previous studies have not been able to not identify a unique cell mediating this *in vitro* function.

The presence of unique markers on NK cells also allowed the evaluation of NK cells *in vivo* during animal studies and in man during clinical trials. In the mouse, the ability to use antibodies against these unique markers for *in vivo* depletion of NK cells[29–31] helped to identify new functions of murine NK cells.

Related to the finding that NK cells had a LGL morphology was the observation of LGL leukemias in both humans and rats.[32–34] These leukemic cells provided a useful source of cellular materials for studies on the characteristics of these cells, including the use of perforin in NK-mediated killing.[33,34]

V. SPECIFICITY OF NK CELLS

Because of the lack of unique markers and the lack of appropriately definitive research tools, early experiments to study the specificity of NK cells were difficult to interpret. This is clearly demonstrated in

FIG. 1: First NK Workshop Collage – chaired by Hillel Koren in NC, USA. As shown, the first NK workshop was small and informal. The photo collage does not represent all of the key attendees' but provides a flavor of these early meetings. Panel A – Herberman (left), Targan (center) Gorelik (right); Panel B – Bonivida (left), Goldfarb (center), Pollack (right); Panel C – Ortaldo (left), Bloom (right), Roder (right most); Panel D – Keissling (left), Pollack (center), Santoni (right); Panel E – Bennett (left), C. Lopez (2nd Left), J. Linna (right), Welsh (right most); Panel F – Keissling (left), Brunda (2nd Left), Wigzell (right).

early studies where some, but not all, lymphoma cells were highly susceptible to NK-mediated lysis.[5,35,36] In addition, analysis of multiple inbred strains of mice added to the complexity of these observations, since different strains were classified as either "high" or "low" for the ability of their leukocytes to kill the prototype murine target Yac-1.[5,6,8,35,36]

In the mouse, one assay that was used to elucidate the specificity of NK-mediated lysis was the competitive cold-target inhibition assay. This assay allowed a panel of target cells to be used with specific indicator targets to identify common patterns of inhibition and perhaps common antigens.[5,35] In humans, similar studies were done using competition assays which indicated a complex pattern of NK target-cell recognition.[37,38]

Another early technique used to study NK specificity was the effector cell adsorption assay, based on the early observations[38,39] that NK cells form strong conjugates with tumor targets.[17] This adsorption assay also demonstrated unique patterns of target cell recognition and suggested multiple "receptor-

ligands" that might be involved in NK lysis.[39,40] The conclusions of these studies were that "human NK cells may be heterogeneous, with each subpopulation recognizing different antigenic specificities on target cells."

Additional early studies based on multiple-target monolayer adsorption analysis suggested that NK cells may utilize a minimum of seven antigenic specificities/receptors.[39]

The demonstration that NK cells can react selectively with some, but not all target cells, ruled out the frequent contention that *in vitro* NK activity simply represented a nonspecific binding or nonspecific membrane interaction. However, major issues remained: (1) Is the specificity of natural reactivity directed toward antigens that are common to a wide variety of cultured cell lines and tumor cells? (2) Does natural cell-mediated cytotoxicity represent a basic immune surveillance mechanism against tumors directed against a series of broadly distributed antigens on tumor cells? (3) How is this activity regulated? Finding the answers to these and other questions regarding NK cells will be the basis of studies for years to come.

VI. NK CELL REGULATION

Like other leukocytes, early studies on the regulation of NK activity indicated that NK cells were highly regulated. Their functional activity could be rapidly increased by a variety of natural agents and pathogens.[41] Several laboratories made the observation that the cytotoxic activities of NK cells were rapidly activated early during virus infection.[42–44] In addition, infection of target cells by a number of viruses rendered these cells susceptible to lysis by NK cells. These studies concluded that NK cells may be important factors in immune surveillance against both virus-induced tumors and virus infections. However, these observations also led to the important observation that virally-induced interferon was a major positive regulator of NK activity.[45–51]

The rapid and potent regulation of NK cells by interferons in the mouse led to early studies that evaluated the potential for interferon therapy in cancer using recombinant interferons.[49] In these studies, "Patients received large doses of interferon to determine (1) whether interferon could induce NK lymphocytes in the peripheral blood of man, and (2) whether there are characteristic kinetics for the appearance, disappearance and reactivation of NK lymphocytes following interferon therapy." These studies demonstrated that the "activation of human NK cells was observed by the systemic inoculation of human subjects with interferon." This observation was followed by numerous additional clinical trials that attempted to manipulate *in vivo* human NK activity with a variety of recombinant proteins.

Another agent that emerged during this period of time, which was both a potent regulator of NK cell function but also a growth factor for NK cells, was interleukin-2 (IL-2). Early studies with T-cell growth factor (later named IL-2) demonstrated its potent effect on NK cells. These studies demonstrated that IL-2 could potently activate NK cells and broaden their range of target cell lysis.[52–54] Where previous studies had focused on the lysis of leukemia and lymphoma targets by NK cells, IL-2-activated NK cells could lyse solid tumors,[52–54] and IL-2-activated NK cells had potent *in vivo* activity.[53]

In addition to positive regulation, it became evident that NK activity could also be rapidly inhibited under certain stress conditions and after certain pathogen insults.[55,56]

A. Functions of NK cells

1. Anti-tumor Activity

The earliest reports of natural killer activity were in murine leukemia and lymphoma models using virus-induced targets. However, initial studies evaluating NK activity in man[1–7] indicated that there was little evidence for direct killing of autologous tumors. This discrepancy led to a large number of studies, in both the human and a variety of animal models, to explain this difference. The total number of citations for studies that evaluated "natural killer" (NK) or "natural cytotoxic" function after 1975 rose

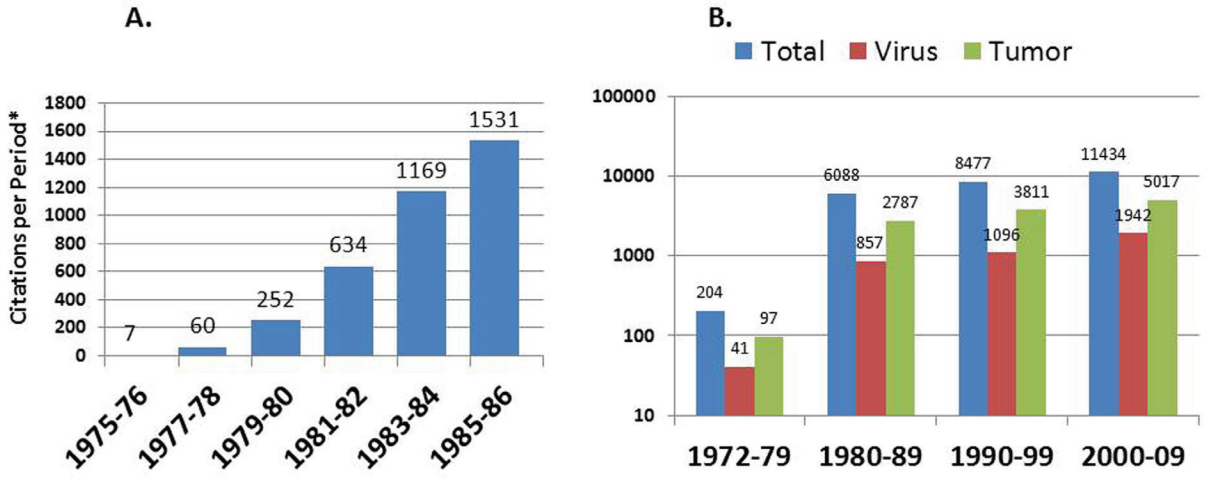

FIG. 2: Graphic represention of PubMed articles published that contained the phrase "natural killer" or "natural cytotoxic". Panel A depicts the period from 1975 thru 1986. Panel B depicts four decades of citations that are further subdivided into those also containing the word "tumor" or "virus".

rapidly after 1975 (Fig. 2a). During the decade from 1980 to 1990, there was an explosion of studies that evaluated NK cells in the tumor setting. Considering the total number of NK references by decade, a vast increasein the number of NK reports can be seen during the 1980s (Fig. 2b). Most of these citations were associated with the keyword "tumor", with the keyword "virus" being a distant second.

Early animal studies employed tumor cell lines to evaluate NK activity *in vitro*. However, evidence for the antitumor immune surveillance role of NK cells *in vivo* was only circumstantial, based primarily on strain variations for tumor incidence and *in vitro* tumor cell killing.[41] Perhaps one of the most important factors leading to the dramatic increase in the number of references regarding NK antitumor function was the discovery that NK cells bear asialo-GM1[57] and express NK1.1[26] antigens on their surface. Ultimately, the use of antibodies to these antigens allowed for the direct evaluation of the role of NK cells in tumors.[42–44,58] These reagents allowed for a large number of studies to be performed studying the *in vivo* role of NK cells in tumor surveillance, viral and parasitic infections, and non-pathogen systems as described in the following reviews.[41,59–62]

2. Anti-viral Activities

As noted above, the *in vitro* ability of murine NK cells to lyse tumor cells that were of viral origin led to the early hypothesis that non-tumor virus-infected cells may also be primary targets for NK cells. Anderson et al.[42] noted, "… The presence of virus infection may be of prime importance in determining the susceptibility of cells to lysis by unsensitized NK lymphocytes. Indeed, preliminary results have been obtained indicating that infection of L cells with the Kunz strain of influenza A renders these cells similarly susceptible to innate cytotoxicity. The importance of NK cells in immune surveillance against both virus-induced tumors and virus infections generally is likely, therefore, to be considerable and worthy of further study." These results were also observed with human NK cells where NK-resistant cells became susceptible NK targets after viral infection.[58,63]

In addition to the *in vitro* lysis of virus-infected targets, an *in vivo* role of NK cells in viral infection was suggested by Welsh et al. [43] in studies which concluded, "The advent of NK cell activity correlated with the synthesis of interferon in LCMV-infected mice. ... These experiments suggest that LCMV induced NK cells via an interferon-dependent mechanism."

Further evidence for the role of NK cells in virus infection was provided by the studies of Biron et al. in a patient lacking NK cells that had recurring severe herpes virus infections.[64]

3. Response to Bacteria and Parasites

Another area of research, which was only partially appreciated from early NK studies, was the potential for NK cells to play a role in a variety of bacterial and parasitic diseases. Early studies with bacterial agents suggested a role for NK cells in the response to these agents.[50] While there was little early evidence regarding the direct role of NK cells in controlling parasitic infections, there were reports of correlations of parasitic infection and NK cell activation. Eugui et al. demonstrated that in response to malaria, "…NK cells were recruited and activated by T lymphocyte-mediated immune responses to parasite antigens."[65,66] In addition, in hemoprotozoan infections, a possible correlation with NK activity was shown, in that "marked activation of NK cells occur, in resistant strains but not in susceptible ones."[65] In summary, these early studies examining the potential for NK cells to play a role in parasitic diseases concluded that "Of the nonspecific factors, macrophage activation, natural killer cells, and serum factors other than antibodies are critical in the battle against parasites."[66]

4. Hybrid Resistance

Early studies on the phenomenon known as hybrid resistance (i.e., F1 anti-parent transplantation resistance) suggested that the effector cells, which mediate this resistance, share characteristics with NK cells.[62] The *in vivo* regulation of F1 bone-marrow transplantation was later confirmed to be mediated by NK cells.[67] As we know today, the receptors responsible for these reactions are the class I recognizing receptors (i.e., Ly49s in mouse and KIR in man). Evidence now suggests that these receptors play an important role in the innate resistance observed in human bone-marrow transplantation.[67]

5. Production of Cytokines

Another function that was associated early on with NK cells was the production of cytokines.[68] Djeu et al. first reported that "The IFN produced by both LGL and monocytes were predominantly IFN-α, as assessed by neutralization assays with antisera …" These studies demonstrated that IFN-γ was also produced by NK cells, presumably for NK cell recognition of the virus used for stimulation. These data suggested an efficient positive self-regulatory mechanism in NK cells that may be readily switched on by viruses. NK cells secrete a high level of cytokines that regulate other leukocytes and NK function. Some important cytokines include but are not limited to IL-1-β, IL-8, TNF-α, IL-10, IL-13, GM-CSF, IFN-α, IFN-γ, and TGF-β.

VII. EARLY SEMINAL MILESTONES

Early studies of NK cells provided a number of key observations regarding the immunological community, which suggested a variety of functions for these cells. Summarized here are important milestone observations from early NK cell studies that have contributed to a better understanding, not only of NK cells, but of immune responses in general.

A. Resistance to tumors. Early reports of recognition of tumor cell lines by NK cells, which were later translated into the demonstration of the important role of NK cells in regulating the metastatic spread of tumors, placed NK cells as a key player of innate immunity in cancer.[69–71]
B. Control of virus infection. Early reports that NK cells were activated by virus infection, and could selectively recognize and kill virus-infected targets, placed NK cells as a critical component of the innate immune response to viral infections.[72–74]
C. Cytokine production. Early reports that activation of NK cells by tumors and viruses led to cytokine production placed NK cells as more than just a cytotoxic effector cell

but as a major player in many aspects of the immune network.[41,72,74] Today, we know that production of cytokines is an important immune-regulatory loop in the *in vivo* function of NK cells.

D. Unique patterns of recognition. Early reports regarding the broad specificity of NK cells led to the identification of a number of receptors used by NK cells.[19–24] Unique among lymphocytes, the NK cells can recognize both pattern recognition domains as well as levels of MHC on target cells.

E. Identification of LGL morphology and LGL leukemias. The discovery of the LGL morphology associated with NK activity guided the initial isolation of purified NK cells and identification of unique markers on these cells that could be used to distinguish them from other lymphocytes.[17,32] In addition, LGL leukemia cell lines provided a critical source of cells that resulted in the definition of lymphocyte perforin-mediated killing.[33,34]

F. Expression of IL2Rβ and response to IL2. The discovery that NK cells could rapidly respond to IL-2 to both proliferate and become activated spurred the discovery of the IL2Rβ chain.[75]

VIII. SUMMARY

In the early 1970s, the spontaneous *in vitro* antitumor activity of normal or unstimulated leukocytes was described as being "non-specific", and possibly just an *in vitro* artifact. Since that time, a large number of studies have resulted in thousands of reports that have defined this activity (NK activity) and the cells associated with the activity (NK cells).

From these seminal early studies between 1970 and1980, it became clear that NK cells were a unique population of large granular lymphocytes (LGLs). We now know that they constitute a unique third major lymphocyte cell type and are a key member of the innate immune system. Today, it is also clear from these early studies that NK cells contribute to a number of immunological responses, including important and rapid responses to viral infection and significant antitumor protection, especially against the development of peripheral metastases. We have now identified many of the positive and negative factors that regulate these cells and their activity. These early studies helped us to understand the specificity of these cells and the basis for this specificity, including the identification of a number of different receptor families on NK cells that recognize MHC and unique pattern recognition domains.

These early studies provided many of the most important and critical observations about NK cells, which later proved to be milestones for understanding the biology of NK cells and their associated activities. While many laboratories contributed to these early studies, Ron Herberman and his laboratory must be considered one of the major contributors and visionary drivers of the NK field during this early period of discovery.

ACKNOWLEDGMENT

The authors recognize Ron Herberman for his vision and perseverance during the early days when NK activity was detected and the cells defined. His tenacity, often in the face of skepticism from the more traditional immunology community, helped to open a new and important aspect of science. The authors are also grateful to Ron for his leadership, mentoring, and staunch support for our own career development and advancement as well as that of many others.

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