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Evidence of a lineage shift between natural (NK) killer cells and T lymphocytes in the spleen and blood of neonatally thymectomized, young adult C3H mice.

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Abstract

The present study was designed to assess the influence of neonatal thymectomy on the proportions of natural killer (NK) lymphocytes and other (non-NK) lymphocytes in the spleen and blood of young adult mice.The progenitors (precursors) of both the T lymphocyte and NK cell lineages are located in the bone marrow, the organ of new cell production for both these lineages and where they both derive from a bi-potential stem cell. Newborn C3H mice were thymectomized when they were 8 – 12 hr old by a process in standard use in our laboratory. Control infant mice, identical in every way, were sham thymectomized. When thymectomized and control mice reached 8 wk of age, their spleens and blood were prepared by our well-established methods. Smears of both organs were analyzed for their proportions of NK cells and other (non-NK) lymphocytes. The results showed that in thymectomized mice, spleen and blood had significantly *increased* proportions of NK cells, *vs* sham-thymectomized controls, while the other (non-NK) lymphocytes in both the spleen and blood were significantly *decreased* in thymectomized mice *vs* control. Moreover, the actual percentage decrease in this group of other (non-NK) lymphocytes in the thymectomized animals was almost precisely the same as the percentage gain in NK cells in both organs.We interpret these findings as evidence suggesting that in the absence of a thymus which would normally convert progenitor T cells into mature functional T lymphocytes, these progenitors which are products of the bi-potential T/NK stem cell, come under the influence of NK cell-stimulating factors, thus causing the shift toward the NK cell lineage in the 2 peripheral organs, i.e., the spleen and the blood.

Keywords: Athymic mice, NK lymphocytes, T lymphocytes, *in vivo*

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Introduction

It has been known for decades, and proven in hundreds of publications, that the hemopoietic system consists of many different cell types that vary in quantity, potential for differentiation, capacity for proliferation and acquity of function. Bone marrow based, multipotential hemopoietic stem cells (HSC) have high self-renewal capacity and can ultimately give rise to all the cell lineages circulating in the blood, including the cells of the immune system. Conversely, the direct progeny of HSC are committed progenitor cells which, although more plentiful, do not have the capacity for long-term self-renewal, and differentiate along lineage defined lines. The Ikaros (Ikzf) family of transcription factors is essential for the commitment of HSC into the common lymphoid progenitor line [1]. Moreover, there is yet another progenitor which is bi-potential, and which, in the presence of the appropriate *in vivo* stimulatory factors, gives rise subsequently to either the T lymphocyte lineage or the NK cell lineage [2].

Cytolytic cells closely resembling NK cells have been a part of innate immunity in non-botanical life forms for approximately 500 million years [3], while T and B lymphocytes arose much more recently in evolution. The long evolutionary conservation of the NK cell lineage indicates that it has been critical for host defense and potentially, species survival. As the bipotential progenitor undergoes sustained exposure to the thymic interior, commitment to the T lymphocyte lineage occurs, while any bipotential progenitors not exposed to the thymic parenchyma are driven into the NK cell lineage [2, 4] In the absence of the thymus, a profound T lymphocyte deficiency occurs [5-7] and indeed, absence of the thymus from birth is not

compatible with a normal life span and leads to early death – this is the fate awaiting human individuals born without a thymus, i.e., a condition known as the di George Syndrome.

NK cell numbers have been well established as being significantly elevated in both congenitally athymic (nu/nu) and neonatally thymectomized mice [8, 9]. NK cells constitute between 5 and 20% of the peripheral blood lymphocytes in mammals including humans [10]. In contrast with T lymphocytes, NK cells have short life span and renew rapidly- with or without having engaged in cytolytic activity - of approximately 2 days [11, 12]. Moreover, NK cells do not require previous sensitization with offensive antigens, do not re-circulate through the blood or lymph, do not require identity with the major histocompatibility locus proteins prior to exerting their cytolytic interaction with the target cells. NK cells, however, do respond to endogenous stimulatory and inhibitory factors which are similar to those to which T lymphocytes respond [13- 15].

Born in the bone marrow [11, 12, 16-18], NK cells leave that organ and travel via the only exit from that organ, i.e., the blood, unidirectionally to the spleen, their primary destiny, never to re-enter the bone marrow or travel elsewhere [11]. In contrast, T lymphocyte progenitors, although generated in the bone marrow, also exit that organ via the blood, and undergo all of their further maturation to immune-competent cytolytic cells in the thymus. Chemokine signaling directs this maturation process as the pre- T lymphocytes migrate through the various microenvironments of the thymus. Upon leaving the thymus, T lymphocytes possess precisely the opposite of all the NK cell features listed above.

Thus, in the absence of the thymus, and given the common (bipotential stem cell) origin of T lymphocytes and NK cells in the bone marrow, we hypothesize that thymus-destined progenitors will fall under the influence of NK lymphocyte-inducing factors. These cells will accumulate ultimately as mature NK cells, in the absence of the T lymphocyte-inducing, thymic microenvironment [1, 2, 19- 23]. The concept that the NK lineage is the evolutionary "tree" off which the T cell "branch" arises much later in evolution has substantial support.

Materials and Methods

Mice

Male pups (Jackson Laboratories, Bar Harbor, ME, USA), were thymectomized or sham thymectomized (control) at approximately 8-12hours of age, under ether-induced anesthesia following our long-standing and well-established methods [9, 24]. All thymectomized and sham operated mice were examined at the time of euthanasia to verify the complete absence (thymectomized), or presence (sham-thymectomized) of both lobes of the thymus in the mediastinal area.

Tissue extraction and preparation for NK cell and T lymphocyte analysis

Thymectomized and sham thymectomized mice were euthanized at 8 wk of age. All were euthanized at mid-afternoon to circumvent physiological changes brought on by circadian rhythms and the known diurnal variations within cell populations.

Single cell suspensions of the spleen were prepared using standard methods in use in our lab [25, 26, 27]. Briefly, the spleen was removed and placed in ice-cold Eagles basal essential medium (GIBCO Invitrogen Corp., Burlington, ON, Canada) which contained 10% heat inactivated newborn bovine serum (NBS) (GIBCO – above). Each spleen was then pressed through a stainless steel mesh into the medium. Through gentle repeated pipetting, cellular suspensions were acquired. Subsequently, these suspensions were layered for 7 min onto 1.5ml of pure NBS to allow the sedimentation of non-cellular debris into the medium. The supernatant, now containing only free single hemopoietic and immune cells, was removed with a Pasteur pipette and centrifuged for 7 min (1100rpm, 4^{o} C). The resulting pellet was then resuspended in a set volume of fresh medium and NBS. Smears were then made from the clean, free cell suspensions onto ©Superfrost Plus microscope slides (Fisher Scientific, Whitby, ON, Canada). Finally, blood smears were prepared directly from a nick in the lateral tail vein immediately prior to euthanasia.

Spleen and blood smears were stained using MacNeal's tetrachrome hematologic stain (Sigma-Aldrich, Oakville, ON, Canada), which allowed for morphological identification of the different cell types [25- 31]. This stain provides a 4-colour discrimination of cellular constituents. NK cells were identified based on their resemblance to all other lymphocytes, with which they are morphologically indistinguishable except for one feature unique only to NK lymphocytes, i.e., 2-5 large, cytoplasmic granules [32- 34]. From this point on, NK cells and all other lymphocytes will be referred to as NK lymphocytes and non-NK lymphocytes.

Each slide (smear) was read by counting the numbers of NK lymphocytes and non-NK lymphocytes in sequential, adjacent microscope fields at x100 magnification. The total number of cells identified and recorded was 1000 in each spleen smear and 500 for each blood smear. NK lymphocytes and non-NK lymphocytes were recorded in separate groups as a percentage of the total nucleated cells encountered in each organ for each mouse, for both the neonatally thymectomized and sham thymectomized groups.

Statistical analysis

The percentages of NK cells and non-NK lymphocytes were calculated for each organ for each mouse (above), and the mean +/- standard error was calculated. The effect of thymectomy was compared with the parallel sham thymectomized groups for each organ, and the two-tailed Student's t-test was applied. Comparisons were considered to be statistically significant at $p < 0.05$.

Results

The data reveal (Table 1) that neonatal thymectomy results in a very significant increase – approximately 5-fold - in the proportions (%) of NK lymphocytes in the spleen over that of sham thymectomized control mice. By contrast, non-NK lymphocytes showed precisely the opposite phenomenon in the spleens of thymectomized mice *vs* control. That is, there was a significant decrease in the proportions of non-NK lymphocytes in the spleens of thymectomized mice *vs* the sham thymectomized controls. Furthermore, the actual *increase* in NK cells in the spleen (approximately 20%) is almost precisely the same as the *decrease* in this organ (approximately 20%) for the other (non-NK) lymphocytes (Table 1).

Table 2 reveals that NK lymphocytes in the blood of neonatally thymectomized mice follow the same pattern observed for these cells in the spleen (Table 1). That is, there is approximately a 5-fold increase in the percentage of NK cells *vs* sham thymectomized controls. Moreover, non-NK lymphocytes recorded in the blood of thymectomized mice were significantly *decreased* relative to their values in the sham thymectomized controls. The actual i*ncrease* in NK cells in the blood of thymectomized mice (approximately 24%) was very similar to the *decrease* (approximately 24%) in the blood of the non-NK lymphocytes (Table 2). Finally, between both organs (spleen and blood), the increase in NK lymphocytes and decrease in the non-NK lymphocytes, was almost identical (Tables 1,2).

Table 1. *The effect of neonatal thymectomy on NK lymphocytes and non-NK lymphocytes in the spleens of young adult, C3H mice*

	Sham thymectomized % [a] (N = 10)	**Thymectomized** % [a] (N = 10)
NK lymphocytes	5.00 ± 0.002[b]	25.15 ± 0.004[b,c]
Non-NK lymphocytes	95.00 ± 0.002[b]	74.85 ± 0.004[b,c]

[a]*Cells were recorded as a proportion (%) of 1000 total cells, identified and enumerated on spleen smears*

[b]*Mean ± standard error*

[c] *$p < 0.0001 \times (10^{-15})$ vs sham thymectomized controls*

Table 2. *The effect of neonatal thymectomy on NK lymphocytes and non-NK lymphocytes in the blood of young adult, C3H mice*

	Sham thymectomized % [a] (N = 10)	**Thymectomized** % [a] (N = 10)
NK lymphocytes	5.88 ± 0.002[b]	30.26 ± 0.003[b,c]
Non-NK lymphocytes	94.12 ± 0.002[b]	69.74 ± 0.003[b,c]

[a]*Cells were recorded as a proportion (%) of 500 total cells, identified and enumerated on blood smears*

[b]*Mean ± standard error*

[c]*$p < 0.0001 \times (10^{-15})$ vs sham thymectomized controls*

Discussion

Non-lymphocytic cells in the thymic parenchyma/stroma play an important role in the production of mature T lymphocytes. These parenchymal cells provide the factors involved in regulating migration from the bone marrow (T lymphocyte progenitors), their differentiation and survival (35). T lymphocyte migration from the bone marrow to the thymus, as well as migration within the thymic epithelial cell niches is facilitated by chemokine signaling (36).

We had hypothesized that in the absence of any T lymphocyte driving factors from the thymus, the bipotential progenitors would come under the influence of molecular factors which would drive these progenitors into the NK cell lineage. The present data demonstrate that indeed, this appears to have been the case in the neonatallythymectomized mice, since one sees a 5-fold increase in NK cells in the spleens of these mice over control (sham thymectomized). The fact that the blood of neonatallythymectomized mice also demonstrates an approximately equivalent 5-fold increase in the percentage of NK cells

vs control further supports this theory. The blood is the only exit from the bone marrow birth site of NK cells. It is however, unknown at present, if the bone marrow derived, progenitor T cells, in the absence of any T lymphocyte-stimulating factors in the thymectomized mice, are (i) coming under the influence of blood-borne, circulating, NK cell-driving, molecular factors, or, alternatively, (ii) whether the bone marrow-derived progenitor T cells, normally destined for the thymus, simply transit through the blood one or more times, and eventually re-entering the bone marrow. If progenitor T lymphocytes, in thymectomized mice, did re-enter the bone marrow, they would fall under the influence of the well-established, NK-cell governing microenvironment of the bone marrow [11, 12, 16- 18].

Further support for our hypothesis of a shift of the T cell progenitor branch of the bipotential progenitor cell toward the NK lineage is evidenced from the data found for the other non-NK lymphocytes. That is, the spleen and blood percentages of this latter group of lymphocytes shows precisely the opposite trend from what we observed in the spleen and blood for NK cells. As was the case with NK lymphocytes, the percentage of blood-borne, non-NK lymphocytes logically is comparable to the splenic values because the spleen is on the direct blood circulatory pathway, i.e., the spleen content will reflect the content of the blood.

Although the bone marrow has not been analyzed in this study, it is possible nevertheless to identify the cells contained in these non-NK lymphocytes. Thus, with the following two features well-established, we can say with virtual certainly that the non-NK lymphocytes are almost exclusively B lymphocytes. First, approximately 90% of the lymphocytes seen in the bone marrow are those of the B lymphocyte lineage, the bone marrow being the unique and central generating site for all virgin B lymphocytes (37- 39). In normal mice, progenitor T lymphocytes and NK cells are contained in the remaining 10%.

In summary, the data support our hypothesis that after neonatal thymectomy, NK lymphocytes which are found in significantly elevated proportions in young adulthood, results from a shift into the NK lineage of *all* progeny arising from the bi-potential lymphocyte progenitor. The concomitant, significant decrease in the frequency of the non-NK lymphocytes in the blood and spleen appears to support a shift in the destiny of all the progeny from the bi-potential progenitor cells into the NK lineage in the absence of a thymus.

Conflict of interest

The authors declare no conflict of interest with any other person, institution or commercial establishment in the execution of this project.

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