DISCUSSION
Natural killer (NK) cells form a subset of lymphocytes distinct from T and B lymphocytes. While cytotoxic T lymphocytes (CTL) as well as NK cells can lyse tumor cells and virus infected cells, unlike CTLs, NK cells require no prior sensitization in order to exert their cytolytic activity. NK cells may play a crucial role as a first line of defense against spontaneously arising tumor cells and for this reason it is important to understand factors which can modulate NK activity.

Modulation of the NK system by a variety of nutritional factors, hormones, alcohol, tumor bearing, aging and beige mutation in mice were studied in details (Saxena et al. 1981a,b, 1982a,b,c,d,e, 1983, 1984a,b,c,d, 1986, 1988a,b). NK cell activity is also modulated by cytokines particularly Interleukin-2 (IL-2), IL-15 (Mrozek et al 1996) and more recently discovered IL-21 (Parrish-Novak et al 2002).

NK cells are generated in bone marrow but the differentiation pathway responsible for their generation is not completely known. Furthermore, these cells can migrate out of bone marrow and can be detected in peripheral blood as well as in spleen. Cytotoxic activity of NK cells can be augmented by treatment with several cytokines like IL-2 and IL-15 and receptors of these cytokines have been demonstrated on NK cells (Waldmann et al 2002, Giri et al 1995). High affinity IL-2 receptors expressed on T cells comprise three chains i.e. α, β and γ chains, but IL-2 receptors on NK cells lack α chain and have poor affinity for IL-2 (Nelson et al 1998). For this reason, large doses of IL-2 are required to activate NK cells. In our studies, we found comparable levels of cytotoxic activity due to NK cells in spleen as well as in bone marrow derived cell preparations. In both cases IL-2 induced a comparable dose dependent increase in cytolytic activity (Figure 1, 2). Enhanced cytotoxic activity could be due to proliferation of NK cells and/or enhanced efficacy.
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of activated NK cells to kill the target cells. We have previously shown that both these mechanisms are responsible for this activation response (Saxena et al. 1984, Sarin et al. 1989).

Interestingly, when spleen and bone marrow cells were exposed to paraformaldehyde fixed tumor target cells during IL-2 induced activation phase, a further boosting of activation response was observed in spleen cells but not in bone marrow cells. NK cells in spleen and bone marrow cell preparations therefore were different in their response to fixed tumor cells. This is not surprising because the stage of differentiation of NK cells may not be identical in the two preparations. Similar differences have been observed for activation of other types of lymphocytes like T and B cells. It is well known that the nature of response to antigenic challenge differs markedly depending upon the stage of differentiation of T and B cells. Mature T and B lymphocytes may be activated by a specific antigenic stimulus resulting in an adaptive immune response. Immature T and B lymphocytes in thymus and bone marrow respectively however undergo an apoptotic response upon exposure to self antigen. Opposite responses of mature and immature T and B lymphocytes to specific antigens ensures that mature T and B cells in periphery are devoid of self reactive T and B cells but are ready to meet the challenge of invading pathogens with non-self antigens. It is possible that a similar situation may exist for NK cells also. We found that activation of mature spleen NK cells by fixed tumor cells further boosted the activation response in a manner reminiscent of the activation of mature T cells by specific antigen. For mature NK cells therefore, activation signals through IL-2 receptors and through NKRss recognizing tumor target cells induced an additive/synergistic activation response. The same may not
occur in case of immature NK cells in bone marrow. Immature bone marrow NK cells may be in a developmental phase where they are learning to discriminate between self and non-self and exposure to any antigen at this stage may result in a qualitatively different response.

The nature of NKR$^*$s which recognize YAC tumor cells is not known. Whatever these receptors are, they are likely to recognize some ligand on the cell membrane of YAC target cells. If this ligand is recognized by NKR$^*$s on spleen NK cells and is responsible for sending a signal to boost NK activation, then isolated YAC cell membranes should also be able to do the same. Our results indeed show that membrane preparations isolated from YAC cells were quite effective in boosting spleen NK cell activation in response to IL-2. This ligands on YAC cell membrane appears to be proteinaceous in nature since the activation response was lost by incubation of membranes with proteolytic enzyme trypsin. Carbohydrate moieties are unlikely to be ligands for NKR$^*$s since destruction of carbohydrate by periodate treatment did not result in a loss of efficacy of these membrane preparations in boosting NK cell activation response.

A large repertoire of NKR$^*$s is encoded in the genome. It is not known how the expression of these receptors is regulated. It is generally believed that the expression of these receptors is a stochastic process, whereby one or more of these receptors may be expressed on a given cell by a process, which is probably a random one. Activating as well as inhibitory NKR$^*$s have been described and the net efficacy of a given NK cell to interact and lyse a particular target cell may depend upon the net signal it derives through its various activating and inhibitory NKR$^*$s as a result of their engagement with appropriate ligands on the target cells. NKR$^*$s responsible for
recognition and lysis of YAC cells by mouse NK cells is/are not known. YAC cells however constitute excellent targets for mouse NK cells and anti YAC cytolytic activity of NK cells is not inhibited by simultaneous inhibition of NKR(s) (Yokoyama et al 1993). Recognition of YAC cells by NK cells may involve unique activating NKR(s) which have high affinity for certain ligands on YAC cells and cytolytic signals transmitted through these receptors may not be susceptible to inhibition by a concurrent inhibitory signal through inhibitory NKR(s). Alternatively, it has been suggested that YAC cells constitutively produce IL-10 which downregulates self MHC I expression on these cells rendering them highly susceptible to NK cells (Petersson et al 1998). Low expression of MHC class I on YAC cells may also explain comparable lysis of YAC cells by NK cells with or without inhibitory NKR(s). Overall, it appears that the lysis of YAC cells by a given NK cell preparation may be proportional to the number of NK cells in that preparation irrespective of the kind of inhibitory NKR(s) expressed on these cells.

Activation of spleen or bone marrow cells by IL-2 induced a significant increase in the expression of inhibitory receptors on NK as well as T cells. This increase was further boosted when paraformaldehyde fixed YAC tumor cells were included in the culture medium. Increased expression of Ly49 on IL-2 activated NK cells may reflect an upregulation of Ly49 gene expression or increased number of Ly49 bearing NK and T cells or both. Additional stimulation with paraformaldehyde fixed YAC tumor cells at a time when NK cells are being activated by IL-2 may further boost the expression of Ly49 molecules as a result of the additional signals that YAC cells may provide. If IL-2 activation of NK cells is considered to be similar to a developmental phase during the ontogeny of NK cells, induction of
inhibitory NKR as a result of exposure to tumor target cells makes sense. It can be argued that during the developmental phase exposure to any antigen may transmit a tolerogenic signal usually associated with self antigens. Enhanced expression of inhibitory NKR like Ly49 family of molecules by paraformaldehyde fixed YAC tumor cells (YAC*) may represent such a tolerogenic response.

It was crucial to determine whether NK cell proliferation was required for the induction of Ly49 on NK cells by IL-2 in presence or absence of YAC*. This was examined by blocking the cell proliferation response by using mitomycin C. In mitomycin C treated spleen or bone marrow preparations IL-2 could induce significant Ly49 expression on NK cells. It therefore appears that IL-2 induced Ly49 expression may not necessarily involve a cell proliferative response. Interestingly when YAC* were added while mitomycin C treated spleen cells were being activated with IL-2, Ly49 expression was completely abrogated. Bone marrow cells behaved quite differently in this respect in as much as Ly49 expression on bone marrow NK cells was not completely abolished by paraformaldehyde fixed YAC tumor cells. Nonetheless, even for bone marrow derived NK cells, Ly49 induction was not different in cultures treated with IL-2 or IL-2 + YAC*. The reason behind the lack of Ly49 response to paraformaldehyde fixed YAC tumor cells in mitomycin C treated spleen or bone marrow cells is not clear. It is possible that during IL-2 induced activation, a simultaneous signal to NK cells by tumor target cells may induce Ly49 expression in cells with normal ability to proliferate in response to IL-2. If proliferative response is prevented (by mitomycin C) an apoptotic response may ensue instead, resulting in a loss of Ly49 expressing cells. This possibility would require further confirmation.
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Induction of Ly49 markers by paraformaldehyde fixed YAC tumor cells in spleen and bone marrow cells was not confined to NK cells but was also seen on T cells. Total abrogation of Ly49 expression in mitomycin C treated spleen cells in response to fixed tumor cells would indicate that the expression was lost on both NK as well as on T cells. While NK cells have receptors which may recognize YAC cells, it is not clear as to how addition of tumor cells influenced Ly49 expression on T cells. Non-sensitized T cells are not known to lyse tumor cells suggesting that the receptors recognizing tumor cells may not be present on resting T cells. T cells are known to express some NKR s and it is possible these receptors belong to NKR family which may interact with YAC tumor cells without sending a lytic signal. Engagement of such receptors may modulate Ly49 expression on T cells. This proposition would also require further confirmation.

Results thus far suggested that interactions between NKR s on NK cells and MHC class I or antigens belonging to the family of class I MHC molecules on target cells may have an important role to play in the process of IL-2 induced NK activation. In these experiments, ligands of NKR s on YAC cells seem to modulate NK cell activation response as well as the expression levels of Ly49 molecules. During normal development of NK cells exposure to tumor cells is not expected. Nonetheless, ligands of NKR s in the form of molecules belonging to MHC class I family would normally be expressed on a wide variety of bystander cells in bone marrow. Our earlier results may be extrapolated to suggest that an interaction of NKR s on developing NK cells and MHC class I molecules on bystander cells in bone marrow may influence the process of NK cell development. It is difficult to study this proposition since all nucleated cells express MHC I and related molecules
and it is impossible to have a system where NK cells are allowed to develop in a milieu devoid of cells bearing MHC I and related molecules. Even if a 100% pure NK cell population is used, exposure to MHC I cannot be avoided since NK cells also express class I MHC molecules. We made an attempt to study the influence of interaction between NKR ligands and MHC I ligands, on NK cell development, by blocking NKR-MHC interaction with the help of specific antibodies. In initial experiments, NK cell activation by IL-2 was studied in the presence or absence of anti MHC alloantiserum (H-2^d anti H-2^b alloantiserum). Addition of alloantiserum significantly boosted the NK cell activation response to IL-2. This activation however turned out to be a consequence of co-activation of CD16 receptor on NK cells by multiple Fc portions of alloantibodies attached to MHC bearing bystander cells. The inference could be drawn because upon addition of (a) antibody coated bystander cells or (b) heat aggregated Ig G or (c) circulating immune complexes derived from mouse serum and (d) anti CD16 antibodies could all boost the activation of bone marrow NK cells by IL2. Addition of F(ab)_2 preparations derived from alloantibodies resulted in a marked inhibition of IL-2 induced NK activation response in bone marrow cells. These results suggested that in absence of interference of Fc portion of alloantibodies, disruption of MHC-NKR interaction inhibited the activation of NK cells by IL-2. From these results we inferred that a normal interaction between NKR ligands on NK cells and MHC molecules expressed on bystander cells could be a crucial requirement for activation of NK cells by IL-2.

Alloantibodies used for blocking MHC-NKR interaction were derived by cross- strain immunization between Balb/C (H-2^d) and C57 Bl/6 (H-2^b) mice and as such these alloantibodies would not exclusively recognize MHC I molecules.
Activation of NK cytotoxicity induced by IL-2 involves two signals, one through IL-2R, other through NKR-MHCI interaction.

No activation of NK cytotoxicity induced by IL-2 due to blockage of signal 2 by anti MHC I monoclonal antibody.

No activation of NK cytotoxicity induced by IL-2 due to blockage of signal 2 by anti NKR monoclonal antibody.

Figure C. Proposed role of MHC I-NKR interaction in NK cell development.
Blockage of NKR-MHC interaction by adding specific anti H-2\(^b\) monoclonal antibodies however also inhibited IL-2 induced NK activation in bone marrow cells. These results suggest that interaction of NKR\(_s\) on NK cells and MHC molecules on bystander cells may be necessary for optimal NK activation response (Figure C). Disruption of NKR-MHC interaction by using anti MHC antibodies results in a sub-optimal NK activation response. Further this hypothesis predicts that disruption of MHC I-NKR interaction by using specific anti-NKR antibodies should also inhibit NK activation response to IL-2 (Figure C). This proposition was tested by using specific monoclonal antibodies against Ly49A, Ly49C and Ly49D receptors. Out of these three receptors or NKRs, only Ly49C is a specific inhibitory receptor for H-2\(^b\) MHC molecules. Since we were using bone marrow cells from C57 B1/6 (H-2\(^b\)) mice, inhibitory NKR-MHC interaction could only be blocked by using anti Ly49C monoclonal antibody (Figure 37, 44). Accordingly our results indicated that anti Ly49C antibody inhibited the activation of NK cells whereas antibodies specific to Ly49A and Ly49D receptors had no effect in this regard (Figure 38, 39).

Taken together, our results suggest for the first time that an interaction between NKR and MHC molecules may be crucial for development of NK cells in mouse bone marrow. This requirement may have important implications about the ontogeny of NK cells. It is now established that a large number of NKR\(_s\) are encoded in the genome and the expression of these receptors on a given NK cell may depend on a random stochastic mechanism. Therefore, as a result of stochastic expression of NKR on NK cells various types of combinations of NKR\(_s\) may be expressed on NK cells. Four basic types of NKR\(_s\) may be conceived:

1. SRA (Self reactive activating) NCR\(_s\). (e.g. NKp46)
2. SRI (Self reactive inhibitory) NKR s (e.g. Ly49C for H-2^b NK cells)

3. NSRA (Non self reactive activating) NKR s (e.g. Ly49D for H-2^b NK cells)

4. NSRI (Non self reactive inhibitory) NKR s (e.g. Ly49A for H-2^b NK cells)

Any combination of the above categories of receptors may be expressed on a given NK cell. A SRA expressing NK cell may be permitted to develop and proliferate only if there is a simultaneous expression of SRI to prevent damage to normal cells (referred to as Type II NK cells by Saxena 1997). In order to ensure that a SRA receptor bearing precursor of NK cell has also expressed a SRI receptor and may therefore be permitted to develop, interaction between SRI and MHC I must send a permissive signal to enable that clone to respond to developmental signals (like IL-2) and proceed towards development. If this permissive signal is blocked by using an anti-MHC I or anti-SRI antibody, the clone would not be permitted to develop and may be aborted. For this reason in our experiments, bone marrow cell cultures activated with IL-2 in the presence of blocking antibodies to prevent SRI-MHC I interaction, show an inhibition of cytotoxicity activation response. By preventing signals through SRI, the NK cell clone would effectively be perceived to be self reactive and may be pushed to undergo apoptosis. Since SRA expressing clones may survive by expressing one of many available SRIs that explains the incomplete inhibition that was observed when anti Ly49C was used to block the MHC I-SRI interaction (Figure D). Since NSRA (Ly49D) or NSRI (Ly49A) receptors are harmless incidentally expressed receptors, their blockage had no effect on NK activation in response to IL-2.
Figure D. Significance of NKR interaction with self MHC I for NK cell function