RESULTS
Chapter 1:
Modulation of NK activation by interaction with tumor target cells
Results

NK cells may constitute a first line of defense against spontaneously occurring tumors. NK cell mediated killing of tumor cells does not require a prior sensitization. A variety of cytokines including IL-2 can activate NK cells and status of NK activation may be a crucial factor in their effectiveness against tumor cells. The basic aim of the present work was to understand if and how tumor target cells can influence the process of NK cell activation. For this purpose we standardized the system of NK cell activation in response to IL-2, so that the effect of adding tumor target cells during NK activation may be studied.

Activation of NK cells by IL-2:

Human recombinant IL-2 was used for activation of murine spleen NK cells. Lytic activity of spleen cells, activated with various doses of IL-2 has been shown in Figure 1. These results indicate that control non-activated spleen cells induced a low yet significant lysis of YAC target cells. A significant increase in lytic activity was induced by IL-2 at all tested doses (10-100 U/ml). For each of the lysis curves, the values of percentage target lysis at different effector to target ratios could be converted to Lytic Units/10^6 effector cells by using a computer program developed for this purpose (David Coggin at National Institutes of Health, Bethesda, MD). At the optimal dose of 50 U/ml of IL-2 a four-fold increase in spleen lytic activity was observed. In subsequent experiment an IL-2 concentration of 50 U/ml was utilized for activating NK cells.

Similar results for IL-2 induced lytic activity in bone marrow derived cells are shown in Figure 2. For bone marrow derived cells also, a significant increase in anti YAC lytic activity was observed at all doses of IL-2. 50-100 U/ml of IL-2 induced optimal activation of lytic activity in bone marrow cells.

Effect of paraformaldehyde fixed tumor cells on IL-2 activation of NK activity:

For studying the effect of adding tumor cells during IL-2 activation of NK cells, live tumor cells could not be used because they would proliferate and overcrowd the culture. YAC tumor cells fixed with paraformaldehyde and washed were used for this purpose. Previous results from our laboratory have shown that the addition of paraformaldehyde fixed YAC target cells during NK cell activation
Figure 1. Dose response of the effect of IL-2 on anti YAC cytotoxic activity of mouse spleen NK cells. Mouse spleen cells were cultured with different doses of IL-2 (0-100 U/ml). After four days, the cells were washed and their cytolytic activity against YAC target cells was determined at several E:T ratios. Each value of target lysis is a mean ± SD of results from three replicate assay wells.

* Values in parentheses show the lytic units/10^6 effector spleen cells.
Figure 2. Dose response of the effect of IL-2 on anti YAC cytotoxic activity of bone marrow NK cells. Mouse bone marrow cells were cultured with different doses of IL-2 (0-100 U/ml). After four days, the cells were washed and their cytolytic activity against YAC target cells was determined at several E:T ratios. Each value of target lysis is a mean ± SD of results from three replicate assay wells.

* Values in parentheses show the lytic units/10⁶ effector bone marrow cells.
Mouse spleen and bone marrow cells were activated in vitro with 50 U/ml IL-2 (top and bottom panel respectively) in the presence or absence of paraformaldehyde fixed YAC tumor cells (YAC *). The ratio of (YAC *) to effector spleen or bone marrow cells was 1:100. After four days of culture, cultured cells were washed and their cytolytic activity against YAC target cells was determined at several E:T ratios. Each value of target lysis is mean ± SD of results from three replicate assay wells. Values in parentheses show the lytic units/10^6 effector cells.
Figure 4. Partial purification of NK cells from bone marrow preparations. NK cells were partially purified by passage through nylon wool column to deplete B cells and depleted of T cells using anti Thy 1.1 antibody and complement (as described in materials and methods) and were subsequently stained with anti NK1.1 antibody. Curve A denotes isotype control, curve B denotes unfractionated bone marrow cells and curve C shows the partially purified NK1.1+ population.
Figure 5. Effect of paraformaldehyde fixed YAC cells on IL-2 activation of NK activity.

NK cells were partially purified (as described in materials and methods) from mouse spleen and bone marrow cell preparations and activated with 50 U/ml IL-2 (top and bottom panels respectively) in the presence or absence of paraformaldehyde fixed YAC tumor cells. Ratio of fixed YAC cells to effector NK cells was 1:100. After four days of culture, cultured cells were washed and their cytolytic activity against YAC target cells was determined at several E:T ratios. Each value of target lysis is mean ± SD of results from three replicate assay wells. Values in parentheses show the lytic units/10⁶ effector cells.
Figure 6. Effect of co-culturing spleen cells with different doses of paraformaldehyde fixed YAC tumor cells on IL-2 activated NK cytotoxicity. Spleen cells were cultured with IL-2 (50 U/ml) either alone or in the presence of paraformaldehyde fixed YAC tumor cells (YAC*) at YAC*: spleen cell ratios of 1:25, 1:50 and 1:100. The cytotoxic activity against YAC target cells was determined at a number of E:T ratios. Each value of target lysis is a mean ± SD of results from three replicate assay wells. Values in parentheses show the lytic units /10^6 effector spleen cells.
phase resulted in a marked increase in the levels of lytic activity generated in response to IL-2. This phenomenon was however restricted to spleen cells and not observed in bone marrow cells (Dhillon et al 1999). Results in Figure 3 confirm the original observation about the effect of fixed YAC cells on NK cell activation. Activation of NK cells by fixed tumor cells could be a direct effect on NK cells or could be mediated through some bystander cell population. To test for this possibility NK cells were partially purified by passage through nylon wool column followed by treatment with anti Thy1.1 antibody and complement. Passage through nylon wool column is known to deplete B cells and anti Thy1.1 antibody and complement treatment would deplete T cells. Flowcytometric analysis of control and NK enriched cell preparations is shown in Figure 4. These results indicate that NK1.1+ cells were less than 5% in control bone marrow and spleen cell preparations. NK enriched preparation were significantly enriched and NK 1.1+ population accounted for 65-70 % in spleen and bone marrow preparations. T cell contamination was less than 2%. These NK enriched cell preparations were activated with IL-2 and the effect of fixed tumor cells was studied. Results in Figure 5 (upper panel) show that the partially purified NK cell preparations had significantly greater cytolytic activity as compared to un-fractionated spleen cell preparation shown in figure 3. Moreover, IL-2 activation of NK cells purified from spleen cells was also boosted significantly by fixed YAC tumor cells. Activation of bone marrow derived NK cells was not influenced by fixed YAC tumor cells (Figure 5 lower panel). Results in Figure 6 show that the effect of paraformaldehyde fixed YAC tumor cells on IL-2 activation was dose dependent. Maximal effect was observed at fixed YAC: spleen cells ratios of 1:100 or 1:50. Higher concentration of fixed YAC produced sub-optimal effect.

Effect of cell membrane derived from YAC cells on the activation of spleen NK cells by IL-2:

Since fixed YAC cells are metabolically inactive, their effect on boosting NK activation may be induced by certain factors on YAC cell membranes. To test this possibility, effect of YAC cell membranes was examined on IL-2 induced activation of spleen NK cells. Results in Figure 7 indicate that the cell membrane
Figure 7. Effect of co-culturing spleen cells with cell membrane preparations derived from YAC tumor cells and C57Bl/6 spleen cells on IL-2 activated spleen NK cytotoxicity. Spleen cells from C57Bl/6 mice were cultured with IL-2 (50 U/ml) either alone or in the presence of membrane preparations derived from YAC tumor cells or C57Bl/6 spleen cells (1μg/ml). After four days, the cells were washed and their cytolytic activity against YAC target cells was determined at several E:T ratios. Each value of target lysis is a mean ± SD of results from three replicate assay wells. Values in parentheses show the lytic units/10^6 effector spleen cells.
Figure 8. Effect of different doses of YAC cell membranes on IL-2 activation of spleen NK cells. Spleen cells were activated in vitro with IL-2 (50 U/ml) either alone or in the presence of different doses of YAC cell membranes. After four days, the cells were washed and their cytolytic activity against YAC target cells was determined at several E:T ratios. Each value of target lysis is a mean ± SD of results from three replicate assay wells. Values in parentheses show the lytic units/10^6 effector spleen cells.
Figure 9. Effect of trypsin treatment of YAC membrane on IL-2 induced cytotoxicity of spleen NK cells. Spleen cells were activated in vitro with IL-2 (50 U/ml) either alone or in the presence of control or trypsin treated YAC cell membranes. After four days, the cells were washed and their cytolytic activity against YAC target cells was determined at several E:T ratios. Each value of target lysis is a mean ± SD of results from three replicate assay wells. Values in parentheses show the lytic units/10⁶ effector spleen cells.
Figure 10. Effect of control or periodate treated YAC cell membranes on IL-2 activation of spleen NK cells. Spleen cells were activated in vitro with IL-2 (50 U/ml) either alone or in the presence of control or periodate treated YAC cell membranes. After four days, the cells were washed and their cytolytic activity against YAC target cells was determined at several E:T ratios. Each value of target lysis is a mean ± SD of results from three replicate assay wells. Values in parentheses show the lytic units/10⁶ effector spleen cells.
preparations derived from YAC cells was capable of boosting NK activation in response to IL-2. Membrane preparations derived from mouse spleen cells were however not effective in this regard, suggesting that some NK activating principle may be present on YAC cell membranes and not on membrane preparations derived from normal lymphocytes. Boosting of IL-2 induced NK activation by YAC membrane was dose dependent (Figure 8). Pre-treatment of YAC membrane with proteolytic enzyme, trypsin abrogated the activating property of YAC membranes (Figure 9). Pre-treatment of the membrane with sodium periodate which is known to destroy carbohydrate rings, however had no effect on the ability of YAC membranes to boost IL-2 induced NK activation (Figure 10). These results clearly indicate that the activating principle associated with YAC membranes is likely to be proteinaceous in nature and is unlikely to be a carbohydrate moiety.
Chapter 2:
Modulation of NKR expression by activation with tumor target cells
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Tumor target cells that escape CTL mediated surveillance by downregulating their MHC class I molecules, become susceptible to NK mediated cytolysis. This phenomenon called 'the missing self hypothesis' proposed by Ljunggren and Karre in 1990. Protection of normal cells to lysis by murine NK cells is mediated by Ly49 family of receptors, which constitute important members of NK receptor (NKR) family of molecules. Ly49 receptors on murine NK cells recognize MHC class I molecules on target cells and initiate an inhibitory signal, thus preventing lysis of target cells by NK cells. Upon down regulation of MHC I on target cells, binding of MHC I molecules to cognate Ly49 receptors is prevented resulting in killing of target cells due to absence of the inhibitory signal. In Chapter 1 of results, modulation of NK activation process by interaction with tumor target cells was examined. In this chapter, influence of interaction with target tumor cells was studied on the expression of Ly49 class of NKR.

Effect of IL-2 and paraformaldehyde fixed target tumor cells on Ly49 expression on NK cells:

Several types of Ly49 receptors are expressed on murine NK cells. In initial experiments mouse spleen cells were activated with IL-2 in the presence or absence of paraformaldehyde fixed YAC cells. Spleen cells were double stained with anti-Ly49A monoclonal antibody and anti-NK1.1 monoclonal antibody. Figure 11 shows that IL-2 activation of spleen and bone marrow cells resulted in a significant increase in Ly49A expression on NK cells (NK1.1+ cells). NK1.1+Ly49A+ cells accounted for about 2% of control spleen or bone marrow cells. Upon IL-2 activation the percentage of NK1.1+Ly49A+ cells went up to 11.6 and 13.4 in spleen and bone marrow cells respectively. When NK cells from spleen and bone marrow were activated with IL-2 in the presence of paraformaldehyde fixed YAC tumor cells, there was a further 2-3 fold increase in Ly49 expression on NK1.1+ NK cells as compared to the spleen and bone marrow cells activated with IL-2 alone (Figure 11).
Figure 11: Effect of IL-2 and fixed YAC tumor cells on Ly49A and NK1.1 expression on spleen and bone marrow cells. Spleen and bone marrow cells were cultured with IL-2 (50 U/ml) either alone or in the presence of paraformaldehyde fixed YAC tumor cells (YAC*) for four days. The tumor to spleen or bone marrow cell ratio during culture was 1:100. The cells were washed and stained with anti Ly49A and anti NK1.1 antibodies as described in materials and methods.
Figure 12: Effect of paraformaldehyde fixed YAC tumor cells on the Ly49A and Ly49C expression on IL-2 activated spleen T cells. Spleen cells were cultured with IL2 (50 U/ml) either alone (left panels) or in the presence (right panels) of paraformaldehyde fixed YAC tumor cells (YAC*) for four days. The tumor to spleen cell ratio during culture was 1:100. The expression of Ly49A and Ly49C and TCRβ was determined by staining the cultured cells with anti Ly49A and anti TCRβ (top panels) or anti Ly49C and anti TCRβ antibodies (bottom panels) as described in materials and methods.
Figure 13: Effect of paraformaldehyde fixed YAC tumor cells on the Ly49A and Ly49C expression on IL-2 activated bone marrow T cells. Bone marrow cells were cultured with IL2 (50 U/ml) either alone (left panels) or in the presence (right panels) of paraformaldehyde fixed YAC tumor cells (YAC*) for four days. The tumor to bone marrow cell ratio during culture was 1:100. The expression of Ly49A and Ly49C and TCRβ was determined by staining the cultured cells with combinations of anti Ly49A and anti TCRβ antibodies (top panels) or anti Ly49C and anti TCRβ antibodies (bottom panels) respectively as described in materials and methods.
Figure 14. Time kinetics of Ly49A expression on mouse spleen cells activated with IL-2 in presence or absence of paraformaldehyde fixed YAC tumor cells. Spleen cells were cultured with IL-2 (50 U/ml) for different time intervals (1-5 days) in the presence (central panel) and absence (left panel) of paraformaldehyde fixed YAC tumor cells. The tumor to spleen cell ratio during culture was 1:100. The expression of Ly49A was determined by staining the cultured cells with anti Ly49A antibodies as described in materials and methods. The extreme right panel summarizes the data of percentage of Ly49A+ cells in spleen cell cultures with IL-2 (open bars) and IL-2 + YAC* (shaded bars).
Figure 15. Time kinetics of Ly 49C expression on mouse spleen cells activated with IL-2 in presence or absence of paraformaldehyde fixed YAC tumor cells. Spleen cells were cultured with IL-2 (50 U/ml) for different time intervals (1-5 days) in the presence (central panel) and absence (left panel) of paraformaldehyde fixed YAC tumor cells. The tumor to spleen cell ratio during culture was 1:100. The expression of Ly49C was determined by staining the cultured cells with anti Ly49C antibodies as described in materials and methods. The extreme right panel summarizes the data of percentage of Ly49C+ cells in spleen cell cultures with IL-2 (open bars) and IL-2 + YAC* (shaded bars).
Figure 16. Time kinetics of Ly49A expression on mouse bone marrow cells activated with IL-2 in presence or absence of paraformaldehyde fixed YAC tumor cells. Bone marrow cells were cultured with IL-2 (50 U/ml) for different time intervals (1-5 days) in the presence (central panel) and absence (left panel) of paraformaldehyde fixed YAC tumor cells. The tumor to bone marrow cell ratio during culture was 1:100. The expression of Ly49A was determined by staining the cultured cells (0.2X10^6) with anti Ly49A antibodies as described in materials and methods. The extreme right panel summarizes the data of percentage of Ly49A+ cells in bone marrow cell cultures with IL-2 (open bars) and IL-2 + YAC* (shaded bars).
Figure 17. Time kinetics of Ly49C expression on mouse bone marrow cells activated with IL-2 in presence or absence of paraformaldehyde fixed YAC tumor cells. Bone marrow cells were cultured with IL-2 (50 U/ml) for different time intervals (1-5 days) in the presence (central panel) and absence (left panel) of paraformaldehyde fixed YAC tumor cells. The tumor to bone marrow cell ratio during culture was 1:100. The expression of Ly49C was determined by staining the cultured cells (0.2X10^6) with anti Ly49C antibodies as described in materials and methods. The extreme right panel summarizes the data of percentage of Ly49C+ cells in bone marrow cell cultures with IL-2 (open bars) and IL-2 + YAC* (shaded bars).
Effect of IL-2 and paraformaldehyde fixed YAC tumor (YAC*) cells on Ly49 expression on T cells:

Results in Figure 11 indicate that there was an enhanced expression of Ly49A on NK1.1 negative cell population in spleen or bone marrow cells activated with IL-2 and the enhancement of Ly49A expression was more pronounced if YAC* cells were added. These results suggest that the expression of Ly49A was not restricted to NK cells alone. Since NKR expression has been shown on T cells, effect of IL-2 and YAC* on Ly49A and Ly49C expression on T cells was also examined. Results in Figure 12 (left panel) shows a significant expression of Ly49A and Ly49C receptors on control IL-2 activated spleen T cells (TCRβ⁺ cells), which was further boosted by adding YAC* cells during the IL-2 activation phase (Figure 12 right panels). Similar boosting of Ly49A and Ly49C expression on bone marrow TCRβ⁺ T cells in response to IL-2 and YAC* was also observed (Figure 13).

Time kinetics of induction of Ly49 expression by IL-2 and paraformaldehyde fixed YAC cells:

Mechanism of Ly49 induction in response to IL-2 and fixed YAC (YAC*) cells is not clear. New cells having Ly49 marker may have been generated in the culture or Ly49 expression could have been induced on existing cells. In order to further probe the mechanism of increased Ly49 expression in response to IL-2 and YAC* cells, time kinetics of change in Ly49 expression was studied. For this purpose mouse spleen cells were cultured with IL-2 in presence or absence of YAC* cells. At different time points spleen cells were stained with anti Ly49A antibody. Flowcytometric data on Ly49A expression on spleen cells cultured with IL-2 alone or with IL-2 + YAC* cells for 0,1,2,3,4 and 5 day time points are given in left two panels in Figure 14. Percentage of Ly49 positive cells derived from flowcytometric data is further plotted as histograms in the right panel. This data indicates that control spleen cells had very low expression of Ly49A receptors and the expression remained low till second day of culture. In cultures activated with IL-2 alone, there was a low yet significant increase in the number of Ly49 expressing cells on third and fourth day of culture. On fifth day the percentage of Ly49 A⁺ cells fell to levels close to control unactivated cells. In culture activated with IL-2 + YAC* cells, Ly49
Figure 18: Effect of YAC tumor cell membranes on the Ly49C expression on mouse spleen cells. Spleen cells were cultured with IL-2 (50 U/ml) either alone or in the presence of YAC tumor cell membrane (3 μg/ml) for four days. The expression of Ly49C was determined by staining the cultured cells with anti Ly49C antibodies as described in materials and methods.
Figure 19: Effect of YAC tumor cell membranes on the Ly49C and expression on bone marrow (BM) cells. Bone marrow cells were cultured with IL2 (50 U/ml) either alone or in the presence of YAC tumor cell membranes (3 μg/ml) for four days. The expression of Ly49C was determined by staining the cultured cells with anti Ly49C antibodies as described in materials and methods.
expression remained low till day 2 of the culture but increased substantially on day 3, 4 and 5. At peak induction (day 4) about 50% of the cultured spleen cells expressed Ly49A receptors.

Figure 15 shows the induction of Ly49C marker in mouse spleen cells in response to IL-2 or IL-2+YAC* cells. The kinetics is very similar to that in Figure 14 for Ly49A expression. IL-2 alone however, seems to be a better inducer of Ly49C marker (Figure 15) as compared to Ly49A marker (Figure 14).

Time kinetics of induction of Ly49A and Ly49C expression on bone marrow cells cultured with IL-2 with or without YAC* cells are given in Figure 16 and 17. Some important differences were noticed in induction of Ly49 expression in spleen and bone marrow cells. Firstly, unlike spleen cells, where a time dependent increase in Ly49A/ Ly49C expressing cells, (peaking on day 4) was observed, IL-2 alone induced a small increase in Ly49A/C expression on bone marrow cells on day 1 to day 5 without any peak of response. A significantly greater expression of Ly49A/ Ly49C was seen in bone marrow cells activated by IL-2 + YAC* cells at all time points. Unlike the case with spleen cells, a significant increase in expression of Ly49A/ Ly49C in response to IL-2 + YAC* cells was observed on day 1. A second peak of Ly49A/C expression in response to IL-2 + YAC* cells was also observed on day 4 (Figure 16, 17).

In the first chapter we had demonstrated that the addition of membrane preparations from YAC cells during IL-2 activation of spleen cells could markedly boost the generation of anti-YAC cytolytic activity. Efficacy of YAC membrane preparations instead of YAC* cells in boosting the expression of Ly49 expression was also examined. Results in Figure 18 and 19 show that YAC membranes could also boost Ly49 expression. These results suggest the same component present in YAC membranes was sufficient to provide a signal for inducing Ly49 expression.

Assessment of role of cell proliferation on Ly49 induction:

Enhanced Ly49 expression induced by IL-2 and IL-2 + YAC* cells in spleen and bone marrow cells could result from proliferation of Ly49 expressing cells. Alternatively, Ly49 marker could be induced on existing Ly49+ cells. For assessing the requirement of cell division for enhanced Ly49 expression, effect of blocking the
Figure 20. Effect of mitomycin C on mitogen induced proliferation of spleen cells. Mouse spleen cells were treated with mitomycin C (5-30 μg/ml) and cultured in the presence concanavalin A for 2 days. Fresh spleen cells were taken as negative control while ConA treated spleen cells was taken as positive control. The cells were pulsed with $^3$Hthymidine for 18 hours and harvested. The CPM was determined as described in materials and methods. Each point represents a mean ± SD value from three replicate assay wells.
Figure 21. Time kinetics of Ly49A expression on mitomycin C (Mit C) treated mouse spleen cells and activated with IL-2 in presence or absence of paraformaldehyde fixed YAC (YAC\*) tumor cells. Spleen cells were pre-treated with Mit C (30 μg/ml) and cultured with IL-2 (50 U/ml) for different time intervals (1-5 days) in the presence (central panel) and absence (left panel) of YAC\* tumor cells. The tumor to spleen cell ratio during culture was 1:100. The expression of Ly49A was determined by staining the cultured cells with anti Ly49A antibody as described in materials and methods. The extreme right panel summarizes the data of percentage of Ly49A+ cells in Mit C treated spleen cell cultured with IL-2 alone (open bars) and IL-2 + YAC\* (shaded bars).
Figure 22. Time kinetics of Ly49C expression on mitomycin C (Mit C) treated mouse spleen cells and activated with IL-2 in presence or absence of paraformaldehyde fixed YAC (YAC*) tumor cells. Spleen cells were pre-treated with Mit C (30 μg/ml) and cultured with IL-2 (50 U/ml) for different time intervals (1-5 days) in the presence (central panel) and absence (left panel) of YAC* tumor cells. The tumor to spleen cell ratio during culture was 1:100. The expression of Ly49A was determined by staining the cultured cells with anti Ly49C antibody as described in materials and methods. The extreme right panel summarizes the data of percentage of Ly49C+ cells in Mit C treated spleen cell cultured with IL-2 alone (open bars) and IL-2 + YAC* (shaded bars).
Figure 23. Time kinetics of Ly49A expression on mitomycin C (Mit C) treated mouse bone marrow cells and activated with IL-2 in presence or absence of paraformaldehyde fixed YAC (YAC*) tumor cells. Bone marrow cells were pre-treated with Mit C (30 μg/ml) and cultured with IL-2 (50 U/ml) for different time intervals (1-5 days) in the presence (central panel) and absence (left panel) of YAC* tumor cells. The tumor to bone marrow cell ratio during culture was 1:100. The expression of Ly49A was determined by staining the cultured cells with anti Ly49A antibody as described in materials and methods. The extreme right panel summarizes the data of percentage of Ly49A+ cells in Mit C treated spleen cell cultured with IL-2 alone (open bars) and IL-2 + YAC* (shaded bars).
Figure 24. Time kinetics of Ly49C expression on mitomycin C (Mit C) treated mouse bone marrow cells and activated with IL-2 in presence or absence of paraformaldehyde fixed YAC (YAC*) tumor cells. Bone marrow cells were pre-treated with Mit C (30 μg/ml) and cultured with IL-2 (50 U/ml) for different time intervals (1-5 days) in the presence (central panel) and absence (left panel) of YAC* tumor cells. The tumor to bone marrow cell ratio during culture was 1:100. The expression of Ly49C was determined by staining the cultured cells with anti Ly49C antibody as described in materials and methods. The extreme right panel summarizes the data of percentage of Ly49C+ cells in Mit C treated spleen cell cultured with IL-2 alone (open bars) and IL-2 + YAC* (shaded bars).
cell proliferation by mitomycin C (Mit C) on this effect was studied. Optimal dose of Mit C for this purpose was determined by its ability to block Con A induced proliferation response in mouse spleen cells. Results in Figure 20 show that all doses of Mit C (5-30 μg/ml) were effective in inhibiting cell proliferation in response to Con A. A dose of 30 μg/ml of Mit C caused maximum inhibition of cell proliferation and was used in subsequent experiments to block the cell division activity.

Effect of mitomycin C on Ly49 expression on mouse spleen cells in response to IL-2 and YAC* cells:

Previous results in Figure 14-17 depicted time kinetics of Ly49 induction in spleen and bone marrow cells in response to IL-2 and IL-2 + YAC* cells. These experiments were repeated using Mit C treated spleen or bone marrow cells. Results in Figure 21 show that IL-2 induced Ly49A expression in mouse spleen cells was not blocked by Mit C. A comparison of results in Figure 14 and 21 indicate that control or Mit C treated spleen cells expressed little Ly49A. Optimum activation of Ly49 expression in response to IL-2 occurred on day 4 in control as well as Mit C treated cells. About 20% of the cells became Ly49A+ in both cases. These results suggest that Mit C treatment had no effect on the process of IL-2 induced Ly49A expression on mouse spleen cells. In case of IL-2 + YAC* cell treatment, optimum activation for control spleen cells at day 4 generated 40-50% Ly49A+ cells. For Mit C treated cells however Ly49 expression was completely blocked and did not even reach the levels achieved by treatment with IL-2 alone (Figure 21). Very similar results were obtained with Ly49C expression as well (Figure 22).

Effect of Mit C on Ly49 expression on mouse bone marrow cells in response to IL-2 and IL-2 + YAC* cells:

Induction of Ly49 expression on Mit C on Ly49 expression on mouse bone marrow cells in response to IL-2 and IL-2 + YAC* cells was studied. Results in Figure 23 indicate that the effect of Mit C treatment differed from what was obtained for Mit C treated spleen cells. A comparison of Figure 16 and 23 indicate that for Mit C treated bone marrow cells, Ly49A induction by IL-2 was significantly
Figure 25. Effect of paraformaldehyde fixed YAC tumor cells on the Ly49A and Ly49C and TCRβ expression on bone marrow cells treated with mitomycin C. Bone marrow cells were treated with mitomycin C (as described in materials and methods) and cultured with IL2 (50 U/ml) either alone or in the presence of paraformaldehyde fixed YAC tumor cells for four days. The tumor to bone marrow cell ratio during culture was 1:100. The expression of Ly49A and Ly49C and TCRβ was determined by dual staining the cultured cells with combinations of anti Ly49A and TCRβ or Ly49C and TCRβ antibodies respectively.
Figure 26. Effect of paraformaldehyde fixed YAC tumor cells on the Ly49A and Ly49C and TCRβ expression on spleen cells treated with mitomycin C. Spleen cells were treated with mitomycin C (as described in materials and methods) and cultured with IL2 (50 U/ml) either alone or in the presence of paraformaldehyde fixed YAC tumor cells for four days. The tumor to spleen cell ratio during culture was 1:100. The expression of Ly49A and Ly49C and TCRβ was determined by dual staining the cultured cells with combinations of anti Ly49A and TCRβ or Ly49C and TCRβ antibodies respectively.
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greater than that observed for control bone marrow cells. For control bone marrow cells, IL-2 induced a sustained low level expression of Ly49A and about 10% cells were Ly49A+ at all time points in IL-2 treated cultures of bone marrow cells. For Mit C treated bone marrow cells, IL-2 treated cultures had about 30-40% Ly49A+ cells at all time points. IL-2 induced Ly49A expression was significantly greater for Mit C treated bone marrow cells as compared to what was seen for control bone marrow cells. Moreover, in Mit C treated bone marrow cells, IL-2 + YAC* cell treatment induced Ly49A expression which was in general comparable to that seen with IL-2 alone (Figure 23). A comparison with results in Figure 16 indicates that Ly49A expression induced by IL-2 + YAC* cells was similar for control and Mit C treated bone marrow cells. For Mit C treated bone marrow cells results for induction of Ly49A (Figure 23) and Ly49C (Figure 24) in response to IL-2 and IL-2 + YAC* were very similar.

Role of T cells in Ly49 induction response in Mit C treated spleen and bone marrow cells:

For Mit C treated spleen and bone marrow cells Ly49A/C induction in response to IL-2 and IL-2 + YAC* cells was studied in T cell compartment (TCRβ+ cells). Results for spleen and bone marrow cells are summarized in Figure 25 and 26 respectively. In case of Mit C treated spleen cells, induction of Ly49A/C was observed in response to IL-2 treatment but T cell compartment was severely depleted when Mit C treated spleen cells were cultured with IL-2 and IL-2 + YAC* cells (Figure 25). In case of Mit C treated bone marrow cells Ly49A/C expression was induced by IL-2 on T cells which remained unchanged if YAC* cells were also included in the culture. These experiments point out another qualitative difference between spleen and bone marrow cells with regards to induction of Ly49 receptors.
Chapter 3:
Effect of blocking NKR-MHC I interaction on NK cell activation by IL-2
Effect of anti H-2\textsuperscript{b} alloantisem on IL-2 induced NK activation in mouse bone marrow spleen cells:

MHC class I molecules are expressed on all nucleated cells including lymphocytes. A class of molecules, which act as receptors for MHC class I molecules, is also expressed on NK cells. When bone marrow spleen cells containing NK cells were activated with IL-2, an interaction between MHC class I molecules expressed on all leukocytes present in bone marrow spleen cells and receptors for MHC I on NK cell, may take place. Since MHC receptors (Ly49 family of molecules) may have activating as well as inhibiting properties depending upon the kind of motifs (ITAM or ITIM) that they have in the cytoplasmic tail and interaction between MHC molecules on bystander cell populations and receptor molecules on NK cells may modulate the activation response induced by IL-2 in NK cell populations.

Attempts were made to study these influences by using antibodies to block the interaction of MHC molecules with their receptors. Alloantisera raised against C57Bl/6 mouse spleen cells in Balb/C (H-2\textsuperscript{d} anti H-2\textsuperscript{b}) was used for this purpose. When this antiserum was added during NK activation by IL-2, a significant increase in activation was noted. Results in Figure 27 show that the anti YAC cytolytic activity generated in response to IL-2 in mouse bone marrow spleen cells was significantly boosted by adding anti H-2\textsuperscript{b} antibodies (Figure 27). The alloantisem that we used is expected not only to block the interaction of MHC I molecules with its receptors but would also generate antibody coated bystander cells in the reaction mixture, which may activate NK cells via CD 16 receptors expressed on NK cells. This proposition was further tested.

Role of Fc-CD 16 interaction in NK activation response:

In order to test the effect of Fc-CD16 interaction bystander antibody coated cells were generated by adding anti mouse IgG antibodies in the activation mixture. Anti Ig antibodies would react with antibody receptors on B-lymphocytes and generate antibody coated B cells. Addition of anti Ig antibodies resulted in a significant increase in NK activation in response to IL-2 (Figure 28). These results suggest that the activating effect of the alloantisem could be non-specifically mediated through the generation of antibody coated bystander cells during the
Figure 27. Effect of anti H-2b alloantiserum on IL-2 induced NK activation in mouse bone marrow cells. Mouse bone marrow cells were cultured with IL-2 (50 U/ml) either alone or in the presence of anti H-2b alloantiserum for four days. Alloantiserum was used at a dilution of 1:1000. The cytotoxic activity against YAC target cells was determined at several E:T ratios. Each value of target lysis is a mean ± SD of three replicate assay wells. Values in parentheses show the lytic units/10^6 cells of the effector cells.
Figure 28. Effect of anti mouse Ig antibodies on cytotoxicity of IL-2 activated bone marrow cells. Bone marrow cells were activated in vitro with IL-2 (50 U/ml) either alone or in the presence of anti mouse Ig (1.25 μg/ml). After four days, the cells were washed and their cytolytic activity against YAC target cells was determined at several E:T ratios. Each value of target lysis is a mean ± SD of results from three replicate assay wells. Values in parentheses show the lytic units/10⁶ effector cells.
Figure 29. Effect of heat aggregated (HA) Ig on IL-2 activation of cytotoxicity of bone marrow cells. Bone marrow cells were activated in vitro with IL-2 (50 U/ml) either alone or in the presence of different doses (2-50 μg/ml) of HA Ig (as described in materials and methods). After four days, the cells were washed and their cytolytic activity against YAC target cells was determined at several E:T ratios. Each value of target lysis is a mean ± SD of results from three replicate assay wells. Values in parentheses show the lytic units/10⁶ effector cells.
Figure 30. Effect of circulating immune complexes (CIC) on IL-2 induced activation of cytotoxicity of bone marrow cells. Bone marrow cells were activated in vitro with IL2 (50 U/ml) either alone or in the presence of different doses (2-50 μg/ml) of CIC (as described in materials and methods). After four days, the cells were washed and their cytolytic activity against YAC target cells was determined at several E:T ratios. Each value of target lysis is a mean ± SD of results from three replicate assay wells. Values in parentheses show the lytic units/10⁶ effector cells.
Figure 31. Effect of anti Fc receptor antibody (anti CD 16) on IL-2 induced activation of cytotoxicity of bone marrow NK cells. Bone marrow cells were activated in vitro with IL-2 (50 U/ml) either alone or in the presence of anti CD 16 antibody at a concentration of 1 ug/million bone marrow cells. After four days, the cells were washed and their cytolytic activity against YAC target cells was determined at several E:T ratios. Each value of target lysis is a mean ± SD of results from three replicate assay wells. Values in parentheses show the lytic units/10⁶ effector cells.
Figure 32. Effect of anti H-2^b alloantiserum and its F(ab)_2 fragment on IL-2 induced activation of NK cells in mouse bone marrow cells. Mouse bone marrow cells were cultured with IL-2 (50 U/ml) either alone or in the presence of anti H-2^b alloantiserum or its F(ab)_2 fragment for four days. Alloantiserum was used at a dilution of 1:1000 while F(ab)_2 fragment was used at a concentration of 1.5 μg/ml. The cytotoxic activity against YAC target cells was determined at several E:T ratios. Each value of target lysis is a mean ± SD of three triplicates. Values in parentheses show the lytic units/10^6 effector cells.
Figure 33. Dose response effect of F(ab)_2 fragment of anti H-2^b alloantibody on IL-2 induced activation of NK cells in mouse bone marrow. Mouse bone marrow cells were cultured with IL-2 (50 U/ml) either alone or in the presence of anti H-2^b alloantiserum F(ab)_2 fragments at different concentrations. After four days, the cells were washed and their cytolytic activity against YAC target cells was determined at several E:T ratios. Each value of target lysis is a mean ± SD of results from three replicate assay wells.
activation phase. These results were further confirmed by using heat aggregated immunoglobulin molecules which may also influence NK cells through CD 16 receptors. Results in Figure 29 indicated that heat aggregated immunoglobulin preparation induced a dose dependent increase in NK activation in response to IL-2. Circulating immune complexes derived from mouse sera had an effect similar to heat aggregated immunoglobulins (Figure 30). Engagement of CD16 receptors using monoclonal antibodies for CD16 also resulted in increase in cytotoxicity by NK cells activated with IL-2 (Figure 31). Taken together, these results suggest that an additional signal through CD16 receptors further boosts the IL-2 induced cytotoxicity response in NK cells. These results however, did not provide any insight into the role of MHC I-NKR interaction on IL-2 activation of NK cells.

F(\text{ab})_2 fragment preparation derived from anti H-2\text{b} alloantibody was used to block the MHC I-NKR interaction because lack of Fe portions would rule out the interference due to CD16 interaction. When MHC I was blocked using F(\text{ab})_2 fragment of anti H-2\text{b} alloantibodies, there was a marked inhibition of IL-2 induced activation of NK cytotoxicity response in contrast to the boosting effect of the whole antibody (Figure 33). F(\text{ab})_2 induced inhibition of NK cytotoxicity response was dose dependent (Figure 34).

**Effect of anti MHC class I monoclonal antibody on NK activation response:**

Alloantiserum raised by immunizing Balb/C mice with C57Bl/6 mice would contain not only antibodies against H-2\text{b} class I MHC molecules but also against several other antigens which are polymorphic and different allelic forms are expressed in the two given strains of mice. Inhibition of IL-2 induced NK activation by H-2\text{d} anti H-2\text{b} alloantibodies is therefore not a sufficient proof that disruption of MHC I-NKR interaction was solely responsible for this effect. In the next set of experiments, we used specific anti H-2\text{b} monoclonal antibody to disrupt the MHC I-NKR interaction. Results in Figure 34 indicate that the anti class I H-2\text{b} monoclonal antibody inhibited the IL-2 induced NK cytotoxicity response. Interestingly, anti class I monoclonal antibody unlike anti H-2\text{b} alloantibodies, did not augment the cytotoxicity response suggesting that the activation mediated by Fc-CD16 interaction did not cause significant interference if monoclonal antibody was used.
Figure 34. Effect of anti MHC class I monoclonal antibody on IL-2 induced cytotoxicity in bone marrow cells. Bone marrow cells were cultured with IL-2 (50 U/ml), alone or in the presence of anti MHC class I monoclonal antibody with or without anti Fe antibody. After four days, the cells were washed and their cytolysis activity against YAC target cells was determined at several E:T ratios. Each value of target lysis is a mean ± SD of results from three replicate assay wells. Values in parentheses show the lytic units/10^6 effector cells.
Figure 35. Effect of anti MHC class II monoclonal antibody on IL-2 induced NK activation in bone marrow cells. Bone marrow cells were cultured with IL-2 (50 U/ml). alone or in the presence of anti MHC class II monoclonal antibody with or without anti Fc antibody. After four days, the cells were washed and their cytolysis against YAC target cells was determined at several E:T ratios. Each value of target lysis is a mean ± SD of results from three replicate assay wells. Values in parentheses show the lytic units/10^6 effector cells.
Figure 36. Effect of monoclonal MHC class I and class II antibodies on IL-2 induced NK activation in bone marrow cells. Bone marrow cells were cultured with IL2 (50U/ml). alone or in the presence of anti MHC class I or anti MHC class II monoclonal antibodies in presence or absence of anti Fe antibody. Anti YAC lytic activity has been represented as Lytic Units/10^6 effector cells. Statistical significance between control and treated sets has been shown by asterisks. * P<0.05
Addition of F(ab)₂ fragment of a rabbit anti mouse Ig Fc antibody to block the Fc portions of the monoclonal antibody however further enhanced the inhibitory effect of the anti MHC monoclonal antibody (Figure 34).

**Effect of anti class II MHC antibody on IL-2 induced NK activation in bone marrow cells:**

We have already shown that the anti MHC I antibody inhibits the NK cytotoxicity response generated by IL-2 in bone marrow cells. Effect of anti MHC II monoclonal antibody was also examined in this system. Results in Figure 35 indicate that anti I-A<sup>b</sup> monoclonal antibody induced marked inhibition of IL-2 induced NK activation. Interestingly however, anti Fc antibody abrogated this effect. This is in contrast to the inhibitory effect of anti MHC class I monoclonal antibody, which was further strengthened by anti Fc antibody (Figure 34). Results of an experiment where effects of anti MHC I and anti MHC class II monoclonal antibodies were simultaneously examined in the presence or absence of anti Fc antibody is depicted in Figure 36. While both anti MHC class I or MHC class II monoclonal antibodies inhibited IL-2 induced NK activation in bone marrow cells, anti Fc antibody had opposite effect in the two cases.

**Effect of blocking Ly49 molecules on IL-2 induced NK activation:**

MHC-NKR interaction can be blocked by using antibody against MHC I molecules or antibody against NKRs. Previous results indicated that anti MHC class I monoclonal antibody could inhibit the NK activation response to IL-2. We attempted to confirm this observation by blocking the NKRs by using specific anti Ly49 monoclonal antibodies. Results in Figure 37 indicate that addition of anti Ly49C monoclonal antibody during NK activation by IL-2, resulted in a significant inhibition of NK cytotoxicity response. Inhibition was more pronounced when anti Fc antibody was also added (Figure 37). Interestingly however, anti Ly49A and anti Ly49D monoclonal antibodies did not modulate the NK cytotoxicity response (Figure 38, 39).

Simultaneous blockage of MHC class I molecules as well as NKRs was also attempted. Results in Figure 40 show that while anti Ly49C monoclonal antibody
Figure 37. Effect of anti Ly49C monoclonal antibody on IL-2 induced activation of cytotoxicity of bone marrow cells. Bone marrow cells were cultured with IL-2 (50 U/ml), alone or in the presence of anti Ly49C monoclonal antibody (1.25 μg/ml) with or without anti Fc antibody (1.5 μg/ml). Panel A shows the cytotoxic activity against YAC target cells at a number of E/T ratios. Each value of target lysis is a mean ± SD of three triplicates. Panel B represents the anti YAC cytolytic activity as Lytic Units. Statistical significance of anti-Ly49C treated sets from control sets is shown by asterisks: * P<0.05, **P<0.01.
Figure 38. Effect of anti Ly49A monoclonal antibody on IL-2 induced activation of cytotoxicity in bone marrow cells. Bone marrow cells were cultured with IL-2 (50 U/ml), alone or in the presence of anti Ly49A monoclonal antibody (1.25 μg/ml) with or without anti Fc antibody (1.5 μg/ml). Panel A shows the cytotoxic activity against YAC target cells at a number of E/T ratios. Each value of target lysis is a mean ± SD of three triplicates. Panel B represents the anti YAC cytotoxic activity as Lytic Units.
Figure 39. Effect of anti Ly49D monoclonal antibody on IL-2 induced activation of cytotoxicity of bone marrow cells. Bone marrow cells were cultured with IL-2 (50 U/ml), alone or in the presence of anti Ly49D monoclonal antibody (1.25 µg/ml) with or without anti Fc antibody (1.5 µg/ml). Panel A shows the cytotoxic activity against YAC target cells at a number of E/T ratios. Each value of target lysis is a mean ± SD of three triplicates. Panel B represents the anti YAC cytolytic activity as Lytic Units.
Figure 40. Effect of anti Ly49A and anti MHC class I monoclonal on IL-2 activation of bone marrow cells in presence or absence of anti Fc antibody. Bone marrow cells were cultured with IL-2 (50 U/ml) either alone or in the presence of anti Ly49A with or without anti Fc antibody and monoclonal MHC class I antibody in presence or absence of anti Fc antibody. The anti YAC cytolytic activity has been represented as Lytic Units/10^6 effector cells.
Figure 41. Effect of anti Ly49D and anti MHC class I monoclonal on IL-2 activation of bone marrow cells in presence or absence of anti Fe antibody. Bone marrow cells were cultured with IL-2 (50 U/ml) either alone or in the presence of anti Ly49D with or without anti Fe antibody and monoclonal MHC class I antibody in presence or absence of anti Fe antibody. The anti YAC cytolytic activity has been represented as Lytic Units/10^6 effector cells.
Figure 42. Effect of anti Ly49C and anti MHC class I monoclonal on IL-2 activation of bone marrow cells in presence or absence of anti Fc antibody. Bone marrow cells were cultured with IL-2 (50 U/ml) either alone or in the presence of anti Ly49C with or without anti Fc antibody and monoclonal MHC class I antibody in presence or absence of anti Fc antibody. The anti YAC cytolytic activity has been represented as Lytic Units/10^6 effector cells. Statistically significant changes in treated as compared to the control cells are shown by asterisks. * P<0.05, **P<0.001.
Figure 43. Purification of NK cells from mouse bone marrow cell preparations. NK cells were first partially purified by passage through nylon wool column and treatment with anti Thyl and complement (as described in materials and methods). Further NK cell purification was done by using StemSep magnetic purification columns (as described in materials and methods). Unfractionated bone marrow cells, partially purified NK cells and affinity purified NK cells were stained with NK1.1 antibody. Curve A denotes NK1.1\(^+\) population in the unfractionated bone marrow cells, curve B shows the NK1.1\(^+\) population in partially purified cell population and curve C shows the affinity purified cell preparations. After final stage of purification 91% cells were NK1.1 positive.
Figure 44. Effect of anti Ly49C and anti MHC class I monoclonal antibodies on IL-2 activation of purified bone marrow NK cells. NK cells were purified from bone marrow preparations as described in materials and methods and were cultured with IL-2 (50U/ml) alone or in the presence of anti Ly49C and anti MHC class I monoclonal antibodies along with anti Fc antibody. Anti YAC lytic activity has been represented as lytic units/10^6 effector cells. Statistically significant difference of treated sets with control set: * P<0.05, **P<0.001.
induced significant inhibition of IL-2 induced NK activation in mouse bone marrow cells, addition of anti MHC I monoclonal antibody and anti Fc antibody had only marginal additional inhibitory effect (Figure 40).

**Effect of anti MHC class I and anti Ly49C monoclonal antibodies on IL-2 induced NK activation in affinity purified NK cell preparation:**

In previous experiments, unfractionated bone marrow cell preparations were used to assess the effect of anti MHC and anti Ly49 monoclonal antibody. In order to ensure that the observed effects of the monoclonal antibodies were direct effects on NK cells and not mediated by bystander cells in bone marrow, we repeated the experiment with highly purified NK cell preparation derived from bone marrow. Bone marrow cells were passed through nylon wool column to get rid of B cells and treated with anti-Thy1 + complement to remove T cells. The resulting partially purified NK cell preparation was further purified by using Stem Sep affinity columns as described in materials and methods. NK 1.1 staining profiles of unfractionated bone marrow cells, partially purified NK cells and affinity column purified NK cells as shown in Figure 43. Affinity purified NK cell preparation were >90% pure. Using highly purified NK cell preparation from bone marrow cells, the effect of anti MHC class I and anti Ly 49C monoclonal antibody were tested on IL-2 induced NK activation. Results in Figure 44 indicate that both anti MHC I and Ly 49C monoclonal antibodies inhibited the NK activation. In addition, using both monoclonal antibodies together further increased the inhibitory effect. These results suggest that the two antibodies exerted their inhibitory effect directly on NK cells.