

The progression of T cells from a quiescent or resting state to a fully activated and proliferating cell is a crucial step in defending against foreign pathogens. T cells require two signals for optimum activation and effector function. One of these signals determines which CD4⁺ cells will respond to a particular antigen: is delivered through the receptor for an antigen on the T cells (TCR), which is recognized in the form of peptide fragments bound to major histocompatibility complex (MHC) class II molecules. The second signal, which is not delivered via the TCR and is not antigen specific, has been termed as a costimulatory signal because while essential it does not by itself induce any response in T cells. However, when a T cell has its receptor ligated and receives costimulatory signal, then only the T cell will proliferate and differentiate into an armed effector cell. T cells that bind antigen but do not receive costimulatory signals are thought to die or to become anergic, a state in which the cell cannot be activated even if it receives both the signals required to activate. Antigen presenting cells viz. B cell, macrophage and dendritic cell express on their surface an array of costimulatory molecules like ICAM-1, HSA, CD40, B7-1, B7-2 and M150. The exact mechanism of sequence of signals delivered by different costimulatory molecules in stimulating T cells is largely unknown. The best defined costimulators till date are B7-1 and B7-2, both of which have been well documented in providing critical costimulatory signals for T cells activation by interacting with their specific receptor, CD28. Recently, we have identified a 150-kDa (M150) molecule on the macrophage membrane. M150 is a potent costimulatory molecule. It preferably activates Th1 cells as evidenced by predominant IFN- γ secretion by anti-CD3 Abs stimulated antigen specific CD4⁺ T cells. IL-12 or IL-1 can also regulate activation of Th1 and Th2 cells respectively. Both Th1 and Th2 cells can proliferate in response to IL-2 and IL-4. IFN- γ inhibits the proliferation of Th2 cells.

In the present study, we wanted to evaluate the possible role of costimulatory molecules B7-1 and B7-2 in the proliferation and cytokine secretion by Th1 and Th2 rested effectors. Further, we also wanted to monitor whether increasing the strength of first signal could by pass the need of costimulation. We also studied the role of cytokines in conjunction with B7-1 and B7-2 costimulatory signals in modulating the activation of rested effectors. Furthermore, we studied the ability of Th1 and Th2 rested effectors activated with anti-CD3 Ab and costimulatory molecules B7-1 and B7-2 to provide help to B cells. Distinct role of B cells, splenic and peritoneal macrophages was also studied in

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the regulation of the proliferation and cytokines secretion by Th1 and Th2 cells in the presence of different cytokines and anti-IL-12 and anti-IFN- γ Abs in the case of Th1 cells and anti-IL-4 Ab in the case of Th2 cells. Finally, the role of IL-2, IL-12 and antigen pulsed splenocytes in the sustenance of Th1 memory was studied.

The study was conducted by employing pGL-10, AE7, D10.10.2 (Th1), D10G4.1 (Th2) clones and EL-4 (Th0) cells. The interesting features observed during the study were that the strength of first signal and second signal could greatly influence the behaviors of the Th1 and Th2 rested effectors. EL-4 cells produced more IL-2 and IL-4 with strong than weak first signal. B7-1 transfectants increased the production of IL-2 with all the doses of anti-CD3 Ab. However, the release of IL-2 was augmented with the increase in the number of B7.1 transfectants. With the delivery of weak first signal, only high concentration of B7-1 transfectants were effective in IL-4 secretion. Both B7-1 and B7-2 transfectants enhanced the secretion of IL-4 by EL-4 cells stimulated with strong first signal. B7-1 transfectants were more potent than B7-2 transfectants.

PGL-10 Th1 rested effectors stimulated with weak anti-CD3 Ab induced marginal proliferation and B7-1 costimulation further increased the proliferation with the highest number of transfectants. Strongest dose of both anti-CD3 Ab and B7-1 transfectants induced maximum proliferation of Th1 rested effectors. As observed in the case of proliferation, significant IFN- γ secretion was also observed with the high dose of B7-1 transfectants when Th1 cells were stimulated with weak first signal. With the increase in the strength of first signal, the level of IFN- γ secretion was also enhanced. The production was further intensified by the addition of B7-1 transfectants. However, the lowest dose of B7-1 transfectants boosted IFN- γ release by Th1 cells stimulated with the strongest dose of first signal. But with the increase in the dose of B7-1 transfectants, not much amplification in the yield of IFN- γ was noticed. Costimulation with B7-2 transfectants showed only minor effect on Th1 cells. To substantiate our results, we also tested our observation on the proliferation and IFN- γ secretion on AE7 Th1 rested effectors. The cells were costimulated using B7-1 and B7-2 transfectants. Stimulation with the strongest first signal, ruled out the need of costimulation for Th1 rested effectors. AE7 Th1 cells showed inhibition in the proliferation when stimulated with the strongest dose of first signal. This dose was 50 times more than the highest dose used for the stimulation of

pGL-10 Th1 cells. As compared to proliferation, when AE7 Th1 cells were stimulated with the strongest degree of first signal, there was no inhibition in the secretion of IFN- γ . AE7 Th1 cells stimulated with moderate strength of first signal showed their requirement for costimulation. The secretion of IFN- γ was further enhanced with the increase in the dose of B7 transfectants. At the weak dose of first signal, IFN- γ production by AE7 Th1 cells was not detected even when the highest numbers of B7-1 transfectants were added into the cultures. However, Th1 cells stimulated with strong first signal produced optimum level of IFN- γ and addition of B7-1 transfectants did not significantly enhanced IFN- γ secretion.

After evaluating the costimulatory activity of B7-1 and B7-2 molecules on Th1 clones, we also utilized D10G4.1 Th2 rested effector to monitor the difference in their requirement for costimulation. Th2 cells stimulated with increased dose of first signal showed enhanced proliferation. However, costimulation with B7-1 transfectants increased the proliferation at weak to moderate dose of first signal. There was no effect of B7-1 costimulation when Th2 cells were stimulated with strong first signal. Surprisingly, highest dose of B7-2 inhibited Th2 proliferation. Only highest dose of B7-1 transfectants induced IL-4 secretion by Th2 cells stimulated with strong first signal. B7-1 costimulation showed increased IL-4 production when Th2 cells were stimulated with strong first signal. B7-2 also helped in the production of IL-4 but was inhibitory at higher doses. Th2 cells produced more IL-5 when stimulated with higher dose of first signal. Addition of B7-1 and B7-2 transfectants further increased the yield of IL-5.

We also wanted to study the influence cytokines in modulating the costimulatory behavior of B7-1 and B7-2 molecules. We therefore, studied the role of cytokines in conjunction with B7-1 and B7-2 transfectants in the regulation of the proliferation and cytokines secretion by Th1 and Th2 rested effectors. Addition of IL-12 in the cultures containing Th1 cells stimulated with anti-CD3 Ab and B7-1 transfectants, induced optimal proliferation. Further, addition of IL-2 alone or in combination with IL-4 marginally increased the proliferation. Addition of IL-2 or IL-4 in the cultures comprising of Th1 cells stimulated with anti-CD3 Ab and B7-1 transfectants further boosted the proliferation. Addition of cytokines and B7-2 transfectants failed to induce any effect. Addition of IL-2 and IL-12 in Th1 cells stimulated with anti-CD3 Ab alone or in context

with B7 transfectants amplified the secretion of IFN- γ . IL-12 induced optimal secretion of IFN- γ and addition of IL-2 and IL-4 could not further induce any change. We also evaluated the role of pro- (IL-4) and anti- (IFN- γ) Th2 cytokines on the proliferation of anti-CD3 Ab stimulated D10G4.1 Th2 rested effectors costimulated with B7-1 and B7-2 transfectants. Addition of IL-2 or IL-4 or both could significantly increase the proliferation of Th2 cells costimulated with B7-1 transfectants. No noticeable change was noted when Th2 cells were costimulated with B7-2 transfectants. Addition of IFN- γ inhibited the proliferation of Th2 cells costimulated with B7-1 transfectants. It also neutralized the stimulatory effect of IL-2 or IL-4 on Th2 cells stimulated with anti-CD3 Ab alone or in conjunction with B7-1 transfectants. In contrast to proliferation, addition of IFN- γ ; moreover, assisted in the secretion of IL-4 by Th2 cells costimulated with B7 transfectants. IL-2 also supported the production of IL-4 by Th2 cells costimulated with B7 transfectants. B7-2 transfectants were more potent in assisting IL-4 production than B7-1 transfectants in presence of IL-2 and IFN- γ . But the addition of IL-2 or IL-4 sustained the production of IL-5 by Th2 cells costimulated with B7-1 but not B7-2 transfectants. Among the cytokines used, IL-2 was more potent than IL-4. The production of IL-5 by Th2 cells mediated by IL-2 or IL-4 was inhibited by IFN- γ . The IFN- γ mediated inhibition of IL-5 was more potent when Th2 cells were costimulated with B7-2 transfectants. Addition of IL-1 in Th2 cells cultures stimulated with anti-CD3 Ab induced optimal proliferation of Th2 cells and addition of B7-1 transfectants further failed to induce any effect. B7-1 transfectants only induced proliferation when they were supplemented with IL-4. B7-2 transfectants could not show any effect. Addition of IL-1 assisted in the secretion of IL-4 by Th2 cells costimulated by B7 transfectants. IL-1 was more potent in the production of IL-4 and IL-5 by Th2 cells stimulated with B7-1 transfectants than B7-2 transfectants. However, addition of IL-4 in the culture decreased the yield of IL-5.

Th1 cells were also costimulated with different antigen presenting cells viz. B cell, splenic and peritoneal macrophages. Peritoneal macrophages induced the proliferation of Th1 cells at low cell number but inhibited when used at a higher density. Antigen pulsed splenic macrophages induced more proliferation of Th1 cells, followed by B cells.

Peritoneal macrophages induced enhanced IFN- γ secretion than splenic macrophages and B cells. B cells were least stimulatory for both proliferation and IFN- γ secretion.

We also evaluated the effect of the mitomycin C treatment and γ -irradiation on the costimulatory function of peritoneal macrophages. Gamma-irradiated peritoneal macrophages failed to support the proliferation of Th1 cells. However, mitomycin C treated macrophages assisted the proliferation at lower dose of cells. Antigen pulsed or un-pulsed mitomycin C treated macrophages produced less nitric oxide than γ -irradiated macrophages cultured with Th1 cells.

We further evaluated the role of costimulatory molecules expressed on the surface of peritoneal macrophages in the proliferation and cytokines secretion by Th1 and Th2 rested effectors. Incorporation of anti-M150, B7-1, B7-2, CD40, ICAM-1 and F4/80 Abs inhibited the proliferation of Th1 cells. However, Abs showed no effect on the IFN- γ secretion by Th1 cells. Only anti-ICAM-1 Ab could inhibit the proliferation of D10G4.1 Th2 cells. There was inhibition in the IL-4 and IL-5 production by Th2 cells when the cultures were incubated with anti-M150, B7-1, B7-2, CD40, ICAM-1 and F4/80 Abs. We also observed that costimulatory molecules expressed on the surface of J774, P388D1 and A20 cells helped in the IL-2 and IL-4 secretion by EL-4 cells stimulated with anti-CD3 Ab. EL-4 cells were stimulated with anti-CD3 Ab in the presence of J774 cells as a source of costimulation. Anti-ICAM-1, B7-1, B7-2 and M150 Abs considerably inhibited the secretion of IL-2 and IL-4. When we used P388D1 as a source of costimulation, we observed decline in the secretion of IL-2 and IL-4 by EL-4 cells when only anti-ICAM-1 Ab was added into the cultures. When we used A20 as a source of costimulation, we observed that only anti-B7-1 Ab could inhibit the secretion of IL-2 and IL-4. This indicates that even cancer cells can efficiently interact and costimulate each other. Further, different APC not only behaved differently as an antigen presenting cell but also utilize distinct costimulatory molecules to stimulate T cell response.

We also evaluated the role of various antigen presenting cells viz. B cells and splenic macrophages in the presence of anti-IL-12 Ab and different Th1 and Th2 type cytokines in the proliferation and IFN- γ secretion by Th1 cells. IL-4 or IL-12 substantially increased the proliferation of Th1 cells stimulated by antigen-pulsed B cell. The response induced by IL-4 or IL-12 was better than IL-2 or IFN- γ . In the absence of B cells, IL-2 and IL-12

induced better response than IL-4 in enhancing the proliferation of Th1 cells. Proliferation of Th1 cells induced by either IL-2 or IL-4 could not be neutralized by anti-IL-12 Ab. We observed maximum secretion of IFN- γ in the cultures stimulated with IL-12, IL-4 and to lesser extent by IL-2. Anti-IL-12 Ab significantly inhibited the release of IFN- γ . We could not detect any blocking in the secretion of IFN- γ in the cultures where IL-2 and IL-4 were added along with anti-IL-12 Ab. The presence of IL-2 could only show marginal increase in the proliferation of pGL-10 Th1 clones. Surprisingly, when we added IL-2 or IL-4 into the cultures containing anti-IL-12 Ab, there was augmentation in the proliferation. In contrast, addition of anti-IL-12 Ab into the cultures along with antigen-pulsed splenic macrophages decreased the proliferation of Th1 clones. In contrast to B cells, no enhancement in the secretion of IFN- γ was observed when IL-2, IL-4 and IL-12 were added into antigen-pulsed splenic macrophages and pGL-10 Th1 clone. However, there was decrease in the secretion of IFN- γ in the cultures where splenic macrophages were used to stimulate Th1 cells in the presence of anti-IL-12 Ab. We also evaluated the role of various antigen presenting cells viz. B cells, splenic and peritoneal macrophages in the presence of anti-IFN- γ Ab and different Th1 and Th2 type cytokines in the proliferation and IFN- γ secretion by Th1 cells. There was not much change in the proliferation of pGL-10 Th1 clones by incorporating anti-IFN- γ Ab into the cultures containing antigen-pulsed B cells. There was however, marginal increase in the proliferation when IFN- γ was added into the cultures in the absence of anti-IFN- γ Ab. There was significant inhibition in the proliferation where anti-IFN- γ Ab was added in the presence of IL-4. Addition of either IL-2 or IL-4 or IL-12 or IFN- γ could not alter the proliferation of Th1 rested effectors stimulated with antigen pulsed splenic macrophages. There was no change in the proliferation of Th1 cells on addition of anti-IFN- γ Ab into the cultures where antigen-pulsed splenic macrophages were used in the presence of either IL-2 or IL-4 or IL-12. There was marginal decrease in the proliferation when either IL-2 or IL-4 or IL-12 was added in Th1 cells cultures stimulated with antigen pulsed peritoneal macrophages. There was decrease in the proliferation of Th1 cells when anti-IFN- γ Ab was added. When Th1 cells were stimulated with IL-2, IL-4 or IL-12, there was augmentation in the proliferation. Addition of either IL-2 or IL-12 further augmented the production of IFN- γ by Th1 cells stimulated with antigen pulsed peritoneal macrophages. In contrast, IL-4 inhibited the release of IFN- γ . When peritoneal macrophages were added

into Th1 cells cultures stimulated with IL-2 or IL-4 or IL-12, there was significant enhancement in the production of IFN- γ . We also noticed that low dose of IL-10 was stimulatory and supported the proliferation of Th1 rested effectors stimulated by antigen pulsed peritoneal macrophages in the presence of aminoguanidine. In contrast, high dose was inhibitory.

We evaluated the role of different antigen presenting cells viz. B cells, splenic and peritoneal macrophages in the proliferation and cytokines secretion by D10G4.1 Th2 cell in the presence of antigen, anti-IL-4 Ab and Th1/Th2 type of cytokines. Addition of IL-2, IL-4 and IL-1 into the cultures augmented the proliferation of Th2 cells stimulated with antigen pulsed B cells. However, these cytokines failed to alter the proliferation of Th2 cells incubated with allogenic B cells. IL-5 and IFN- γ also could not show any effect on the proliferation. Addition of either IL-2 or IL-1 augmented the proliferation of Th2 cells in the presence of anti-IL-4 Ab. The extent of proliferation was lesser as compared to the cultures where no anti-IL-4 Ab was added. Similar observations were also noticed with Th2 cells stimulated with allogenic B cells. Addition of IL-2, IL-5 and IL-1 could not magnify the secretion of IL-4 by Th2 cells stimulated with antigen pulsed B cells. Interestingly, IFN- γ significantly increased the release of IL-4. Addition of IFN- γ and IL-1 could also boost the secretion of IL-4 by Th2 cells stimulated by allogenic B cells. Addition of IL-1, IL-2, IL-4 and IFN- γ intensified the secretion of IL-5 by Th2 cells stimulated with antigen-pulsed or allogenic B cells. Maximum enhancement in the secretion of IL-5 was demonstrated by the addition of IL-2. In the presence of anti-IL-4 Ab, IL-2 and IL-1 augmented the production of IL-5 by Th2 cells stimulated with antigen-pulsed or allogenic B cells. This effect was more prominent with IL-2. IFN- γ could not influence the production of IL-5 in the presence of anti-IL-4 Ab. However, IFN- γ induced the production of IL-5 by Th2 cells stimulated by allogenic B cells in the presence of anti-IL-4 Ab. Th2 clone when stimulated with allogenic B cell produced more of IL-4 and IL-5 as compared to the cultures where antigen-pulsed B cell was used as a source of APC. IL-2, IL-4 and IL-1 also aided the proliferation of Th2 cells stimulated by antigen-pulsed splenic macrophages. However, only IL-1 induced marginal increase in the proliferation of Th2 rested effectors stimulated by allogenic splenic macrophages. IFN- γ inhibited the growth of Th2 rested clone stimulated by antigen pulsed or allogenic splenic macrophages. IL-2 and IL-1 also enhanced the proliferation

of Th2 cells even in the presence of anti-IL-4 Ab. However, the extent of proliferation was less as compared to the cultures deprived of anti-IL-4 Ab. Th2 cells showed better proliferation with allogeneic splenic macrophage than antigen-pulsed splenic macrophages. Addition of IL-2, IL-5, IL-1 and IFN- γ into the cultures further compounded the production of IL-4 by Th2 cells stimulated with antigen pulsed splenic or allogeneic splenic macrophages. Addition of IL-2, IL-4 and IL-1 augmented the secretion of IL-5 by Th2 cells stimulated by antigen-pulsed splenic macrophages. Similar observations were noted with allogeneic splenic macrophages. IL-2 was most effective in enhancing the production of IL-5. IL-2, IL-4 and IL-1 augmented the production of IL-5 by Th2 cells stimulated with antigen un-pulsed splenic macrophages. In the presence of anti-IL-4 Ab; IL-2, IL-1 and IFN- γ enhanced the production of IL-5 by Th2 cells stimulated by antigen pulsed splenic and allogeneic splenic macrophages. As compared to antigen-pulsed peritoneal macrophages, allogeneic peritoneal macrophages induced better proliferation of D10G4.1 Th2 clone. IFN- γ inhibited the proliferation of Th2 clone stimulated with antigen pulsed or allogeneic peritoneal macrophages. Addition of IL-1 and IL-2 in the cultures containing anti-IL-4 Ab augmented the proliferation of Th2 rested effectors. The extent of proliferation was significantly less in the cultures where anti-IL-4 Ab was added. Addition of IL-2, IL-5, IL-1 and IFN- γ augmented the production of IL-4 by Th2 cells stimulated with antigen pulsed peritoneal macrophages. However, IFN- γ and IL-1 considerably supported the production of IL-4 when Th2 clone was stimulated with allogeneic peritoneal macrophages. Addition of IL-2, IL-4 and IL-1 boosted IL-5 secretion by Th2 clone stimulated with antigen-pulsed peritoneal macrophages. IL-2 substantially upregulated the production of IL-5 by Th2 cells stimulated with allogeneic B cells. In the presence of anti-IL-4 Ab, IL-2, IL-1 and IFN- γ increased the IL-5 production by Th2 cells stimulated with antigen pulsed and allogeneic peritoneal macrophages. Since D10G4.1 Th2 clone was also allogeneic to the IA^b haplotype bearing APC, we therefore further wanted to substantiate our results using allogeneic APCs. Allogeneic B cell increased the proliferation to greatest extent as compared to splenic or peritoneal macrophages. High number of peritoneal macrophages induced inhibition in the proliferation of Th2 clone. B cells induced maximum IL-4 secretion, followed by peritoneal macrophages and splenic macrophages. Unlike IL-4, peritoneal macrophages induced maximum IL-5 secretion, followed by B cells and splenic macrophages.

It is very crucial to explore the factors responsible for sustenance of memory cells. We therefore also studied the role of IL-2, IL-12 and APC in regulating the sustenance of memory cells. IL-12 showed major role in supporting the sustenance of memory response for short duration. IL-2 synergistically acted with IL-12 but also showed independent impact in the continuance of memory Th1 cells. However, incorporation of antigen-pulsed APC inhibited the memory response mediated by IL-12. Interestingly, by day 56, IL-12 in combination with IL-2 successfully maintained the memory Th1 cells.

Finally, we studied the ability of Th1 and Th2 cells costimulated with either B7-1 or B7-2 to provide help B cells. Anti-CD3 Ab stimulated Th1 cells costimulated with either CHO-B7-1 or CHO-B7-2 induced nearly similar level of B cells proliferation. Costimulation of Th1 cells with either B7-1 or B7-2 transfectants, could gain only marginal competence to help B cells in the IgM secretion. Similar type of results were observed when Th2 cells were costimulated with B7 transfectants in providing help to B cells. When anti-CD3 Ab stimulated Th1 rested effectors costimulated with B7-1 or B7-2 transfectants were utilized to deliver help to B cells, there was inhibition in the production of IgG1, IgG2a, IgG3 Abs. In contrast, when Th2 rested clones were costimulated with B7-1 and B7-2 there was marginal amplification in the yield of IgG1, IgG2a, IgG3 Abs. Enhancement in the production of IgA was observed when B cell help was provided by Th1 clone that was costimulated with B7-1. Similar case was observed with B7-1 costimulated Th2 clones but no change was noticed when Th2 clone was costimulated with B7-2 molecules. However, as compared to the help extended to B cells, Th2 clone's performance was better than Th1 clone; both in the proliferation and antibodies secretion. It may be inferred from these results that costimulation by B7-1 and B7-2 could influence the ability of Th1 and Th2 cells to help B cell.