Combination Chemotherapy and IL-15 Administration Induce Permanent Tumor Regression in a Mouse Lung Tumor Model: NK and T Cell-Mediated Effects Antagonized by B Cells

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Combination Chemotherapy and IL-15 Administration Induce Permanent Tumor Regression in a Mouse Lung Tumor Model: NK and T Cell-Mediated Effects Antagonized by B Cells

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Previous studies have demonstrated that IL-15 administration after cyclophosphamide (CY) injection of C57BL/6J mice bearing the i.m. 76-9 rhabdomyosarcoma resulted in a significant prolongation of life. In the present study, we investigated the immune response against the 76-9 experimental lung metastases after CY + IL-15 therapy. Administration of CY + IL-15, but not IL-15 alone, induced prolongation of life and cures in 32% of mice bearing established experimental pulmonary metastases of 76-9 tumor. The CY + IL-15 therapy resulted in increased levels of NK1.1+/LGL-1+ cells, and CD8+/CD44+ T cells in PBL. In vitro cytotoxic assay of PBL indicated the induction of lymphokine-activated killer cell activity, but no evident tumor-specific class I-restricted lytic activity. Survival studies showed that the presence of NK and T lymphocytes is necessary for successful CY + IL-15 therapy. Experiments using knockout mice implied that either αβ or γδ T cells were required for an antitumor effect induced by CY + IL-15 therapy. However, mice lacking in both αβ and γδ T cells failed to respond to combination therapy. Cured B6 and αβ or γδ T cell-deficient mice were immune to rechallenge with 76-9, but not B16LM tumor. B cell-deficient mice showed a significant improvement in the survival rate both after CY and combination CY + IL-15 therapy compared with normal B6 mice. Overall, the data suggest that the interaction of NK cells with tumor-specific αβ or γδ T lymphocytes is necessary for successful therapy, while B cells appear to suppress the antitumor effects of CY + IL-15 therapy. The Journal of Immunology, 1998, 161: 6977–6984.

Interleukin-15, a 14- to 18-kDa cytokine, has biological activities similar to those of IL-2 (1, 2). IL-15 has been shown to stimulate the growth of NK cells (3), activated peripheral blood T lymphocytes (4), γδ T cells (5), and B cells (6). It has been reported recently that IL-15 induces the production of proinflammatory cytokines from macrophages (Mφ) (7) and activates human neutrophils (8). Reports that IL-15 induces the expression of mRNA for perforin and granzymes in murine lymphocytes (9), activates human PBL for perforin-mediated lysis of melanoma and lung cancer tumor cells (10, 11), and induces the generation of CTL (1) and the maturation/differentiation of cytokytic NK cells (12, 13) suggest that this cytokine may play an important role in antitumor immunity. Indeed, it was shown that administration of IL-15 prolonged survival of lymphoma-bearing mice (14) and suppressed pulmonary metastases induced by i.v. injection of sarcoma cells (15).

It was shown previously in this laboratory that IL-15 acted as an adjuvant when administered in combination with CY, significantly prolonging the life of mice bearing the i.m. implanted 76-9 rhabdomyosarcoma (16). Combination therapy was seen to induce an increase in NK cells in vivo. These were shown to be cytotoxic in vitro against YAC-1 cells, and to exert antitumor effects when adoptively transferred to CY-treated tumor-bearing (TB) mice. Their lack of cytotoxic activity in vitro against the 76-9 tumor, together with little or no evidence for IL-15-induced MHC class I-restricted lysis, suggested that NK cell involvement in antitumor activity was probably indirect and mediated via its secretory products. However, the mechanisms of the combined action of CY and IL-15 on tumors still need to be clarified. It is established that CY augments delayed-type sensitivity reactions by eliminating suppressor T cells (17) or by increasing the production of Th1-related cytokines (18). It has been reported that CY increases the localization of effector cells in the tumor mass (19), to augment the antitumor action of adoptively transferred tumor-infiltrating lymphocytes in clinical trials (20), and increases therapeutic efficacy of IL-2 (21). In addition, as shown in this laboratory, CY injection resulted in an increase in tumor-associated Mφ, as well as NK cell and granulocyte precursors (22, 23). Thus, because of its reported ability to react with NK cells, Mφ, granulocytes, T cells, and B cells, as cited above, it seems plausible to suggest that antitumor adjuvant activity of IL-15 may be mediated by activation of any or all of these cellular compartments following CY chemotherapy.

In this study, we examined the impact of IL-15 as an adjuvant to cancer chemotherapy using CY in an experimental pulmonary metastasis model. In addition, we explored the cellular compartments most likely to be involved in successful CY + IL-15 therapy.

Materials and Methods

Mice

Male C57BL/6J, C57BL/6-Ly5+ (B6.beige), C57BL/6J-Prkd−/−/Sz3 (B6.scid), C57BL/6-J-Hha1nu (B6.nude), C57BL/6J-TCRδ−/−/Mom
using the Becton Dickinson FACScan.

FITC-labeled streptavidin and washed in PBS. Stained cells were analyzed.

mAb, washed in PBS containing 5% FBS. Cells treated with medium, as required, and used in experiments.

for 5 min. After centrifugation, cells were washed three times to remove debris that contained red cell ghosts and residual platelets that sedimented above the cell pellet. The remaining white cells were suspended in medium, as required, and used for i.v. injection into mice.

Preparation of PBL

PBL were obtained by modification of the methods described previously (26). In brief, blood was collected from the tail vein, diluted immediately in serum-free RPMI 1640 containing 50 mM EDTA, and washed by centrifugation for 10 min at 170–190 × g. The pellet was lysed using ice-cold lysing buffer (154 mM NH₄Cl, 1.5 mM KHCO₃, 0.1 mM EDTA, pH 7.2) for 5 min. After centrifugation, cells were washed three times to remove debris that contained red cell ghosts and residual platelets that sedimented above the cell pellet. The remaining white cells were suspended in medium, as required, and used for i.v. injection into mice.

In vivo treatment studies

Preparatory experiments indicated that injection of B6 mice with 10⁵ 76-9 tumor cells was a minimal dose that resulted in the development of pulmonary metastases that could not be cured with CY alone, but were sensitive to therapy with CY + IL-15. Pulmonary metastases developed after injection of 5 × 10⁵ or fewer 76-9 cells were cured with CY alone. Thus, in further experiments, we used 10⁷ 76-9 tumor cells as a minimal dose for development of pulmonary metastases not sensitive to chemotherapy alone.

On day 0, mice were injected i.v. into the tail vein with 76-9 tumor cells (5 × 10⁵) to establish pulmonary tumors. Ten days later, mice were treated i.p. with single dose of 200 mg/kg body weight of CY (Cytotoxan; Bristol Myers Squibb, Princeton, NJ). Human rIL-15 (sp. act. of 4.45 × 10⁶ U/mg; Immunex, Seattle, WA) was given by i.p. injection for 20 days at a dose of 10⁵ μg/mouse/day starting 24 h after CY treatment. Survival of TB mice was monitored every day. Mice that became moribund due to lung tumors (usually between 35 and 40 days after tumor inoculation for TB mice) were killed for humane reasons. Mice surviving the course of therapy, mice were bled (200 μl of blood from mouse) at day 35 after 76-9 tumor inoculation, and lungs were infused with a 15% solution of india ink and bleached by Fekete’s solution (27).

Statistics

All data were analyzed by using the Student’s t test (SigmaPlot), or χ² test for survival studies, whereby p < 0.05 indicated that the value of the test sample was significantly different from that of the relevant controls.

Results

IL-15 administration after CY injection prolongs the life and cures TB mice

Lung metastases were established in B6 mice, as described. Ten days later, TB mice received an i.p. injection of CY (200 mg/kg) and daily i.p. injections of IL-15 (5 × 10⁶ U/mouse/day) beginning 24 h after CY. The data in Fig. 1 summarize five independent experiments and show that 32% (8 of 25) of CY + IL-15 mice were cured, while the remaining mice showing significant prolongation of life compared with mice receiving CY treatment alone, in which 6.7% (2 of 30) were cured. The difference between groups of mice treated with CY alone or CY + IL-15 was statistically significant (p < 0.005), as calculated at 100 days after tumor inoculation. Fig. 2 shows that by day 35 after tumor inoculation, there were no visible tumor nodules in the lungs of mice receiving CY + IL-15 therapy in contrast to lungs from CY-treated controls. Mice that were cured by either CY alone or combined CY + IL-15 therapy were resistant to a subsequent i.m. challenge with 10⁴ 76-9 tumor cells, while challenge with the irrelevant syngeneic B16LM tumor resulted in tumor growth (data not shown), indicating the presence of immunologic memory.

IL-15 induces an increase in the number of NK1.1⁺/LGL-1⁺ cells and CD8⁺/CD44⁺ cells

PBL from TB mice that had been treated with CY and 20 daily injections of IL-15 (10 μg/day) were analyzed by flow cytometry.
for the expression of multiple Ags, including NK1.1, LGL-1, CD4, CD8, CD44, B220, Gr-1, MAC-1, and F4/80 as markers of the major types of potential effector cells. As was shown previously, injection of CY alone decreased the absolute number of PBL (22). Multiple injections of IL-15 into CY-treated TB mice did not significantly change the absolute number of PBL, but increased the proportions of NK1.1, LGL-1, CD8, and CD44 cells. The data presented in Fig. 3 is a typical dot plot of PBL isolated from TB mice injected with CY or CY + IL-15. Cells were double stained for the expression of NK1.1 and LGL-1 (upper panel) or CD8 and CD44 (lower panel). It is seen that 20 daily injections of IL-15 induced increase in NK1.1\(^{-}\)/LGL-1\(^{-}\} cells (sixfold) and NK1.1\(^{+}\)/LGL-1\(^{-}\} cells (17-fold). The percentage of CD8\(^{-}\)/CD44\(^{-}\} cells in PBL from CY + IL-15-treated mice was also five times higher than in control mice (injected with CY alone), while the percentage of CD8\(^{-}\)/CD44\(^{-}\} was the same in both groups of mice. The above changes in NK1.1, LGL-1, CD8, and CD44 expression were also seen in non-TB mice after injection with CY + IL-15, indicating that this was not related to the presence of tumor and depended on IL-15 administration (data not shown). Administration of IL-15 into normal or TB mice that did not receive CY treatment resulted in lower levels of NK1.1\(^{-}\)/LGL-1\(^{-}\} and higher levels of CD8\(^{-}\)/CD44\(^{-}\} cells compared with mice treated with CY + IL-15 (data not shown). The changes in the expression of the other Ags relative to the appropriate controls were not significant and are not shown.

It was evident the maximum accumulation of NK and CD8\(^{-}\)/CD44\(^{-}\} T cells in peripheral blood was dependent on the number of IL-15 injections after CY injection. This is shown in Fig. 4. TB mice were injected i.p. with CY, followed 24 h later by daily injections of 10 \(\mu\)g of IL-15. PBL (from a pool of five mice per group) were collected 24 h after 5, 10, 15, and 20 injections of IL-15. As shown, the number of NK cells in PBL reached the maximum level after 10 injections of IL-15 and declined slightly after 15 injections. By day 7 after the twentieth injection of IL-15,
the percentage of NK cells returned to the level seen in control normal B6 mice (data not shown). In contrast, the percentage of CD8^+CD44^+ cells increased in parallel with the number of IL-15 injections (Fig. 4B) and remained high for at least 21 days after the last injection of IL-15 (data not shown).

**IL-15 induces LAK cell, but not tumor-specific, class I-restricted activity in vivo**

To determine whether the increased levels of IL-15-induced NK1.1^+ and CD8^+ cells were associated with increased cytotoxicity, PBL isolated as above were also tested for cytotoxicity in a standard 4-h ^51 Cr release assay. Fig. 5 shows that PBL isolated from TB mice treated with CY and IL-15 were highly cytotoxic against NK cell-sensitive targets (YAC-1). Similar to the accumulation of NK1.1^+ cells in PBL, the peak of NK-mediated cytotoxic activity occurred by 10–15 injections of IL-15 and declined thereafter. Cytotoxicity above background levels was not detectable 7 days after the twentieth injection of IL-15 (data not shown).

PBL isolated after 10 injections of IL-15 expressed high levels of cytotoxicity against YAC-1, and a lower level of lytic activity toward 76-9, C26, and B16LM target cells (Fig. 6), suggesting NK and LAK cells activity. The cumulative data from eight independent experiments indicated wide variability in 76-9 cell lysis (e.g., in one experiment, lysis was 40–50%; in four experiments, lysis was 10–30%; and in three experiments, lysis was 0–10%). Variability was also seen in the lysis of BALB/c C26 colon carcinoma target cells (0–20% lysis) and B6 B16LM melanoma cells (0–30%). The increased cytotoxicity against syngeneic B16LM melanoma cells suggested the presence of LAK cells, but not Ag-specific cytolytic activity. In addition, no significant increase in tumor sp. act. in PBL from CY and IL-15-treated TB mice was seen in tumor growth-inhibition assays, in which effector cells were cultured with target cells (YAC-1, 76-9, C26, B16LM) for 96 h.

**Cellular requirements in vivo for successful CY + IL-15 therapy**

To determine whether NK, T, or B cells were responsible for the antitumor action of CY + IL-15 therapy, survival studies were conducted using B6 mice with impaired NK cell activity (B6.beige), T and B cell deficient (B6.scid), T cell deficient (B6.nude), lacking of B cells (B6.IgH-6), and induced mutants deficient in ab T cells (B6.TCR-b^−/−) or gd T cells (B6.TCR-d^−/−) or both ab and gd T cells (TCR-bd^−/−). Mice were inoculated i.v. with 5 × 10^5 76-9 tumor cells. Ten days later, they were injected with CY (200 mg/kg), followed 24 h later by 20 daily injections of IL-15. Fig. 7 summarizes the survival data. It is seen that IL-15 in combination with CY did not improve the survival rate in B6.beige, B6.nude, or B6.scid mice, but resulted in cures in 30% of the B6.TCR-b^−/− mice and in 40% of the B6.TCR-d^−/− mice. In the double knockouts B6.TCR-bd^−/−, therapy with CY + IL-15 had no effect on survival compared with treatment with CY alone. The most effective CY + IL-15 therapeutic effect was seen...
Those mice deficient in abCY1 mice were cured, suggesting a suppressor role for B cells toward bone marrow-associated NK cell precursors. Puzanov et al. (12), IL-15 induces maturation and proliferation of resulted in the reappearance of peripheral NK cells. As reported by and splenic NK cells was successful, the administration of IL-15 therapy. Unfortunately, although depletion of circulating and splenic NK cells was successful, the administration of combination CY injection inhibits suppressor cell activity associated with spleen cells (17). In addition, it was shown previously that spleen cells from 76-9 TB mice could be used as a potent source of sensitized T cells that were therapeutically active when adoptively transferred to CY-injected TB mice (28). Other reports similarly suggest that in some tumor models, suppressor cells are absent or exert minimal effects in CY-injected mice (29). Third, it

cell populations in all strains, including beige. The high levels of NK1.1+ cells in B6.scid and B6.IgH-6/- B cell-deficient mice can probably be explained by the absence of the major population of B cells, which account for up to 70% of the total PBL in B6 mice. This explanation serves to explain the increase in CD8+/CD44+ cells in B cell-deficient mice (Fig. 8B). The absence of CD8+ cells in PBL from B6.scid, B6.nude, and B6.TCR-β5/- mice confirms the T cell deficiency in these mice. Fig. 9 summarizes the NK cytotoxicity data. An increase in cytotoxic PBL (versus YAC-1 cells) was seen in all mutant mice, relative to background levels seen in untreated B6 mice or CY-treated TB mice, with the exception of B6.beige PBL, which expressed background levels only. PBL from B cell-deficient mice treated with CY and IL-15 show the highest cytotoxic activity, presumably because of the absence of B cells that increased the proportion of NK cells. All PBL that showed cytotoxicity against YAC-1 cells were also able to lyse 76-9, C26, and B16LM targets, but significant variation in killing of these targets was seen, as described above.

Discussion

The current data indicate that treatment of mice bearing established experimental 76-9 rhabdomyosarcoma pulmonary metastases with CY + IL-15 induced cures in 32% of mice, while only 6.7% of mice were cured with CY alone. Since the complete eradication of experimental pulmonary metastases could be achieved only by treatment with the combination of CY and IL-15, but not with either agent alone, the data imply that IL-15 potentiated the antitumor action of CY. First, the oncolytic action of CY may result in a smaller tumor mass that may be more amenable to rejection by host-mediated immune responses. While CY induces a reduction in the size of the 76-9 lung tumors, it is unlikely that tumor size alone determines whether IL-15 induces antitumor activity, since tumors developing from smaller tumor inocula were not more sensitive to IL-15 administration. Second, it has been reported that CY injection inhibits suppressor cell activity associated with spleen cells (17). In addition, it was shown previously that spleen cells from 76-9 TB mice could be used as a potent source of sensitized T cells that were therapeutically active when adoptively transferred to CY-injected TB mice (28). Other reports similarly suggest that in some tumor models, suppressor cells are absent or exert minimal effects in CY-injected mice (29).
has been shown in the 76-9 tumor model that CY injection was followed by an increase in the expression of Th1-related cytokine genes such as IFN-γ, IL-2, and TNF-α at the tumor site (24, 30). Since IL-15 has been reported to induce the production of TNF-α and IFN-γ from T and NK cells (2, 31, 32), its administration after CY injection may further promote the production of Th1-related cytokines. This in turn may augment T cell immune reactions at the tumor site, including the generation and activation of CTL and LAK cells. Finally, it has been reported that CY injection resulted in an increase in Mφ, NK cells, and polymorphonuclear precursor at the tumor site (22, 23). In view of the reports that IL-15 may activate each of these cell types (7, 8, 12, 13), the administration of IL-15 in combination with CY therapy clearly has the potential to accentuate the antitumor roles that each or all of these cells express.

The flow cytometry data indicated that when TB mice received combination CY + IL-15 therapy, there was a substantial increase in the proportions of NK and CD8+ T lymphocytes. Increases in peripheral blood CD4+ T lymphocytes, B cells, Mφ, or granulocytes were not seen. The question raised was whether the increased levels of NK cells or CD8+ cells, or both, were responsible for the observed in vivo antitumor effects. Although high cytotoxic PBL activity was generated toward YAC-1 cells, only relatively low cell cytotoxic activity was generated against the 76-9 targets. Moreover, the specific tumor targets were no more susceptible to cytotoxic cells than the B16LM melanoma or C26 targets, suggesting LAK but not T cell cytotoxicity in PBL. In some experiments, the data suggested significantly higher cytotoxic activity toward 76-9 cells compared with the other two targets, but this was not reproducible over the full range of experiments. This low level of NK cell activity observed in PBL was induced in the various natural and induced mutant mice and did not correlate with in vivo antitumor effects induced by CY + IL-15 therapy. Nevertheless, previous data indicated that NK1.1+/LGL-1+ cells expanded in vitro with IL-15 expressed potent antitumor effects in vivo when adoptively transferred to CY-treated 76-9 TB mice (16). These expanded cells showed considerable NK cell activity in vitro, but only low LAK cell activity. Clearly, in vivo activity was not reflected by in vitro cytotoxicity data. Similarly, it seems unlikely that CD8+/CD44+ T cells detected in PBL, putative memory cells (33) played a direct role in the antitumor effects generated by CY + IL-15 therapy since IL-15 administration induced an increase in non-TB mice. If within this population there is a tumor-specific subset of memory T cells, this was not evident based on the in vitro cytotoxicity data. However, the findings that those αβ and γδ T cell-deficient mice that were cured by CY + IL-15 therapy were shown to be resistant to a challenge with 76-9 cells, but not to the syngeneic B16LM melanoma cells, indicated that tumor-specific effectors had been generated. As discussed previously in the context of spleen cells (16), to what extent the in vitro activity of PBL reflects events occurring at the tumor site during the generation of antitumor activity remains to be elucidated.

In an attempt to determine what cells are required for successful CY + IL-15 therapy, the survival of mutant mice in response to combination therapy was evaluated. The overall data suggested that NK cells and T cells expressing either αβ-TCR or γδ-TCR were required for a positive antitumor effect, while B cells appeared to be antagonistic to positive antitumor responses. The evidence concerning NK cells based on the use of B6.beige mice is somewhat equivocal. First, unsuccessful therapy in B6.beige mice may be explained on the basis that other defective cells play important roles. For example, it has been reported that lysis mediated by cytolytic T cells is defective in B6.beige (34). Second, IL-15 administration resulted in increased numbers of NK cells and NK cell-mediated cytotoxicity in B6.scid, B6.nude, and B6.TCR-βδ-/- mice that failed to respond to CY + IL-15 therapy. This would indicate that if NK cells were required for antitumor activity, they did not appear to act independently of T cells and probably did not exert their effects toward 76-9 tumor cells by direct lytic activity. There is no question that the NK cells are activated, as measured by increased cytotoxicity and by expression of the activation marker B220 (16). Thus, as discussed previously (16), it seems more plausible to suggest that the involvement of activated NK cells in antitumor effects will be via their secretory products acting on other cell types, such as T cells or Mφ. It is proposed that the therapeutic efficacy of IL-15-expanded NK cells adoptively transferred to CY-treated 76-9 TB mice is likely to be mediated by their secretory products orchestrating the generation of antitumor effectors.

On the other hand, the collective data from the experiments involving B6.scid, B6.nude, and TCR-deficient mice were compelling in that there was also an absolute requirement for T cells for successful CY + IL-15 therapy. The apparent alternative roles of αβ and γδ T cells in this regard are intriguing, since these two cell populations have different mechanisms of Ag recognition. It is well documented that αβ T cells can kill tumor cells in an MHC class I-restricted manner (35). It also has been reported that γδ T cells can lysis tumor target cells in an Ag-specific manner (36, 37). Reports that γδ T cells may localize in the lung, as well as other epithelial tissues such as skin and intestine (38), suggest that γδ T cells might be important in protecting the host against lung metastases. As cited above, IL-15 activates both αβ and γδ T cells (5, 39, 40). The findings that cured TB mice deficient in αβ or γδ T cells resisted a challenge with 76-9 cells, but not with the B16LM melanoma cells, indicated that tumor-specific effectors had been generated in vivo. As discussed above, the failure of the in vitro cytotoxicity assays to show the presence of tumor-specific T cells would suggest that cytolytic T cells are not generated systemically, but only at the tumor site.

The exciting finding that the most successful antitumor effects induced by CY + IL-15 therapy were seen in the TB B6.IgH-6-/- mice deficient in B lymphocytes provides for the first time a likely pathway by which therapeutic efficacy is regulated. The role of B cells in antitumor immunity is rather controversial. In several mouse models and in melanoma patients, it has been reported that the clinical outcome of immunotherapy was associated with B cell immune responses (41, 42). In addition, it was shown that B cells play an essential role in host protection against virus-induced tumors (43). However, it is evident that depletion of B cells by Abs against mouse IgG or IgM enhanced rejection of allogeneic or chemically induced tumors (44, 45). Our current data indicate that the absence of B cells is associated with enhanced antitumor effects, suggesting that in replete B6 mice, the presence of B cells antagonizes antitumor effects. We can only speculate at this time as to the mechanism of action involved. It was shown that cell-mediated antitumor immunity can be blocked by Ab or Ab-Ag complexes (46, 47), and in the absence of B cells this inhibition did not occur. In view of the proposed dependence of successful CY + IL-15 therapy on NK cells and T cells, a more plausible candidate may be based on reports that B cell-deficient mice are unable to mount significant Th2 responses, while Th1 responses are reported to be enhanced (48, 49). Th2-related cytokines such as IL-4 and IL-10 were shown to suppress IL-15-induced activation of T lymphocytes and NK cells (31, 50). Thus, in the absence of B cells and suppressive Th2 factors, IL-15 may amplify Th1-dependent reactions, including the generation of antitumor cytotoxic effectors.
In conclusion, we have shown that the combined treatment of CY and IL-15 induced a significant incidence of permanent regression of experimental metastases of the 76-9 rhabdomyosarcoma. This was associated with an increase in activated peripheral blood NK cells and CD8+/CD4+ memory T cells. Successful therapy required the presence of either αβ or γδ T cells, and the absence of both subsets abrogated the therapeutic efficacy. Of considerable interest in the context of understanding how the therapy works was the finding that the most effective therapeutic benefit was seen in B cell-deficient mice, suggesting that B cells or their products antagonize potential antitumor effector function. While neither the positive effects of CY + IL-15 therapy nor the negative effects of B cells have yet to be fully elucidated, in future experiments we will test the hypothesis that NK cells mediate their effects by amplifying the effects of Th1 cells whose products activate effector αβ or γδ T cells. From a practical standpoint, the antagonistic effect of B cells would suggest that depletion of B cells may improve the clinical outcome of combination CY + IL-15 therapy.

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