IMMUNOLOGY RESEARCH: INTERCONNECTED ENDEAVOURS IN RELATION TO LOW DOSE RADIATION, NATURAL PRODUCTS AND CANCER

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Abstract

Immune response is central to our existence, and after perturbation, be it physical, chemical, or biological, the first responders will be the cells of the immune system. Over the years, immunology research at BARC has been focused on the following areas: immune alterations following radiation exposures, immunomodulators, basic research on cancer-immune system interactions, and epigenetic changes in immune cells. Radiation exposure can be too low or high doses, acute or chronic. Diagnostic procedures are the primary source of low dose acute exposures, and nuclear workers or people living in high background radiation areas are exposed to low dose fractionated or chronic exposures. Acute high doses could be from accident scenarios, and high dose fractionated exposures are usually administered to the tumors in cancer patients. Changes in immune responses can occur in each of these scenarios, and these changes could also reflect inter-individual variations. Over many years, we have studied different aspects of immunological alterations following radiation exposure. Extensive research has been carried out on immunomodulators derived from both natural products and synthetic chemicals. For example, G1-4A, a polysaccharide

isolated from *Tinospora cordifolia*, MAMPDM, a red pigment isolated from *Serratia marcescens*, and several others. Immunomodulatory effects of synthetic chemical inhibitors discovered through fundamental research were also investigated, for example, COX-2 inhibitor NS-398 and TGF-β inhibitor SB431542. Apart from the cancer cells, the tumor microenvironment comprises several other cell types, like infiltrating immune cells, fibroblasts, and pericytes. We have been studying the relationship between cancer and immune cells like dendritic cells, macrophages, B cells, and T cells to identify immunosuppressive mechanisms employed by tumor cells and develop strategies to overcome them. Many immune cells have several subtypes, and based on the surrounding microenvironment, cells can differentiate from one type to another, this process is regulated by epigenetic changes, such as DNA or histone modifications or miRNA. For example, miR365 was found to negatively regulate IL-6 secretion; EPZ004777 and FG2214 were identified as epigenetic inhibitors of Treg cells. Understanding the intricate interplay between immune cells and tumor cells is crucial for developing effective cancer immunotherapies. By targeting specific epigenetic modifications, or microRNAs, researchers hope to enhance anti-tumor immune responses and improve patient outcomes.

1. Preamble

The immune system is the backbone of our daily defense against apparent and invisible threats. These can include a variety of agents like microbes, their products, transformed cells, ionizing radiation, pollutants, and diverse chemicals. Immunosuppression is associated with exposure to acute high doses of radiation. But the dose-effect relationship for immune responses at lower doses has been found to be different, and even stimulation has been reported. The Bhabha Atomic Research Centre (BARC), Department of Atomic Energy, emphasizes research into the consequences of low doses of radiation exposure. This is especially relevant in the light of the linear-no threshold (LNT) model of radioprotection, which is used to evaluate the risk of different stochastic health effects due to exposure to ionizing radiation. The LNT model states that exposure to radiation, regardless of how small the dose, is an increased cancer risk for humans. However, animal data on low-dose exposures are contradictory. Interestingly, epidemiological studies in high-natural-background radiation areas and nuclear power workers have shown decreased cancer mortality compared to controls. Therefore, we investigated the effects of low-dose radiation on the immune system in radiosensitive and resistant mouse strains and the associated signaling mechanisms. Another important area of research is the identification of novel immunomodulators. Cells of the immune system are differentiated quiescent cells and serve as the human body's defense mechanism. The signaling mechanisms are extremely sensitive, and any slight alterations might trigger an alert. This could result in the activation of diverse immune cells to varying degrees. This could impact their proliferation or differentiation, causing changes in their cytokine

secretion pattern. Cytokines are soluble mediators that circulate in the blood and reach all organs. Most of these effects are transient, and the cytokines remain elevated for a short time before returning to their resting condition. There are other regulatory mechanisms in the body that ensure that immune activation is temporary. However, in situations such as malignancies, both the immune system and the inflammation that occurs in the microenvironment play important roles in cancer growth and progression. In the early stages of cancer, the immune system can exert control and fight against the cancer cells. Several immune cells, like dendritic cells, macrophages, NK cells, and T cells, are involved in the detection and elimination of the transformed cells with the help of soluble mediators like tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), perforins, and granzymes. However, if tumor cells proliferate rapidly, the immune cells are unequipped to stop them. In addition, tumors generate a variety of immune evasion and immunosuppressive strategies to promote their proliferation. At this stage, the tumor cells completely overwhelm the immune system. At BARC, we have been studying each of these research areas to better understand the underlying mechanisms and find targets that can be blocked by drugs in order to restore the immune system. Additionally, we have also studied immunomodulatory agents of plant origin, microbial origin, and synthetic chemicals identified through mechanism-based investigations.

2. Brief history and scope of immunology activities

In the early 1960s, it emerged that the hematopoietic system was highly vulnerable to the deleterious effects of ionizing radiation, and death in irradiated animals could be prevented by reconstitution with syngeneic bone marrow cells. Since hematopoietic stem cells are also the progenitors of the cells of the immune system, it was also recognized that immunological responses would be adversely affected by exposure to ionizing radiation. The science of immunology was still in its infancy in the 1960s, and the pivotal role of the lymphocyte was beginning to be established. In the Bio-Medical group of BARC, studies on the immune system were initiated by Dr. K. Sundaram and Dr. G. P. Phondke around this time. Their initial work was related to the biophysical characterization of the lymph node cells (LNC) from normal and *S. typhi* immunized Wistar rats in terms of their electrophoretic mobilities (EPM) as measured using the technique of 'Cell Electrophoresis'. It was shown for the first time that immunization produced a reduction in the EPM of LNC. Interestingly, the heterogeneity among lymphocytes in terms of T cells and B cells was being reported in the literature around this time, and in our studies too, such a heterogeneity was demonstrated in LNC in terms of their mean EPM, with T cells being more electronegative than B cells. The technique of cell electrophoresis was extensively harnessed in the subsequent years to monitor cell-mediated immune phenomena like delayed type hypersensitivity, allograft rejection, and the development of spontaneous lymphoblastic leukemia in AKR mice.

Studies on the electrokinetic properties of normal (unimmunized), immune, and malignant lymphocytes from rats and mice led to two interesting lines of research involving interactions of lymphocytes with ligands like antibodies and a polyclonal lectin

mitogen, Concanavalin A (Con A). In the first set of investigations, heterologous antilymphocyte sera (ALS) were prepared against normal (ALS-N) and immune lymphocytes (ALS-I). It was demonstrated that the sera against nonimmune lymphocytes (ALS-N) suppressed the humoral as well as cell mediated responses in rats only when administered prior to immunization with a specific antigen (sheep red blood cells-SRBC) and not after antigen exposure. On the other hand, administration of sera against immune lymphocytes (ALS-I) specifically suppressed the humoral and cellular responses when administered after antigen exposure due to the presence of anti-idiotypic/anti-clonotypic antibodies. This was the beginning of the later extensive studies on immunomodulation, which till today, involve several different contrivances or agents like acute and fractionated ionizing radiation, plant-derived natural products, antioxidants, and drugs.

Until monoclonal responses of T and B cells could be evaluated experimentally, responses to polyclonal mitogens served as a model for understanding the events associated with antigen-induced stimulation of lymphocytes. A requirement for such stimulation was the induction of redistribution of their receptors on lymphocytes after interaction with such ligands. Cell electrophoresis was used to study the redistribution of receptors to the polyclonal T cell mitogen Con A in the splenic lymphocytes of AKR mice. The EPM increased in conditions favoring redistribution of ligand-receptor complexes at mitogenic concentrations and decreased at supra-mitogenic concentrations of Con A. A detailed assessment of this phenomenon led to the demonstration, for the first time, of two sets of receptors for Con A on the surface of normal, healthy lymphocytes. One that underwent redistribution, like the formation of clusters, patches, and caps, which led to an increase in EPM. The second set appeared consequently. Using Con A labeled with two different fluorochromes and fluorescence microscopy, these two sets could be vividly seen. Since at that time Con A-induced enhanced agglutination was being described as a characteristic of malignant cells, similar studies were performed on the lymphocytes of malignant AKR mice. They showed the presence of only one type of receptor, which showed inhibition of lateral mobility at supra-mitogenic concentrations. It was proposed that the Con A-receptor interaction profiles of normal and leukemic cells could represent those of mature and immature T cells. Since the thymus is an organ in which maturation of T lymphocytes takes place during development, similar studies were carried out on adult thymus cells and hydrocortisone-resistant thymocytes, which supported such a proposition.

In the mid-1970s, studies in tumor immunology were also initiated by Dr. P. K. Ray and colleagues. It was observed that mouse tumor cells (fibrosarcoma) treated with the enzyme neuraminidase, which removed surface sialic acid residues, rendered the tumor cells susceptible to the cytotoxic action of lymphocytes and autologous serum. Even the normal cells thus treated were found to be susceptible to lysis by normal sera, as the treatment with neuraminidase probably exposed xenogeneic neoantigens. Immunotherapy of murine fibrosarcoma by immunization with neuraminidase-treated cells initially yielded positive results. Another aspect of the tumor immunology work was related to the curative effect of BCG administration on tumor growth in mice.

In the early 1980s, a detailed investigation of the effects of whole-body irradiation on delayed type hypersensitivity (DTH) was undertaken to decipher the radiosensitivity of naïve lymphocytes, antigen-presenting cells, DTH effector T cells, and the cells migrating at the site of the inflammatory response. While the naïve cells were found to be most radiosensitive, the effector T cells were shown to be most radioresistant. Thus, radiation effects on various cellular players in a specific immune response to a single antigen were evaluated. In the same system later, the effect of fractionated exposure to low-dose whole body gamma radiation was also studied.

In collaboration with Dr. R. S. Kamat's group at Haffkine Institute Mumbai, it was established that the route of antigen administration influenced the outcome of the immune response to *Mycobacteria*. While intradermal administration of the *Mycobacteria* induced a strong delayed type hypersensitivity response, intraperitoneal administration of the same antigen was immunosuppressive. It was further shown that this suppression was on account of the activation of $CD8^+$ T cells, which competed with the $CD4^+$ effector T cells for the cytokine interleukin-2 (IL-2).

3. Immunomodulation by low dose radiation and bystander effects

Exposure to both acute and fractionated whole-body low dose (<50 cGy) ionizing radiation exposures (LDR) alters immunological markers in mice. It remained unclear, however, if the immunological responses elicited by LDR would be universal and not influenced by genetic background. Many proteins, including p53, are activated in response to radiation, but the significance of p53 in the context of activation-induced apoptosis in LDR-induced immunomodulation was not understood. To answer these problems, two different strains, viz., C57BL/6 and BALB/c mice, were irradiated (4 cGy every day for 5 days a week, amounting to a total dose 20 cGy) to evaluate physiologically important functional responses. Delayed type hypersensitivity (DTH) and spleen cell polyclonal mitogen response were chosen as endpoints, and the antigens used for DTH were *Mycobacterium vaccae* or dinitrofluorobenzene (DNFB) and concanavalin A (Con A) as the mitogen for spleen cell response. Low-dose irradiated C57BL/6 mice had significantly increased Con A-induced spleen cell proliferation and suppressed DTH response to antigens as compared to sham-irradiated controls. In contrast, low-dose irradiated BALB/c mice had suppressed Con A-induced spleen cell proliferation and increased DTH response to antigens. The increase in Con A induced proliferation was primarily seen in $CD4^-(CD8^+)$ T cells in C57BL/6 mice, along with a decrease in p53-expressing cells and decreased apoptosis **(Fig. 1)**. A reverse pattern was observed in BALB/c mice spleen cells. Hence, it was concluded that, following LDR exposure, changes in the immune response are dependent upon the antigen, type of response, and mouse strain employed. These results also highlighted the important role of p53 and activation-induced apoptosis. The expression of several proteins involved in cell cycle and apoptosis was also studied. Increased expression of cyclins D and A, proliferating cell nuclear antigen (PCNA), and a decrease in caspase activity were observed in Con A-stimulated spleen cells of C57BL/6 mice **(Fig. 1)**. Further, it was confirmed that the

decrease in apoptosis was not due to changes in the expression of death signaling molecules like Fas or FasL but because of an increase in mitochondrial stability.

Apart from cell cycle changes, the responses of macrophages and T cell subpopulations were also evaluated in low dose irradiated C57BL/6 mice. Macrophage function was enhanced with low-dose radiation, as seen by increased nitric oxide release and phagocytosis. With respect to T cell subpopulations, there was activation of $CD8^+$ T cells as seen by increased expression of early activation marker CD69, proliferation response to alloantigens in a mixed lymphocyte reaction (MLR), and cytotoxicity response against tumor cells. Such an effect of LDR on $CD8^+$ T cells was demonstrated for the first time. These investigations also found that fractionated exposures resulted in a stronger long-term recovery response after challenge radiation dose, whereas acute radiation exposure resulted in a short-term, immediate adaptive response.

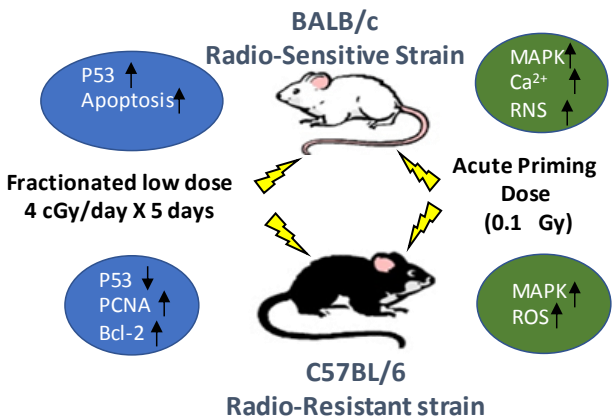

Fig. 1: Schematic representation of different pathways upregulated in radiosensitive and radioresistant strains of mice exposed to acute (0.1 Gy) or fractionated (0.2 Gy) low doses of radiation

Since spleen cells from LDR-irradiated mice showed an increased proliferation response, we explored if comparable effects could be found in low dose irradiated spleen cells. The transfer of conditioned medium (ICM) from such *in vitro* irradiated lymphocytes to unirradiated lymphocytes increased the proliferation response to Con A, with the highest increase observed with 0.5 Gy ICM. Treatment with ICM increased the generation of reactive oxygen species (ROS) as well as the release of nitric oxide (NO), along with increased expression of markers CD25 and cyclin D. Pre-treatment with ICM also resulted in an adaptive response to a challenge dose of radiation in lymphocytes. These studies demonstrated that soluble factors produced by irradiated lymphocytes activated a reactive oxygen/nitrogen species-mediated signaling cascade through medium transfer in control lymphocytes not exposed to radiation. This in turn resulted in enhanced mitogen

response as well as radio resistance in the control lymphocytes, which could play a significant role in radiation-induced immunomodulation.

Recent research has expanded on these findings to gain a deeper understanding of the nature and mechanism of adaptive responses (RAR) caused by prior exposure to low-dose radiation. After irradiating C57BL/6 and BALB/c mice with a low priming dose (PD, 0.1 Gy) or a high challenge dose (CD, 2 Gy) at a 4 h interval (P+CD) in the combination group, the inhibition of mitogenic responses in splenic lymphocytes was examined. The radio-adaptive response was evaluated in terms of DNA damage, early activation markers CD69 and CD71, cytokines IL-2, IFN-γ, and proliferation. The radiosensitive strain of mice, BALB/c, had a transient adaptive response 24 hours after CD, which was found to be due to LDR induced hyperactivation of MAPK signaling pathways in lymphocytes. These results, along with abrogation of the adaptive response by ERK and p38 inhibitors, indicated that LDR-induced MAPK signaling was responsible for the radio-adaptive response. On the other hand, the radioresistant strain, C57BL/6 mice, showed no RAR, either transient or late. However, MAPK activation was observed in spleen cells from both strains after exposure to LDR. Therefore, upstream signaling molecules such as reactive oxygen and nitrogen species (ROS, RNS) and calcium levels were assessed. LDR exposure increased intracellular calcium (Ca^{2+}) and nitric oxide (NO) in lymphocytes of BALB/c mice, while intracellular reactive oxygen species (ROS) levels were increased in lymphocytes of C57BL/6 (**Fig. 1**). In BALB/c mice, NO inhibition and calcium chelation abolished RAR. In both BALB/c and C57BL/6 mice, *in vitro* stimulation of spleen cells with a combination of NO donor and Ca^{2+} ionophore generated an adaptive response after 2 Gy, mirroring the action of PD, demonstrating their critical function in RAR. These data imply that low-dose radiation-induced differential activation of Ca^{2+} and NO signaling together with MAPK was responsible for differing RAR with respect to the immune systems of BALB/c and C57BL/6 mice.

4. Immunomodulation by an acidic arabinogalactan from *Tinospora cordifolia*

Medicinal plants are a rich source of several compounds, and many of them are immunomodulatory in nature. In the initial studies, alkaloid-rich extracts of leaves of *Tylophora indica* (Anantmul) used in Ayurveda for treatment of asthma and catarrh were studied and were found to be highly toxic to lymphocytes at high concentrations but at very low concentrations enhanced the T cell mitogenic response. In another study, oral administration of the crude extracts obtained from dried stems of *Tinospora cordifolia* (Guduchi), a well-known Indian medicinal plant, to mice increased antibody response against *Streptococcus pneumoniae* vaccine and sheep red cells. It was also mitogenic to mouse spleen cells *in vitro*. In an innovative effort, an immunomodulatory large-molecular-weight-polysaccharide from *Tinospora cordifolia* was purified based on its biological activity. This compound, named G1-4A, is an acidic arabinogalactan and was found to act on mouse B cells and increase B cell proliferation as well as antibody production (**Fig. 2a**). Studies into the underlying mechanism of action of G1-4A in B

cells, revealed an increased expression of early activation marker, CD69, Akt, ERK, JNK, IKK phoshorylation, IκB degradation, and NF-κB nuclear translocation. The PI3K inhibitor Ly294002, the mTOR inhibitor rapamycin, and the NF-κB inhibitor plumbagin all suppressed G1-4A-induced B cell proliferation, confirming the involvement of this signaling cascade. In addition, antibody mediated neutralization of the TLR4-MD2 complex also suppressed this increased B cell proliferation and IκB degradation, indicating that theTLR-4 receptor could be the binding site for G1-4A on B cells. In addition to B cells, G1-4A also activated macrophages via ERK and NF-κB-mediated signaling, resulting in increased phagocytosis (**Fig. 2b**). These experiments indicated that G1-4A is a TLR4 agonist with non-microbial origins with potential applications as an immunomodulator and adjuvant.

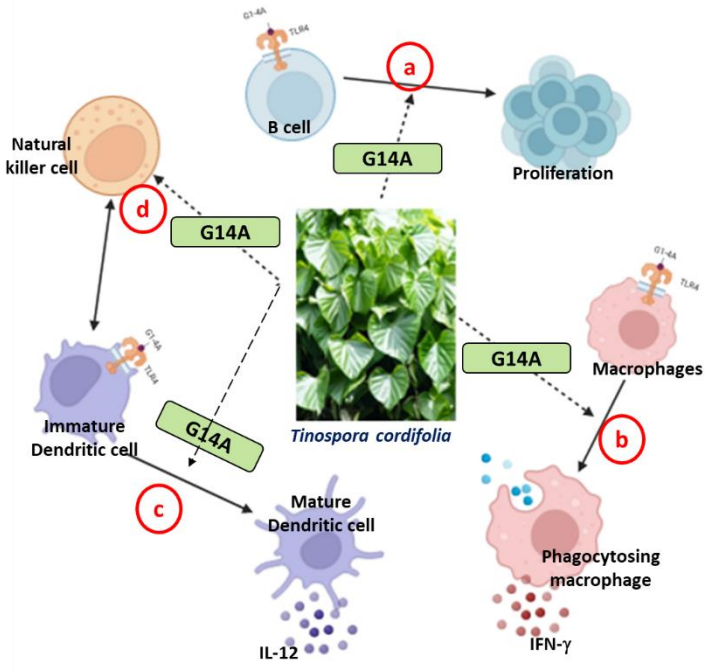

Fig. 2: G1-4A, a polysaccharide derived from *Tinospora cordifolia* has distinct effect on different immune cells like (a) B cells, (b) macrophages, (c) dendritic cells and (d) NK cells

Next, studies were focused on the application of G1-4A to treat diseases like tuberculosis, endotoxin-induced septic shock, and cancer. Being a TLR4 agonist, treatment of *Mycobacterium tuberculosis* (MTB)-infected RAW264.7 macrophages with G1-4A resulted in enhanced expression of co-stimulatory molecules, secretion of nitric oxide, and several proinflammatory cytokines. Consequently, intracellular survival of drug-sensitive as well as multi-drug resistant strains of MTB was decreased upon

treatment of macrophages with G1-4A. This was partially explained by the fact that G1-4A stimulated TLR4-MyD88 signaling, resulting in increased NO generation. These effects were observed *in vivo* also. Similar to this, the lungs of G1-4A-treated MTB-infected BALB/c mice had a significantly lower bacillary burden along with an upregulation of pro-inflammatory cytokines and nitric oxide synthase in the lung tissues. Increased T_{H1} and decreased T_{H2} cytokines were observed in infected mice treated with G1-4A. Additionally, compared to individual isoniazid (INH) or G1-4A, the combination treatment demonstrated superior protection against MTB, indicating the possibility of G1-4A as an adjuvant treatment. Our findings imply that G1-4A regulation of host immunity may improve the therapeutic effectiveness of currently known anti-mycobacterial prescription drugs, providing a compelling strategy for developing new tuberculosis treatments.

G1-4A pre-treatment offered complete protection in a lipopolysaccharide (LPS) model of sepsis in mice, prompting studies to investigate the mechanism of this protection. G1-4A treatment modestly increased pro-inflammatory cytokines TNF-α and IL-1β, whereas LPS increased the levels of these cytokines in serum several times more. So, following LPS challenge, G1-4A pre-treated mice showed considerably lower TNF-α, IL-10, and higher levels of TNF-RII, IL-6, IL-1β, and IFN-γ in serum as compared to mice treated only with LPS. G1-4A also affected the nitric oxide release by murine and human macrophages. Thus, G1-4A appeared to induce'resistance' to septic shock by modulating the cytokines and nitric oxide levels. These studies revealed that G1-4A conferred protection against endotoxin-induced sepsis.

As powerful antigen presenting cells, dendritic cells are crucial for the development of an adaptive immune response to malignancies. Dendritic cells interact with the cancer cells, process the cancer antigen, and prime and activate effector T cells. DC are known to become more immunogenic with maturation, which is stimulated by microbial products such as lipopolysaccharide (LPS). We also investigated the possibility of G1-4A as a maturation agent of murine bone marrow-derived dendritic cells (BMDC) and the possibility of using these G1-4A-treated DC's in cancer immunotherapy in preclinical models. G1-4A induced dendritic cell maturation, allowing them to activate cytotoxic T cells capable of killing cancer cells (**Fig. 2c**). Tumor lysate pulsed G1-4A-treated DC was administered in a preclinical lymphoma model, and the results of both preventive as well as therapeutic tumor challenge experiments showed a decrease in tumor burden. Aside from their function as antigen-presenting cells, DCs have direct cytotoxic effects against tumor cells. We discovered that G1-4A-treated BMDC killed tumor cells multiple times more efficiently via a nitric oxide-mediated mechanism. These data demonstrate that G1-4A-treated mBMDCs develop a killer phenotype during maturation and may be a safe non-microbial origin maturation agent for use in DC-based immunotherapy of tumors. In addition, these G1-4A-activated dendritic cells also activated natural killer (NK) cells by crosstalk in NK cell co-culture systems with either *in vitro* G1-4A-matured BMDC or splenic DC purified from G1-4A-administered mice. In addition, G1-4A also directly activated NK cells in DC-depleted splenic cells and purified NK cells (**Fig. 2d**).

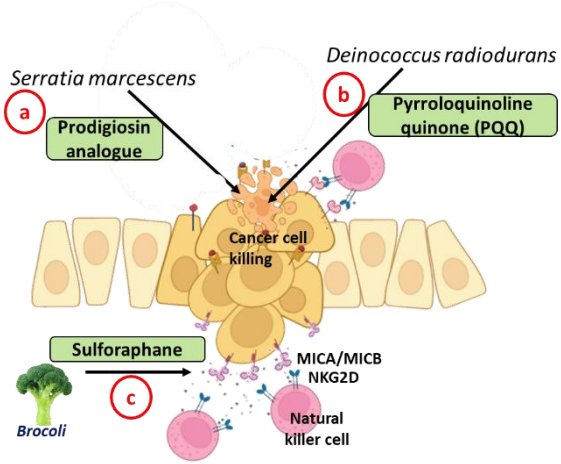

Fig. 3: Anti-tumor effects of natural products isolated from (a) *Serratia marcescens* (b) *Deinococcus radiodurans* (c) Broccoli

To summarize, G1-4A treatment activates B cells, macrophages, dendritic cells, and NK cells and has the potential to be used as an immunotherapeutic drug. However, since the purification procedures may not be that cost-effective, we tested the repeated dosage effects of polysaccharide-rich stem extract (PRE) of *T. cordifolia* and found it to have comparable effects to pure polysaccharide G1-4A. Hence PRE therapy also has the potential to be used as an immunotherapeutic adjuvant.

5. Anti-tumor and immunosuppressive effects of natural products and synthetic molecules.

The anti-tumor effects of several other natural compounds and synthetic agents chosen based on the deregulated pathways in the cancer microenvironment were investigated. These studies are summarized below:

(a) A new prodigiosin analogue was isolated as a red pigment from an organic solvent-tolerant strain of *Serratia marcescens* and identified as 2,2'-[3-methoxy-1'amyl-5'-methyl-4-(1"-pyrryl)] dipyrrylmethene (MAMPDM). MAMPDM had significant cancer cell cytotoxic activity and reduced the proliferation of cancer cell lines, inducing necrotic and apoptotic cell death in murine fibrosarcoma, S-180 cells, and lymphoma EL-4 cells, respectively (**Fig. 3a**). This might be advantageous for eliminating tumor cells that are deficient in the apoptotic pathway and thus resistant to conventional therapy. The effect of MAMPDM was also investigated in splenic lymphocytes stimulated with mitogen Con A. MAMPDM treatment resulted in increased IL-2 secretion, apoptosis, and decreased CD71 expression, proliferating cell nuclear antigen (PCNA), and cyclin D, resulting in the conclusion that MAMPDM selectively inhibited pro-mitogenic signaling but not pro-apoptotic signaling.

(b) The immunomodulatory effects of plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), present in Plumbago zeylanica (also called Chitrak) roots, were also investigated. Plumbagin inhibited lymphocyte proliferation by modulating cellular redox, resulting in glutathione depletion and an increase in reactive oxygen species.

(c) The water-soluble sodium-copper salt chlorophyllin (CHL), an analogue of the natural pigment chlorophyll, was identified to be an antioxidant that protects lymphocytes against oxidative stress and radiation-induced apoptosis. Mice treated with chlorophyllin developed splenomegaly due to increased lymphocytes and macrophage infiltration. Additionally, increased peritoneal exudate cells (PEC) obtained from CHL-treated mice also showed elevated phagocytic activity. Administration of CHL to mice immunized with the antigen sheep red blood cells (SRBC) significantly increased both T and B cell responses.

(d) Pyrroloquinoline quinone (PQQ), an antioxidant and redox co-factor, obtained from *Deinococcus radiodurans*, was also studied for its cytotoxic potential. Treatment with PQQ led to depletion of key cellular antioxidant glutathione, resulting in increased intracellular reactive oxygen species (ROS) and apoptosis of U937 cells (human promonocytic leukemia) (**Fig. 3b**). The cellular redox status was found to have a significant impact on PQQ-induced cytotoxic activity. An increase in intracellular GSH promoted PQQ-induced apoptosis, while depletion changed the manner of cell death to necrosis. Our results showed that modulating intracellular GSH can be an effective technique for increasing the cytotoxicity of quinones such as PQQ.

(e) The anti-tumor effects of sulforaphane (SFN), present in Broccoli, were investigated in combination with ionizing radiation (IR) (**Fig. 3c**). Higher doses of SFN induced cytotoxic effects. But lower concentrations (10 μM) of SFN up-regulated natural killer group 2, member D (NKG2D) ligands, increasing tumor cell sensitivity to natural killer (NK) cell-mediated death. Blocking this with an anti-MICA/MICB antibody prevented this effect. Increased cellular glutathione levels with N-acetyl cysteine treatment abrogated SFN induced effects such as expression of MICA/MICB and increased susceptibility of lung and breast cancer cells to NK cell mediated killing. Our findings showed that SFN can be used as an immunomodulatory adjuvant in cancer therapy.

(f) Cancer progression is associated with an evolving interaction between the immune system and the tumor cells. Many of the soluble molecules that promote immunosuppression in the microenvironment have been identified, but the means by which the tumor impacts the bone marrow progenitors is unknown. We identified that the tumor cell-derived prostanoids affected the distant progenitor cells, inhibiting the expression of transcription factor Zbtb46, which is specific to the classical dendritic cell DC (cDC) lineage-specific and therefore influenced its development. Tumor-induced DC dysfunction was abrogated when tumor cells were treated *in vitro* with the COX-2 inhibitor NS-398. Tumor-bearing mice treated with NS-398 developed immunocompetent DC and had a lower tumor burden. The absence of such an effect in SCID mice supported the hypothesis that NS-398's effects were attributable to immunomodulation. These results illustrate that Zbtb46 expression is a marker of

immunocompetent DC and show that COX-2 inhibitors may be effective in cancer immunotherapy.

(g) Soft tissue sarcomas (STS) have a largely unexplored immune milieu, and understanding it is crucial for designing immunotherapy approaches. Murine fibrosarcoma triggered the development of B regulatory cells (Breg) with $CD19^{+}CD25^{+}PD\text{-}L1^{hi}$ phenotypes that secreted TGF-β. These tumor-evoked Bregs decreased the proliferation of T cells in response to anti-CD3/CD28 stimulation, which was reversed by SB431542, a small-molecule inhibitor of TGFβ receptor type I. When tumor bearing mice (TBM) were administered SB431542, the number of Treg cells reduced significantly with the restoration of the T cell proliferation response. Additionally, this treatment significantly reduced the tumor load. Our findings indicate that the tumor-induced Breg cells inhibit immunity through a TGFβ-mediated mechanism. Immunotherapy drugs targeting the Breg-Treg axis may thus have potential benefits in soft tissue sarcomas.

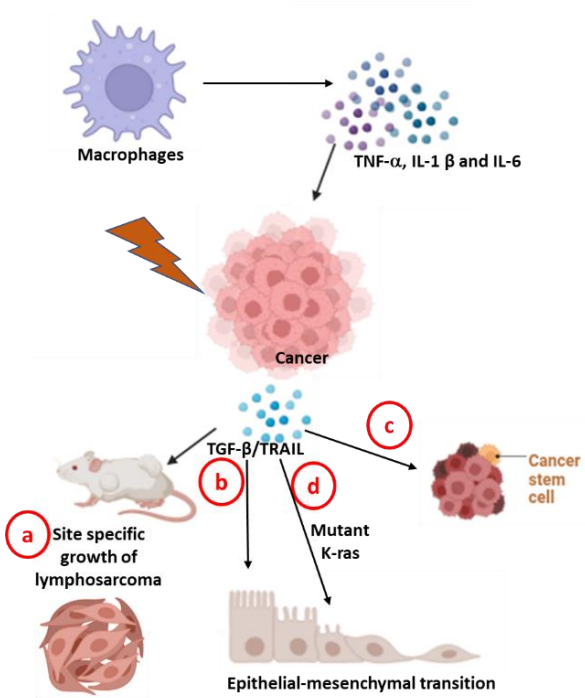

Fig. 4: Pro-tumor effects of TGF-β resulting in (a) site specific growth of lymphosarcoma (b) macrophage mediated epithelial-mesenchymal transition (EMT) (c) enrichment of cancer stem cells (d) TRAIL induced EMT responses in K-ras mutated cancer cells

6. Pro-tumor effects of immune cells and cytokine microenvironment

Earlier studies demonstrated that after intraperitoneal (i.p.) transplantation, ascitic lymphosarcoma (LS-A) showed rapid progression leading to host mortality in Swiss mice. However, this was found to be site dependent, and the tumors showed spontaneous regression after subcutaneous (s.c.) transplantation. Studies were undertaken to understand this mechanism. Studies conducted *in vitro* revealed that at increasing cell densities, neutralization with an anti-TGF-β antibody greatly suppressed LS-A proliferation. Mice with i.p. ascites tumors exhibited increased serum TGF-β1, reduced hemoglobin levels, transferrin receptor (CD71) expression, splenic cellularity, and compromised T cell mitogen response. However, these changes were not found in mice with spontaneous regression of s.c. transplants. These studies indicated that tumor growth sites and the host immune system have a significant impact on tumor progression. These findings suggest TGF-β1-secreting human tumors may have comparable pathophysiological consequences in the host based on their anatomical location (**Fig. 4a**).

Within the tumor microenvironment, macrophages have pro-tumor effects. They can also affect tumor cell proliferation, invade normal tissues, and disseminate to both local and distant locations. We studied the impact of monocytes and macrophage conditioned media (MϕCM) on breast cancer cell proliferation and migration. Twenty-four-hour monocyte and macrophage conditioned media were collected. Pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, present in high concentrations in the macrophage-conditioned medium, stimulated TGF-β1 synthesis in tumor cells, increased CREB phosphorylation, epithelial-mesenchymal-transition (EMT) responses, and migration (**Fig. 4b**). Proteomics studies of these conditioned media identified MϕCM to be enriched in integrin and matrix metalloproteinases. The gene signature identified by macrophage-tumor interactions was significantly associated with mutation, deletion, amplification, and differential expression of some prominent candidate genes in the TCGA database. These genes together formed a 15-gene signature found in >60 % of samples in TCGA database and were linked to high breast cancer risk and poor overall survival ($p < 0.05$). These findings emphasize the crucial role of macrophage signaling in breast cancer. Prognostic indicators based on tumor-macrophage interaction could thus be beneficial for tracking cancer progression.

Breast cancer is a very heterogenous disease with several clonal populations, and the tumor microenvironment affects the plasticity of cancer stem cells. In MCF7 cells and mammospheres, MϕCM-activated ERK/TGF-β1 signaling led to EMT and the enrichment of cancer stem cells (CSC) (**Fig. 4c**). It was discovered that these enriched stem cells exhibited both mesenchymal ($CD44^+$ $CD24^-$ cells) and hybrid ($ALDH1^+$) properties. At the single cell level, the hybrid E/M state was characterized by elevated expression of the mesenchymal marker vimentin (M) and the epithelial marker claudin-1 (E). These effects could be reversed by either inhibiting TGF-β1 synthesis with MEK inhibitor PD98059 or downstream signaling with TGF-βR1 inhibitor SB431542. Prior to implantation in SCID mice, a number of CSC and EMT markers were monitored in the cancer cells. There was no growth benefit of MϕCM-treated cells in SCID mice, and

evaluation of CSC and EMT markers in cells recovered from the tumor showed reversal. The ERK /TGF-β signaling leading to CSC enrichment was abrogated by either removing the MϕCM or antibody-mediated neutralizing of pro-inflammatory cytokines present in MϕCM. This underscores the significance of the requirement of continuous signaling for maintenance of cancer stem cells. Thus, ERK/TGF-β1 signaling plays a major role in MϕCM-induced EMT and CSC plasticity, both of which are completely reversible.

TRAIL (tumor necrosis factor-α-related apoptosis-inducing ligand), also called Apo2L or TNFSF10, is an important cytokine that regulates cell survival and death in the tumor microenvironment. Bioinformatics analyses revealed that the expression of TRAIL had different impacts on disease-free survival in adenocarcinoma (AC) and squamous cell lung carcinoma (SCC), two distinct forms of lung cancer. Genomic analysis showed that AC had considerably higher KRAS mutation rates along, with enhanced TRAIL expression and metastasis, whereas SCC had more TRAIL gene amplifications. *In vitro* studies showed that TRAIL only stimulated ERK phosphorylation in AC cell lines that have mutant KRAS (**Fig. 4d**). This in turn led to enhanced migration, which was abrogated by the MEK inhibitor PD98059. The effects of TRAIL-induced migration were accentuated when combined with ionizing radiation exposure. These results advance our knowledge about TRAIL signaling in metastasis, which is important for creating strategies to convert these signals into pathways that promote apoptosis.

7. Immune involvement in radiotherapy and chemo/radio resistance

Several types of lymphoid and myeloid tumor cells are known to be more resistant to radiation-induced apoptosis than normal lymphocytes. Our research identified that tumor cells have greater inherent radio resistance than normal lymphocytes due to enhanced levels of antioxidants and lower production of reactive oxygen species (**Fig. 5a**). Alterations in mitochondrial membrane potential and cytoplasmic Ca^{2+} concentration were detectable in lymphocytes even at a dose of 1 Gy, while no such variations were observed in tumor cells. After 1 Gy irradiation, approximately 65% of spleen cells died within 24 h. However, under the same conditions, the tumor cells EL-4 and P388 failed to undergo cell death and instead accumulated in the G2/M phase. This constitutive radio resistance of EL-4 cells was found to be due to the activation of the Nrf-2/ERK pathway. In response to radiation, EL-4 cells altered their thiol redox circuits, GSH, and thioredoxin. Pharmacological ERK and Nrf-2 inhibitors significantly increased radiosensitivity of EL-4 cells. Unirradiated lymphoma cells accumulated Nrf-2 in the nucleus, with an increase in the expression of its downstream genes. Interestingly, Nrf-2 nuclear translocation was blocked by MEK inhibitors, indicating that ERK plays a role in basal and radiation induced Nrf-2 activation in tumor cells. This was confirmed by a further increase in radiosensitivity in ERK/Nrf-2 double knockdowns as compared to individual knockdowns. Importantly, EL4 cells lacked even basal-level NF-κB

expression, a protein known to be constitutively active in many tumors. As a result, NF-κB inhibition did not affect EL-4 radiosensitivity.

In addition to natural or inbuilt radio resistance, cancer cells also acquire resistance during radiotherapy. Identifying the underlying mechanisms of therapy-induced radio resistance and activated pathways will result in more effective combination therapies. Breast cancer cell lines were subjected to 6 Gy that was followed by a 7-day recovery period. These cells (D7-6G) demonstrated enhanced proliferation and apoptosis. The cytokine known to induce such dual effects, transforming growth factor β, all its isoforms 1-3, along with their receptors R1, and R2, were expressed at higher levels in these cells. TGF-β downstream transcription factors Zeb1, Snail, and HMGA2 were also enhanced. These cells also displayed a phenotype that had hybrid epithelial-mesenchymal (E/M) characteristics, with increased motility and expression of E/M markers. When challenged with radiation, these cells exhibited resistance to killing and had an increased proportion of cancer stem cells (**Fig. 5b**). SB431542, a TGF-βRI inhibitor, significantly reduced the proliferation of D7-6 G cells. Thus, blocking TGF-β signaling can be a promising way to combat radio resistance produced by radiation exposure.

We also explored the potential of these cells to form tumors in severe combined immunodeficiency (SCID) mice and used proteomic techniques to characterize these tumors. Larger tumors with a shorter latency period were produced by these radioresistant cells (**Fig. 5c**). Expression of TGF-β isoforms, downstream genes pSMAD3, Zeb1, Snail, HMGA2, hybrid epithelial/mesenchymal phenotype, motility, and cancer stem cells were all increased in these tumors. Radioresistant breast cancer cells showed enhanced TGF-β signaling and increased metabolism with both oxidative phosphorylation and glycolysis. We also studied the effects of prolonged treatment of breast cancer cells with the TGF-βR inhibitor SB431542 on radiation-induced signaling. Radioresistant cells had higher levels of TGF-β1 and TNF-α signaling as seen by enhanced phosphorylation of SMAD3, NF-κB, and ERK. Pre-treatment of radioresistant cells with the TGF-βR inhibitor SB431542 lowered phosphorylation of SMAD3, and increased proliferation, apoptosis, and motility. Downregulation of TGF-β downstream genes, Snail and HMGA2, and hybrid E/M phenotype, was also observed. TGF-β independent effects were also observed, whereby SB431542 treatment itself led to increased expression of some E/M genes. This was most likely caused by increased phosphorylation of pSTAT3 and CREB1 genes in addition to enhanced production of cytokines IL-6 and IL-10. These findings indicate that primary signaling pathways in radioresistant cells are mediated by TGF-β/pSMAD3 and TNF-α/pNF-κB and that prolonged SB431542 treatment may result in TGF-β/Smad3 independent effects.

We investigated the influence of tumor necrosis factor-α (TNF-α), a pro inflammatory cytokine, and insulin-like growth factor 1 (IGF-1), a growth factor, present in the tumor microenvironment, on the response of lung cancer cells to radiation. A bioinformatics analysis of 982 lung cancer patients showed that increased TNF-α expression was linked to a lower risk of cancer growth, while IGF-1 overexpression was linked to a higher risk. TNF-α treatment reduced cell motility and increased radiosensitivity, by activating the

MAP kinases such as stress-activated protein kinases (SAPK), jun amino-terminal kinases (JNK), and p38 kinases (**Fig. 5d**). IGF-1 treatment increased mitotic index, reduced DNA repair, and caused abnormal chromosomal segregation, resulting in increased cell proliferation and motility. Collectively, these findings show that the cytokines and growth factors in the tumor microenvironment influence radiation therapy by activating many signaling pathways.

In addition to radiotherapy, cytokines in the tumor microenvironment also influence the effectiveness or failure of molecular- targeted therapies. We investigated the effects of TRAIL and IGF-1 on sirtinol cytotoxicity. Sirtinol or SIRT1 knockdown increased the expression of death receptors DR4 and DR5 and sensitized A549 cells to TRAIL mediated cell death. This was found to be iNOS-mediated, caspase-independent, with classical characteristics of necroptosis (**Fig. 5e**). Inhibiting iNOS increased caspase activity and altered the manner of cell death to caspase-mediated apoptosis. IGF-1 reduced sirtinol cytotoxicity and increased cell survival by preventing ligand-induced IGF-1R downregulation and therefore activation of the PI3K-AKT pathway (**Fig. 5f**). To summarize, these findings indicate that the tumor microenvironment influences drug cytotoxicity and that combination therapy with drugs that inhibit the IGF-1 pathway and increase TRAIL signaling may improve anticancer efficacy.

Despite being the most commonly used cancer treatment, little is known about how radiation affects dendritic cell development. We found that in tumor-bearing mice exposed to localized irradiation, the tumor-induced suppression of splenic and bone marrow-derived DC (BMDC) function was reversed. This was not due to the effect of radiation on tumor cells, because DC derived from normal mice exposed to whole-body irradiation (WBI) also showed higher immunocompetence. This increased immunocompetence was also observed when DC were generated from *in vitro* irradiated progenitor cells. It was shown that this effect was related to STAT5/Zbtb46 signaling, which was mediated by the irradiation-induced apoptotic bodies (ABs). The involvement of this pathway was demonstrated by the reversal of these effects upon annexin-bead-mediated depletion of these ABs. In addition, these DCs generated from irradiated progenitors (IP) were resistant to the suppressive effects of tumor conditioned medium (TCM). For the first time, we demonstrated that ABs formed due to IR exposure at specific doses can enhance DC's capacity to activate the immune system. This may affect the choice of appropriate IR dosages for cancer patients undergoing radiotherapy.

8. Epigenetics in immune cells

The functional plasticity of immune cells in the tumor microenvironment (TME) determines the fate of tumor progression. There are various factors that regulate the characteristics of immune cells in the TME, among which epigenetic regulation plays a major role in modulating immune-immune and immune-tumor cell interactions. In general, epigenetic modifications are alterations in gene expression without altering the primary DNA sequences. Histone modifications, DNA methylation, and translational regulation by non-coding RNAs that have the ability to activate or suppress target gene

expression are examples of these epigenetic alterations. Epigenetic modifications in the tumor cells and immune cells can reprogramme the TME and influence tumor progression and metastasis. Tregs are one of the major immunosuppressive cells in the TME. In the tumor milieu, the cytokine transforming growth factor-β converts naïve T cells into Treg cells, which is largely regulated by epigenetic modifications.

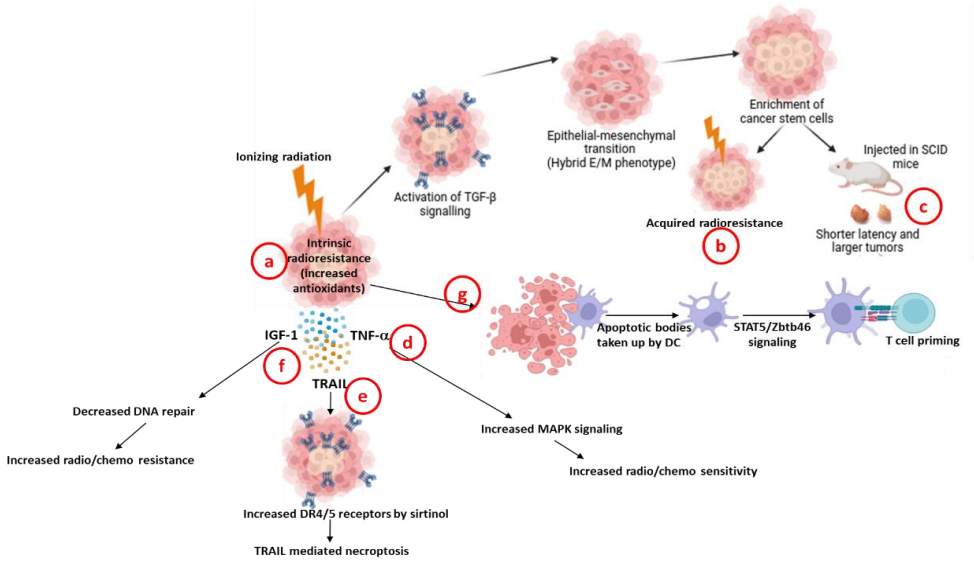

Fig. 5: Radio resistance and immune system: Cancer cells have (a) intrinsic radio resistance or that which is (b) acquired during the course of therapy (c) radioresistant cells grow rapidly in SCID mice. Presence of cytokines like (d) TNF-α and (e)TRAIL increase radio/chemo sensitivity whereas (f) IGF-1 increase radio/chemo resistance. (g) Irradiated apoptotic bodies released from the tumor cells can be taken up by dendritic cells resulting in their activation.

We have extensively studied the epigenetic modifications involved in regulatory T cell (Treg) generation. We generated a detailed epigenetic landscape (DNA methylation and 14 histone modifications) of TGF-β-induced changes in three regulatory regions of the Foxp3 gene, the master transcription factor of Treg generation. By validating in physiologically relevant conditions, we found out that increased levels of histone methylation H3K4me3 and decreased histone methylation H3K27me3, along with increased acetylated H3K27 or decreased DNA methylation, are indispensable for TGF-β induced Foxp3 gene expression and Treg generation and form an epigenetic signature for Treg cells. Currently, there are some epigenetic drugs that are approved for clinical use in hematological malignancies. While the anti-tumor effects of these drugs are demonstrated, how these drugs reprogram the immune cells in TME is unknown. To identify potential epigenetic drugs that can reprogram Tregs in the TME, we developed a

screening strategy using TGF-β-mediated immune responses as an endpoint. We identified two compounds, EPZ004777 and FG2214, that could inhibit TGF-β-induced Treg generation by reversing the epigenetic signature of TGF-β in the Foxp3 gene locus. While these two drugs show promise in the *in vitro* studies, further preclinical and clinical studies are required to establish their efficacy and suitability as potential immunotherapeutic agents, with ongoing research efforts aimed at this goal.

MicroRNAs are among the major regulators in the TME. It is well known that the dysregulation of microRNAs contributes to oncogenic transformations leading to onset and progression of cancer. In 4T1 murine mammary cancer, dendritic cell dysfunction is mediated by the tumor-derived prostaglandin PGE2, which acts through EP2/EP4 receptors present on the immune cells. We have identified that PGE2-induced DC dysfunction is mediated through IL-6/STAT3 signaling. Dysregulation of microRNAs can also exert immunosuppressive functions in the TME. Our studies on dendritic cell dysfunction in the TME identified the differential regulation of multiple microRNAs in dendritic cells generated in the presence of tumors. For example, mmu-mir-155-2p, mmu-mir-146, and mmu-mir-365-5-2p. Using synthetic mimics and inhibitors of the above miRNAs, we observed that miR 365 negatively regulates PGE2-induced IL-6 generation in DCs, and use of miR 365 mimics could reverse PGE2 induced DC dysfunction. Our studies show novel insights on the potential use of miRNA regulators as immunotherapeutic agents in cancer treatment.

9. Summary and Way forward

The focus of immunology research at BARC has been in the following areas: (a) low dose radiation studies; studies so far have been carried out in animal systems, which have revealed changes in several markers but also have shown that this could vary between different strains. Future work will be focused on studying the effect of low dose diagnostic exposures in cancer patients, with particular emphasis on DNA damage response and immune response. (b) Research on *Tinospora cordifolia:* studies so far have clearly established the mechanism of action of G1-4A, the polysaccharide from *T. cordifolia*, to activate antigen presenting cells and NK cells, preclinical studies on animals also have shown three potential areas of application: as an adjuvant to treat tuberculosis, sepsis, and cancer. In the future, efforts will be undertaken to prepare formulations that can be used to treat these diseases\conditions. (c) Research on several immunomodulators and anti-cancer compounds, of both natural and synthetic origin: Amongst the various immunomodulators studied, chlorophyllin has been prepared as a formulation and approved as a food supplement/nutraceutical. Other promising molecules like COX-2 inhibitors or TGF-β inhibitors will be taken up for their potential use as an adjuvant in cancer chemo/radio/immunotherapy. (d) role of immune mediators in radio/chemoresistance: studies so far clearly confirm the role of cytokines like IL-6 and TGF-β in radio resistance, so efforts in the future will be directed to identify the prognostic value of TGF-β/IL-6 in radiotherapy and the application of TGF-β/IL-6 inhibitors or siRNA to overcome radio resistance in cancers. (e) epigenetic changes in

immune cells: studies so far show that miR-365 negatively regulates IL-6, a proinflammatory cytokine involved in several diseases. Future studies will be focused on developing miR-365 mimics that can abrogate IL-6 and therefore can be used in inflammatory disorders.

In addition, our expertise in understanding the biology of differentiation and reprogramming of immunosuppressive cells, especially regulatory B cells and regulatory T cells, would provide greater insight into potentiating them as beneficial therapeutics. Our epigenetic screens have identified two molecules, EPZ004777 and FG2214, as potential inhibitors of T regulatory cells. Application of these compounds or related compounds in inflammatory disorders will be tested. Autoimmune diseases caused by uncontrolled immune activation are often associated with the dysfunction of these regulatory cells and currently do not have any effective treatment against them. With our understanding of these immunosuppressive cells, identifying ways to improve their differentiation and functions in such conditions may provide potential benefits to patients with autoimmune diseases. The research in this area may also help in developing effective treatment modalities, which are lacking as of today.

Overcoming chemo and radiotherapy resistance is a major challenge in cancer treatment. Our recent studies have shown increased fatty acid metabolism associated with chemo and radioresistance in human cancers. This metabolic reprogramming of cancer cells in the TME will be studied in depth to identify novel molecules to reverse them and thereby improve the clinical outcome. Extensive studies in this field are necessary to identify more promising TME modulators that can be potential therapeutics either on their own or as adjuvants to chemo/radio/checkpoint blockade therapy.

Most of the conventional cancer treatments also destroy the immune system, which is replenished by the hematopoietic stem cells. However, recent studies have shown that the soluble mediators secreted by cancer cells can affect the distal progenitors also, affecting their ability to differentiate into functional cells. These defective progenitors may thus have a role in inducing immunosuppression and poor outcomes with immunotherapeutics in the majority of the patients. Another future prospect is to carry out research on the effect of the cancer derived soluble mediators on the hematopoietic stem cells and approaches to prevent their negative impact on differentiation of progenitor cells.

While extensive studies on immunomodulation in tumors and the development of advanced immunotherapies are ongoing globally, the role of co-morbidities as contributory factors remains poorly understood. Co-morbidity is defined as the co-existence of a disorder in addition to a primary disease of interest. The interaction of co-morbidities such as diabetes, cardiovascular diseases (CVD), obesity, and other non-communicable diseases is one of the major challenges in cancer treatment and survival. The involvement of these factors in delaying diagnosis and affecting the efficacy of treatment is being studied globally. But whether and how they affect the immune interactions in the TME and their role in the outcome of cancer immunotherapeutics is still unclear. Considering our country's growing cancer incidence and increasing CVD, diabetes, and hyperlipidemia, it has become the need of the hour to delineate the

interactions of these diseases with each other. Our major focus of research in the coming years would be to study this aspect, particularly diabetes and obesity, in modulating tumor biology. With the current preliminary research, it is evident that obesity in fact negatively modulates anti-tumor responses in at least some strains of mice. These studies can be extended to include drug-induced or dietary reprogramming to manage these diseases with better anti-tumor responses.

10. Acknowledgements:

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