ImmunoTools *special* Award 2014

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The role of CIK purinome in cell expansion and killing of tumor cells: a new strategy for tumor therapy

Background:

Human Cytokine Induced Killer (hCIK) cells are an heterogenous population of immune cells sharing phenotypic and functional properties of $CD8^+$ T lymphocytes and natural killer (NK) cells. They are generated by *in vitro* stimulation of peripheral blood mononuclear cells and due to their high cytotoxic activity against transformed haematopoietic cells they represent a very valuable and save tool for antitumor therapy. Clinical trials have shown safety and efficacy of CIK cell-based therapy for patients with different malignancies and many efforts are ongoing to enhance the potency and specificity of CIK therapy.

Nucleotides (ATP, UTP, ADP, etc.) are recognized as a new class of extracellular mediators acting at specific plasma membrane receptors named P2 receptors (P2R). ATP and its products of hydrolysis ADP, AMP, adenosine play a role in conditioning tumor survival and killing ability of NK and $CD8^+$ cells. No information on P2R expression and function are available for CIK cells so far. Therefore, in view of their clinical use against cancer cells, a thorough investigation of the P2R repertoire of human CIK cells would be highly desirable and could represent an important advancement in the understanding of CIK biology and more important a potential new way to increase their number and efficiency to better counteract tumor progression.

Hypothesis:

Immune killing response to cancer cells results from the balance between activating and inhibitory signals delivered to effector cells, no data are available on this topic for the clinically relevant CIK cells. It is thus of great importance to know the full P2R profile of CIK cells with the aim of modulate their P2R repertoire to potentiate tumor killing capacity. To this purpose: an in deep molecular, pharmacologic and functional analysis of P2 receptors expression and modulation will be performed by taking advantage from the expertise of leading laboratories in the different fields: CIK biology, nucleotide receptors pharmacology, molecular genetics and cell physiology.

Team Composition:

Davide Ferrari, University of Ferrara, Ferrara, Italy.

Dario Sangiolo and **Giulia Mesiano**, Istitute for Cancer Research and Treatment, Candiolo, Turin, Italy.

Rossella Manfredini, University of Modena and Reggio Emilia, Modena, Italy.

Gennady Yegutkin, Turku University and National Public Health, Turku, Finland.

Roberto Gambari and **Nicoletta Bianchi**, University of Ferrara, Ferrara, Italy.

Specific Aims:

Aim 1: CIK cell P2 receptors repertoire.

No information on P2 receptors expression and function is available for cytokine-induced killer cells (CIK), so far. Therefore, the first purpose of our study will be to identify P2 subtypes expressed by CIK. The mRNA of specific P2 subtypes will be examined by using RT-PCR specific primers for all cloned P2Y subtypes: P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, P2Y14 and P2X subtypes: P2X1, P2X2, P2X3, P2X4, P2X5, P2X6, P2X7. For most of these receptors, specific polyclonal antibodies are also available, thus we will evaluate protein expression in whole cell lysates by Western blot analysis and in isolated intact cells by flow cytometry or immunofluorescence analysis. Pharmacological characterization of P2 receptors expressed by CIK cells will be performed by analyzing alteration of the calcium homeostasis as a consequence of the pharmacological stimulation of CIK cells with P2 receptor agonists. For cytosolic $Ca2^{+}$ concentration measurements, cells will be loaded with the $Ca2^{+}$ indicator fura-2/AM and stimulated with different nucleotides (ATP, ADP, UTP, UDP-glucose), nucleotide analogs (BzATP, 2-metSATP, ATP-

gammaS) or inhibited by appropriate_antagonists/inhibitors (KN-62, oxidized-ATP, brillant blue, PPADS). Analysis of morphological changes following to incubation of CIK with nucleotides will also be carried out by both phase-contrast and fluorescence microscopy.

Aim 2: Modulation of CIK purinome to improve killing of cancer cells.

Purinome modulation during killing of tumor cells by CIK might provide new tools to potentiate efficacy of CIK-mediated killing of tumor cells. Therefore, the investigation and evaluation of the process will start in vitro and then prosecuted *in vivo.*

The experimental design will include a first phase dedicated to explore the functional role of purinome at the interface between CIK and tumor cells. As second step we will attempt modulating the purinome pathway to potentiate the killing activity of CIK cells and reduce or counteract the inhibitory influence by tumor cells and microenvironment. We will employ an autologous patient specific model, with both CIK and tumor cells derived from our patients, accounting from intrinsic biologic variables. Tumor killing experiments will be performed in vitro following exposure of either CIK or tumor cells to P2R agonists or inhibitors. Results will be compared in parallel with untreated controls. In vivo essays will be based on tumor xenografts generated by implanting immunocompromised mice with tumor samples directly collected from our patients. To assess the antitumor activity, autologous CIK cells, previously treated with PR agonists or inhibitors, will be adoptively infused by tail vein injection. Treatments will be repeated weekly for a total of 8 infusions or up to a maximum of 2 cm in tumor main diameter. Tumor volume, proliferative Ki67 index, lymphocyte infiltration will be evaluated as endpoints.

Aim 3: Effects of ATP or UTP treatment on gene expression profile of CIK cells.

mRNA and micro RNA (miRNA) expression analysis of nucleotide treated and untreated cells could lead to identify the impact of ATP or UTP treatment on gene expression profile of CIK cells. In particular, the pathways that are involved in response to nucleotide treatment will be highlighted. One major limitation in the understanding of mRNA/ miRNAs role in response to different drugs is the difficulty of correlating deregulated expression of miRNA with that of target genes. To overcome this issue, we are going to perform an integrative analysis of mRNA and miRNA expression data in the same sample of CIK cells. This integrated analysis will allow a

more reliable identification of miRNA target genes, thus enabling a better understanding of the role played by these mRNA/miRNAs in response to nucleotide treatment.

Aim 4: Evaluation of extracellular purine homeostasis in CIK and its tuned regulation via membrane-bound ecto-nucleotidases.

By employing the combination of enzyme-coupled luminometric and fluorometric sensing assays (*Helenius, 2012*), the whole spectrum of extracellular ATP, ADP, AMP, adenosine and inosine levels will be simultaneously determined at basal and various stimulated conditions. Furthermore, we intend to measure the decay rates of exogenously added ATP and other nucleotides and in this way, to evaluate the activities of major purine-converting converting ectoenzymes, NTPDase1 and ecto-5'-nucleotidase/CD73. Depending on the results obtained, these experiments could be independently ascertained by using thin-layer chromatographic assays with 3H-labelled ATP, ADP and AMP as preferred enzyme substrates (*Yegutkin et al., FASEB J 2001*).

Aim 5: Nucleotide-stimulated CIK expansion.

The efficient ex vivo expansion of CIK cells is a crucial issue for their prospective clinical translation. We will evaluate whether purinome modulation might affect the ex vivo expansion rate and final phenotype of CIK cells. In particular it will be interesting to assess the effect on the $CD3^+$ $CD56^+$ subset of CIK cells, considered the main responsible for the tumor killing activity.

Aim 6: Modulation of CIK purinome: effects on CIK cytokine secretion.

The effects of CIK on cytokine secretion will be evaluated following isolation of the supernatant and employing the multiplex approach allowing the simultaneous detection and quantification of 27 cytokines, chemokines and growth factors.

Expected results:

Current clinical trials show that CIK cells are a promising and safe way to treat malignancies. Since the validity of this therapeutic approach has been warranted by encouraging results on many patients, this method will be adopted alone or in

combination with chemotherapy, radiotherapy or other immunotherapy approaches to improve cancer treatment and prolong patients survival.
However, this technique should be further improved by increasing number, efficiency and specificity of CIK cells. Identification of P2 receptors expressed by CIK cells would shed light on mediators that negatively regulate these cells and hopefully increase their performance against tumors. This will be done by modulating purinergic-medited responses of CIK cells thus controlling their proliferation, cytokine repertoire and killing response. An impact of this study might be represented by the opportunity to modify the therapeutic approach to cancer by a pharmacological purinergic-based new protocol applied to CIK cells.

ImmunoTools *special* AWARD for **Davide Ferrari** includes 16 reagents

FITC - conjugated anti-human CD3, CD4, CD8, CD45RA, CD56, HLA-ABC,

PE - conjugated anti-human CD8, CD56, CD62L,

APC - conjugated anti-human CD3, CD56, CD62L,

recombinant human cytokines: rh IFN-gamma, rh IL-2, rh IL-15, rh IL-21

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