T cell activation and apoptosis: the molecular background of decision

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1. INTRODUCTION

The maintenance of homeostasis is the essential term of the organism's normal function. Birth, death and the functional activity of individual cells within a particular environment in the body is under strict temporal and spatial control. This is particularly true for the cells of the immune system.

The primary role of lymphocytes is the recognition and elimination of the foreign substances (antigens) or potentially harmful self materials from the organism. This task is carried out by the T and B lymphocytes in collaboration with other immunocompetent cells in a well coordinated manner. The homeostasis of immunocompetent cells is maintained by the regulation of renewal, activation, clonal expansion and cell death. The proliferation and differentiation of the peripheral T cells exiting the thymus is induced by the interaction with antigen presenting cells (APC). The interaction of the $CD4^+$ T helper lymphocytes to their specific ligands presented by the APC-s activates multiple signaling pathways, that are integrated by membrane bound and intracellular adaptor molecules. The binding affinity of T to the MHC-peptide complex on the APC, the nature and concentration of the presenting peptide and the duration of the TCR activation are key factors in the regulation of the subsequent biochemical processes. These parameters essentially determine the outcome of T cell activation.

We have designed a model system to study the role of APC function on the activation of T lymphocytes. We used the IP12-7 effector/memory T cell hybridoma, and several well characterized cell lines and primary cells as APC-s, that alowed us to study cell proliferation, differentiation and apoptosis induced by different T cell stimuli.

Previous data in our laboratory verified, that the thymocytes express retinoid acid receptor α (RARα) and γ (RARγ) and the two receptors have opposite effects on thymocyte activation and activation induced cell death. While ligand binding of RARα prevents the negative selection of thymocytes and activation induced cell death (AICD), stimulation of RARγ starts thymocyte apoptosis and promotes negative selection. We described previously, that these opposing effects of RARα and RARγ is mediated by cell surface expression of the apoptotic protein FasL. The surface expression of FasL is regulated by the function of the transcription factor Nur77.

2. AIMS

By using the IP12-7 T cell hybridoma system we designed experiments to identify the regulatory signals leading to T-cell activation or to apoptosis by antigen induced cell death. To achieve this we systematically followed individual steps of the events induced by TCR stimuli. In addition, using kinetic studies to determine the immediate, the early, and the late events of TCR signaling upon activation by different APC-s. We seeked to study how the amount of the presented antigen, the duration of T cell-APC interaction and the nature of the given APC influence the outcome of T-cell activation and cell fate.
Activation of peripheral T lymphocytes, besides specific antigen stimuli, can be influenced by other factors. Previously we defined some of the effects of retinoids on tymocyte function. In these experiments we examine the effects of the same compounds on activated peripheral T lymphocytes.

Our questions were:

1. What is the efficiency the different APC-s to activate IP12-7 T cell hybridoma?
2. Do differences in the stimulation of IP12-7 T cell hybridoma correlate with the adhesion features and/or the cell surface molecules on the given APC?
3. Is it possible to define checkpoints that regulate the fate of T cells towards activation induced cell proliferation and effector function or the activation induced cell death?
4. How retinoids influence peripheral T cell proliferation and activation induced cell death
5. Which signaling pathways mediate the regulatory role of retinoids in peripheral T lymphocytes.

To answer the questions we used the following methods:

1. *in vitro* cell culturing and isolation of primary cells from blood
2. activation of T cells with different APC-s, anti-CD3 monoclonal antibody or phytohemagglutinin
3. monitoring the cell proliferation with [^{3}H]thymidine incorporation
4. investigation of cell-cell interaction by flow cytomerty
5. detection of cell surface molecules, apoptosis and cell cycle phase by flow cytomerty
6. quatitation of the level of the intracellular free calcium ($[Ca^{++}]_{ic}$) by flow cytomerty
7. determination of level of secreted cytokines by bioassays, RT-PCR or ELISA
8. detection DNA binding capacity of transcription factors by EMSA (*e*lctro*m*obility *s*hift *a*ssay)
9. determination of changes in the expression of relevant genes by RT PCR
10. determination of changes in the level of regulatory phosphorylation events and the level of protein expression by Western blot analysis
11. transient transfection assay

Completion of our work should provide further information that will help to understand our central question: how signals coming through the same receptor (TCR) can induce opposing effects (proliferation or apoptosis) within the cell and how the same compound can generate the opposite effects through ligation by different receptors (RARα or RARγ). The outcome of such regulatory signals will determine the size and quality of the of the peripheral T cell pool and immunological memory.

3. RESULTS AND DISCUSSION

Investigation of the requirements of $CD4^+$T cell activation and differentiation has developed the concept of a progressive model, which proposed that signal strength was able to regulate the response of T cells through hierarchical thresholds of proliferation, differentiation and activation induced programmed cell death. The strength of T cell stimulation was shown to be determined by (1) the concentration of peptide antigen displayed on the APC surface in the context of MHC class II molecules, which determines the rate of TCR triggering, (2) the degree of co-stimulatory signals provided by the APC, which regulates the extent of signal amplification, and (3) the duration of the interactions between antigen-specific T cells and professional APC, which depends on the adhesive properties of the two cells and determines the duration of the signaling process. Most of these experiments were performed in model systems where T cells were activated by stimuli of strong strength, which generated rapid and full responses.

In the present work we compared the thresholds of immediate, early and late signals as well as the induction of AICD in $CD4^+$ peptide-specific effector/memory T lymphocytes stimulated in the presence of various professional APC. Our system is specific, because the effects of different strength of TCR signal and every phase of the T cell activation may follow in the same cell line. Our results revealed that the response of $CD4^+$ effector/memory T lymphocytes, which play a crucial role in regulating both humoral and cellular immune responses, is highly sensitive for the signals delivered by the APC they contact.

The most efficient 2PK3 APC was shown to be unique in that it was able to present exogenous peptides promptly and was highly active to make contact with T lymphocytes. The full T-cell response, which was manifested by stable T cell – APC contact, sustained increase of intracellular calcium, activation of transcription factors, down regulation of TCR, increase of Fas ligand, CD69 and Nur77 expression, up-regulation of IL-2 and TNFβ mRNA and protein secretion as well as AICD, could be achieved by the most potent 2PK3 B cells, while the others induced quantitatively and qualitatively different responses. The rank order of APC efficiency, however, was similar in all assays suggesting that the APC-dependent signals acted at an early stage of T-cell activation and were regulated in concert. This was in line with the kinetic studies, which demonstrated the rapid activation of T cells in the presence of 2PK3 APC as compared to the others.

2PK3 APC induced prompt activation of the whole population of T cells. In the presence of A20 APC the magnitude of the early intracellular calcium response, measured in a fraction of T cells, was similar to that of 2PK3-induced T cells but about half of the T lymphocytes did not get activated. In contrast to 2PK3 and A20 cell TA3 APC did not induce measurable calcium signaling in T cells. This provides additional evidence for regulating the calcium response by the duration of initial cell contacts between APC and T cells. The increase of contact time by long lasting sedimentation instead of short centrifugation could not compensate for the reduced T cell activating capacity of A20 or TA3 APC. Similar results were obtained with partial peptide agonists, which induced calcium signaling in a smaller fraction of T cells than the agonist peptide. The intensity, frequency and duration of calcium signaling is known to influence the activation of transcription factors involved in regulating genes mediating T cell effector functions such as cytokine production, expression of membrane proteins and AICD. These results suggest that the increase of $[Ca^{++}]_{ic}$ is modulated by the number of serial TCR contacts with specific MHC-peptide complexes controlled by the strength and duration of APC-T cell interactions.

The intensity, frequency and duration of calcium signaling is known to influence the activation of transcription factors involved in regulating genes mediating T cell effector functions such as cytokine production, expression of membrane proteins and AICD. Our

results showed rapid activation of NF-AT in IP12-7 T cells after 15 min contact with 2PK3 cells but not in the presence of other B cells. The most efficient activation of AP-1, NF-κB and NF-AT was detected in T cells activated in the presence of 2PK3 APC, while the two other B cells were less efficient. These results revealed similar, strong correlations between the intensity of APC-T cell contact, calcium signaling and the activation of transcription factors related to T cell activation.

The contact of IP12-7 T cells with all the selected APC resulted in measurable IL-2 and TNF secretion and in the cell surface expression of CD69, which demonstrated that T cell activation was initiated. However, rapid up regulation of NF-AT, NF-κB and AP-1, TCR down regulation, TNF-β and nur77 gene expression and the appearance of Fas ligand, followed by AICD could be achieved by the most potent 2PK3 APC, only. In concert with the secretion of high amounts of IL-2 and with the expression of FasL the contact with peptide-loaded 2PK3 APC rapidly rendered the majority of IP12-7 cells apoptotic.

The functional activity of 2PK3 cells was similar to that of bone marrow derived DC and was substantiated by the nanomolar concentration of peptide or by the low number of peptide-pulsed cells required for cytokine synthesis. 2PK3 cells were characterized by the high expression of B7-1 and B7-2, CD40 and CD44 co-stimulatory molecules.

Monitoring T-cell triggering by kinetic studies in this well characterized system allowed us to compare the threshold and the requirements of various T cell responses. In line with the kinetic proofreading model [42] our results demonstrated that the kinetics and intensity of conjugate formation between APC and T-cell affected the threshold of various signaling events to a different extent. Hence, TCR down regulation, FasL expression and AICD requires the contact with the most efficient 2PK3 APC, calcium signaling can be induced by both 2PK3 and A20 while CD69 expression is induced by 2PK3, A20 and also TA3.

While the retinoids regulate the proliferation and activation induced cell death of the thymocytes, we justified to investigate the influences of these compounds on peripheral T lymphocytes. In our experiments were applied retinoid receptor agonists and antagonists to promote or inhibit one or more receptors.

To use the IP12-7 T cell hybridoma and the Jurkat T cell line were demonstrated, that the RARα and the RARγ have opposite effects on the trans-activating capacity of the Nur77 molecule ant regulate this way - among others – the develop of biologically active FasL form.

Since demonstrated, that the retinoids have a central role of the signaling events followed by TCR stimulation, in our further experiments we investigated the influence of different retinoid receptors on the T cell proliferation.

The ligation of RARα promoted proliferation, this mechanism seems to be related to the enhanced IL-2 production. since addition of the RARα selective CD336 promoted PHA-induced IL-2 production, but did not affect proliferation at saturating concentrations of IL-2. Enhanced IL-2 production in the presence of the RARα agonist led to increased expression of JAK3, enhanced phosphorylation of Stat5, accelerated cell cycle entry (as detected by the phosphorylation of pRB), increased expression of Bcl-2, and inhibition of apoptosis. These observations are in line with previous studies, which also indicated that the growth promoting effect of retinoids is related to the regulation of IL-2 production.

While ligation of RARα promoted proliferation of T cells by enhancing the production of IL-2, ligation of RARγ inhibited T cell growth by interfering with the signaling pathway of IL-2. In addition, ligation of RARγ slightly reduced the production of IL-2, without significantly affecting the expression of IL-2Rα. Ligation of RARγ inhibited the expression of JAK3, and consequently the phosphorylation of Stat5, a transcription factor, which plays a key role in mediating the proliferating effects of IL-2. As a result, Stat5-mediated events on cell cycle progression, and induction of Bcl-2 were also inhibited without induction of apoptosis. This

might be related to the fact that IL-2 mediates a JAK3-independent cell survival pathway as well.

We have previously shown the opposite role of RARα and RARγ in the regulation of T cell apoptosis. The data presented here demonstrate the opposite role of these receptors in the regulation of T cell proliferation as well. It seems that retinoids regulate the level and the signal transduction pathway of key proteins in both processes. One of these key proteins is IL-2 in the case of T cell proliferation and, as we show here, ligation of RARα promotes its TCR-induced production, while ligation of RARγ inhibits the IL-2 mediated signaling. In the case of T cell apoptosis the apoptosis-linked Nur77 transcription factor is regulated. Ligation of RARγ promotes the TCR-induced expression of nur77, while ligation of RARα inhibits the nur77-mediated transcription. All together our data imply an opposite role of RARα and RARγ in the regulation of the size of the available T cell pool. Ligation of RARα will promote the expansion of the T cell pool by inhibiting negative selection of thymocytes and by promoting the proliferation and inhibiting activation-induced cell death of peripheral T cells. This latter was suggested to negatively regulate the size of the proliferating T cell pool. Ligation of RARγ, on the other hand, will reduce the size of the available T cell pool by promoting apoptosis of thymocytes, and by inhibiting proliferation and enhancing activation-induced death of peripheral T lymphocytes.

The summary of these works presented in this Dissertation is as follows:

1. Our previously established *in vitro* T cell activation system allowed us to vary signal strength that resulted in partial to full activation and AICD in the same cell line (effector/memory $CD4^+$ T cell hybridoma).
2. We demonstrated, that the T cell response against TCR signaling is highly dependent on APC phenotype and the concentration of the presented peptide.
3. We proved, that in our model, the intensity of T cell activation mediated by TCR-derived and co-stimulatory signals determine cell fate, as strong activation signals inevitably coincide with AICD.
4. To investigate the influence of retinoids in activated peripheral T lymphocytes on T cell activation and apoptosis, we documented, that the stimulation of the RARα and γ receptors have the opposite effects. Ligation of RARα promotes the expansion of the T cell pool by inhibiting activation-induced cell death of peripheral T cells. RARγ activation, on the other hand, reduces the size of the available T cell pool by inhibiting proliferation and enhancing activation-induced death of peripheral T lymphocytes.
5. We showed, that these effects are mediated by the regulation of Nur77 trans-activation capacity, by the cell surface expression of FasL and by the regulation of the interleukine-2 signalling pathway.

4. PUBLICATIONS

4.1 Articles use in this dissertation

Ludanyi K, Gogolak P, Rethi B, Detre C, Matko J, Rajnavolgyi E. Antigen presenting cell-induced fine tuning of murine helper T cell activation and death. *Cell Signal* 2004 (IF: 4.362)

Ludanyi K, Nagy Zs, Alexa M, Reichert U, Michel S, Ancian P, Fesus L, Szondy Z. Ligation of retinoic acid receptor α promotes IL-2 production, while ligation of retinoic acid receptor γ inhibits IL-2 signaling in phytohaemagglutinin-stimulated T cells. (send fpr publicaton)

Toth R, Szegezdi E, Reichert U, Bernardon JM, Michel S, Ancian P, **Kis-Toth K**, Macsari Z, Fesus L, Szondy Z. Activation-induced apoptosis and cell surface expression of Fas (CD 95) ligand are reciprocally regulated by retinoid acid receptor α and γ and involve nur77 in T cells. *Eur J Immunol* 2001;31:8. (IF: 4.832)

4.2 Another articles

Hajas Gy, Zsiros E, Laszlo T, Hajdu P, Somodi S, Rethi B, Gogolak P, **Ludanyi K**, Panyi Gy, Rajnavolgyi E. New phenotypic, functional and electrophysiological characteristics of KG-1 cells. *Imm Letters* (IF: 1.847)

Toth B, **Ludanyi K**, Reichert U, Michel S, Fesus L, Szondy Z. Retinoid-induced Fas (CD95) ligand expression is mediated via RARγ and involves Nur77 in T cells. *Eur J Immunol* (IF: 4.832)

Kovesdi D, Paszty K, Enyedi A, Kiss E, Matko J, **Ludanyi K**, Rajnavölgyi E, Sarmay G. Antigen receptor mediated signaling pathways in transitional immature mouse B cells. *Cell Signal* (IF: 4.362)

Miklos I, Benko Zs, **Ludanyi K**, Sipiczky M. Point mutation in the D-loop of praline tRNA can cause cell division defect in Shizosaccharomyces pombe. (send for publication)

4.3 Conference abstracts and presentations

2003: 12th Symposium on Signals and Signal Processing in the Immune System, Sopron: **Ludanyi K**, Gogolak P, Rethi B, Detre C, Horvath A, Matko J, Rajnavolgyi E. Antigen presenting cell-induced fine tuning of murine helper T cell activation and death. (poster)

2003: 12th Symposium on Signals and Signal Processing in the Immune System, Sopron: Detre C, Kiss E, **Ludanyi K,** Paszty K, Enyedi A, Rethi B, Rajnavolgyi E, Matko J. Membrane ceramide release may control the sensitive balance of activation, survival and death signals in T cells: implications in rescuing from AICD. (presentation)

2003: EFIS, Rhodos: Detre C, **Ludanyi K**, Gombos I, Rethi B, Vamosi G, Rajnavolgyi E, Matko J. Lipid rafts in APC and ceramide in T cell membrane modulate activation threshold and death signaling in APC (B cell) – T cell conjugates/synapses. (poster)

2003: The 33th Congress of Membrane Transport, Sumeg, Hungary; Detre C, **Ludanyi K**, Gombos I, Kiss E, Rethi B, Rajnavolgyi E, Matko J. The role of ceramide in the activation and cell death of the antigen-specific T lymphocyte. (presentation)

2003: International summer school on: From transcription to physiology; regulation of gene expression and protein function in an integrated context, Spetsai, Greece; Toth B, **Kis-Toth K**, Szondy Z. Role of Fas ligand and Nur77 in the T cell apoptosis induced by retinoids. (poster)

2003: The 8th Congress of the Hungarian Society of Biochemistry, Sopron, Hungary; Toth B, **Kis-Toth K**, Fesus L, Szondy T. Role of Fas ligand and Nur77 in the T cell apoptosis induced by retinoids. (poster)

2002: The 32th Congress of Hungarian Society of Immunology, Kaposvar, Hungary; **Ludanyi K**, Rethi B, Gogolak P, Kurucz I, Rajnavolgyi E. The molecular effects of IL-7 depletion. (poster)

2001: The 31th Congress of Hungarian Society of Immunology, Eger, Hungary; **Kis-Toth K**, Gogolak P, Detre C, Rajnavolgyi E. T cell activation and apoptosis: the molecular background of the decision. (poster)

2001: The 6th Congress of the Hungarian Society of Biochemistry, Sopron, Hungary; Rethi B, Gogolak P, Horvath A, **Kis-Toth K**, Detre C, Kolonics A, Magocsi M, Rajnavolgyi E. Antigen presenting cell induced fine tuning of murine helper T cell activation. (poster)

2000: The 30th Congress of Hungarian Society of Immunology, Budapest, Hungary; **Kis-Toth K**, Rethi B, Gogolak P, Rajnavolgyi E. T cell activation and apoptosis: the molecular background of the decision. (poster)

2000: The 30th Congress of Hungarian Society of Immunology, Budapest, Hungary; Rethi B, Gogolak P, Detre C, Kolonics A, **Kis-Toth K**, Magocsi M, Rajnavolgyi E. The study of T cell activation in the presence of different antigen presenting cells. (presentation)

2000: The 14th European Immunology Meeting, EFIS, Poznan, Poland; Rethi B, Gogolak P, **Kis-Toth K**, Derte C, Kolonics A, Magocsi M, Rajnavolgyi E. Antigen presenting cell-induced fine tuning of murine helper T cell activation. (presentation)

2000: The 5th Congress of the Hungarian Society of Biochemistry in Sarospatak, Hungary; **Kis-Toth K**, Rethi B, Gogolak P, Rajnavolgyi E. T cell activation and apoptosis: the molecular background of the decision. (poster)

1999: The 4th Congress of the Hungarian Society of Biochemistry, Eger, Hungary; **Kis-Toth K**, Szondy Z, Fesus L. The effects of retinoids on activated T-lymphocytes. (poster)