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Short Communication

Cytokine-induced killer cells are type II natural killer T cells

Zytokin-induzierte Killerzellen sind Typ II natürliche Killer-T-Zellen

Abstract

Background: Until now, cytokine-induced killer (CIK) cells were assumed to be part of the type I natural killer T (NKT) cell population, but it was not yet investigated if this is correct.
Methods: For analysis, CIK cells were generated by various culture conditions. Human type I NKT cells express a T cell receptor (TCR) composed of an invariant Vα24-JαQ chain combined with one of several Vβ chains. The Vα24 is a reliable marker for the presence of these TCRs.
Results: While comparing cultures stimulated with different substances, we observed the lack of any Vα24 on the surface of CIK culture cells.
Conclusion: We conclude that CIK cells do not belong to the type I NKT cells.

Keywords: cytokine-induced killer, natural killer T, Valpha24-JalphaQ

Zusammenfassung

Hintergrund: Bis jetzt wurde angenommen, dass Zytokin-induzierte Killer(CIK)-Zellen Teil einer Typ I natürliche Killer-T(NKT)-Zellpopulation sind, aber bisher fehlten die Untersuchungen, die dies bestätigten.
Methoden: Zur Analyse wurden CIK-Zellen unter verschiedenen Kulturbedingungen hergestellt. Humane Typ I NKT-Zellen exprimieren einen T-Zellrezeptor (TCR), der zusammengesetzt ist mit einer invariaten Vα24-JαQ-Kette kombiniert mit einer von verschiedenen Vβ-Ketten. Vα24 kann als zuverlässiger Marker für das Vorhandensein von diesen TCRs verwendet werden.
Ergebnisse: Beim Vergleich von mehreren CIK-Zellkulturen fanden wir keine Vα24-Expression auf der Oberfläche von CIK-Zellen.
Schlussfolgerung: Wir schlussfolgern, dass CIK-Zellen nicht zum Typ I NKT-Zellen gehören.

Schlüsselwörter: Zytokin-induzierte Killerzellen, natürliche Killer-T-Zellen, Valpha24-JalphaQ

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Introduction

Cytokine-induced killer (CIK) cells have recently been shown to be highly effective in targeting viruses to tumor target cells [1]. CIK cells consist of a heterogeneous population of effector cells after stimulation with a mixture of interferon-gamma (IFN-γ), anti-CD3 (αCD3), interleukin-2 (IL-2) and interleukin-1beta (IL1β). CIK cells possess a cytolytic activity while it is stated that their killing is non-major histocompatibility complex (MHC)-restricted [2], [3]. This makes them different from normal cytotoxic T lymphocytes (CTL) as these need MHC molecules on antigen presenting cells (APCs) which presents antigenic peptides to the T cell receptor (TCR) on CTL. The major cytotoxic subset of the CIK cells are $CD3^+$ and $CD56^+$, which will be the most important property to identify these cells by fluorescence activated cell sorting (FACS) in this work. This NKT cell like subset derives from cytotoxic T lymphocytes [4].
Natural killer T (NKT) cells form a subpopulation of T cells with interesting properties. NKT cells express both natural killer (NK) cell markers, like CD56, as well as T cell markers, like CD3. NKT cells are believed to play crucial roles in several diseases, inhibiting one and exacerbating another. Amongst others it is stated [5] that NKT cells inhibit diabetes and several infectious diseases while they exacerbate allergic asthma, lupus and atherosclerosis. The most important fact to know about NKT cells is their special α/β T cell receptor. There are two types of NKT cells: Most of them express an invariant TCR while some do not. The first are called type I NKT cells and the latter type II NKT cells. Usually type I NKT cells are referred to as NKT cells. The invariant TCR of these human

cells is composed of the Vα24-JαQ α-chain and one of several β-chains, but mainly Vβ11. This TCR does not recognize peptide antigens like the α/β TCR of a normal CTL, but instead glycolipid antigens presented by the non-classical MHC molecule CD1d on APCs. Because it is assumed that the physiologically recognized antigens are endogenous, it is believed that the NKT cells are autoreactive: Upon stimulation, the APCs present the proper endogenous antigen to the TCR of the NKT cells and thus activate those cells. As stated in literature it is not possible to stimulate NKT cells without CD1d expressing cells [6]. NKT cells are referred to in this work as $V\alpha24^+$, $CD3^+$ and $CD56^+$. Once NKT cells are activated they release high amounts of cytokines, like IFN-γ, which also activate other cells, for example NK cells. Several subtypes of these NKT cells exist, distinct by their expression of CD4 and CD8 and their cytokine profile. The already mentioned following activation of other cells may play a crucial role in the cytolytic activity of NKT cells. Because of the activation of other cells, it seems that NKT more resemble regulatory cells which activate effector cells rather than being effector cells themselves [7].

Type II NKT cells are different from type I in that they do not express the invariant Vα24 TCR but a diverse TCR and do not respond to the stimulator of type I NKT cells KRN7000. But still these cells respond to other lipid antigens presented to them by CD1d on APCs. The functions and antigens of type II NKT cells seem to differ, as some of these cells are autoreactive. Thus the type II NKT cells are no homologous population of cells [8], [9].

For stimulation of NKT cells two glycosphingolipids were used. As mentioned before, these antigens are presented to the NKT cells by the molecule CD1d on certain APCs. This happens in a way that the glycosphingolipid is bound with its lipid part to the hydrophobic groove of CD1d while the carbohydrate portion interacts with the TCR of the NKT cell. KRN7000 was the first substance used. This glycosphingolipid is an exogenous stimulator for NKT cells which was originally isolated from a marine sponge. Because it is stated to activate NKT very strongly, this substance was used as positive control. Additionally we used the glycosphingolipid globotrihexosylceramide-3 (Gb3). In contrast to KRN7000 this glycosphingolipid has no stimulating effect on NKT cells and was used as negative control.

CIK cells were assumed to be part of this type I NKT cell population, but this was not yet investigated.

Material and methods

Isolation of peripheral blood leukocytes

Blood from buffy coats was mixed 1:2 with PBS/1%BSA (both PAA, Cölbe, Germany) and used for a ficoll gradient (LymphoPrep, PAA, Cölbe, Germany). After the centrifugation at 800 g for 30 minutes, the leukocyte layer was removed and collected in new tubes. These cells were then washed 3 times with PBS/1%BSA and afterwards resuspended in fresh medium, consisting of RPMI medium (PAA, Cölbe, Germany) with 10% fetal calf serum (Invitrogen, Karlsruhe, Germany), 2.5% hepes buffer solution (PAA, Cölbe, Germany) and 1% penicillin/streptomycin (PAA, Cölbe, Germany). An adhesion period of 1 hour was performed in a culture flask and the non-adherent cells were removed and used for culture subsequently.

Generation of cytokine-induced killer cells

The non-adherent cell population from blood was cultured in complete RPMI medium at a density of 2-3 x 10^6 cells mL^{-1}. The CIK culture was generated as previously described [10] but with minor changes: At the day of isolation IFN-γ was added in a final concentration of 1000 U mL^{-1} medium. On day 1 of the culture, anti-CD3 (Cilag GmbH, Sulzbach, Germany) and IL1β (Boehringer Mannheim Biochemica, Mannheim, Germany) were added, resulting in a final concentration of 100 ng mL^{-1} anti-CD3 (instead of 50 ng mL^{-1} as stated in literature) and 100 U mL^{-1} IL1β in the culture. On the days 1, 3, 6, 9 and 12 the CIK culture was supplemented with 300 U mL^{-1} IL-2. Additionally fractions of the culture were removed on these days and fresh medium was added.

Stimulation of type I natural killer T cells

To the αGalCer culture 100 ng mL^{-1} αGalCer (KRN7000; Kirin brewery, Gunma, Japan) in methanol were added at the day of isolation. To the negative control, the Gb3 culture, 100 ng mL^{-1} Gb3 (Alexis, Lausen, Switzerland) were added at the day of isolation. The working solution of Gb3 had the same concentration as the KRN7000 working solution and was also dissolved in methanol. To both cultures 300 U mL^{-1} IL-2 were added on the days 1, 3, 6, 9 and 12. Additionally fractions of the culture were removed on these days and fresh medium was added. The cultures will be named by the type of their initial stimulus through out this work, i.e. CIK, KRN7000 and Gb3 as is shown in Table 1.

Analysis of cell surface markers

Cells from the different cultures were counted and analysed in flow cytometry with fluorescein isothiocyanate (FITC) labelled antibody and/or phycoerythrin (PE) labelled antibody against various surface markers. The following labelled antibodies from Coulter Immunotech were used: CD3 PE and CD8 FITC, CD14 FITC, CD19 FITC, Vα24 FITC and the isotype controls IgG1 mouse FITC and IgG1 mouse PE, as well as the antibodies from BD Pharmingen: CD3 FITC, CD4 FITC and CD56 PE. The measurement was done with an Epics XL-MCL System II Version 3.0 Instrument (Coulter Immunotech, Krefeld, Germany). For each measurement, data from 20,000 cells was collected. The negative controls were set to 1.0%-2.0% using the isotype controls.

Table 1: Definitions of the three culture conditions

Name of culture	Stimulus on day 0	Stimulus on day 1	IL-2 addition every 3 days
CIK	IFN-γ	αCD3, IL1β, IL-2	Yes
KRN7000	Natural αGalCer (Kirin brewery)	IL-2	Yes
Gb3	Gb3	IL-2	Yes

Results

The results from the flow cytometric analysis (Figure 1) demonstrate that all cultures achieved high CD3 values between 94% and 99% while the CIK culture achieved an higher value than the other cultures. The amount of $CD56^+$ NK cells was more or less similar at about 20% and the amount of $CD3^+/CD56^+$ NKT cells was also similar at about 14% in all 3 cultures. The amount of $Vα24^+$, $CD3^+/Vα24^+$ and $CD56^+/Vα24^+$ type I NKT cells differed between the 3 culture types. Only in the KRN7000 culture we could detect Vα24 positive cells at 23.6% +/- 5.9% while the CIK and the Gb3 cultures seem to be negative for Vα24. The $CD4^+$ and $CD8^+$ cell amount also differed: The CIK culture was high in $CD8^+$ cells and lower in $CD4^+$ cells while the KRN7000 and Gb3 cultures had higher CD4 than CD8 values. As expected $CD14^+$ and $CD19^+$ cells were only present in low amounts.

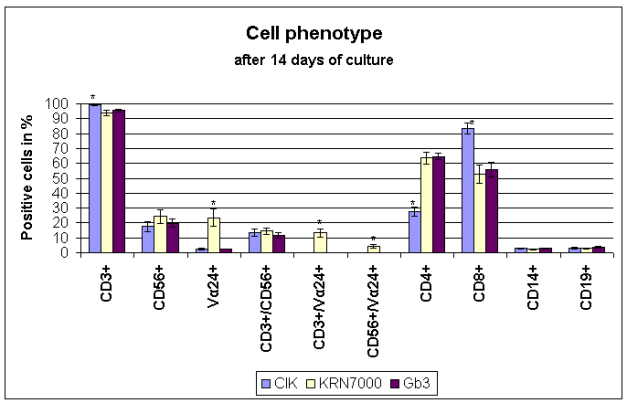

Figure 1: Mean percentage of tested surface molecules of the different culture types after 14 days as outlined in the material and methods section. The results are shown in mean of six experiments ± SEM (*P<0.05 compared with the Gb3 culture).

Discussion

As described above, we were able to characterise the different subpopulations in each culture. The CIK culture as shown in Figure 1 was composed almost entirely of $CD3^+$ cells (~99%). The mean percentage of $CD56^+$ cells in the CIK culture was 17.5% +/- 3.5%. $CD3^+/CD56^+$ cells, which are the classical effector cells of the CIK population [3], reach up to 14% of the CIK culture. Earlier we could show that NKT cells are the major cytotoxic subset of the CIK cells [11]. All of the cells in the CIK culture were negative for the invariant TCR Vα24 usually found on NKT cells. Thus they may not be assumed to be normal type I NKT cells. Probably these double positive NKT cells are $CD3^+$ cells with variant TCRs, which express additionally the NK cell marker CD56.

The culture also contained only very little monocytic $CD14^+$ cells (2.8%) or other cells expressing CD1d (APCs) or $CD19^+$ B cells (3.3%). This was found in all cultures analysed. The CIK culture consisted of 83% $CD8^+$ cells and 28% $CD4^+$. The biased ratios of CD4 and CD8 in the CIK culture result from the addition of IFN-γ. IFN-γ inhibits the growth of T_H2 cells, which are less effective against tumours than T_H1 cells [11], and thus inhibited a large fraction of $CD4^+$ cells. This favoured the growth of $CD8^+$ and T_H1 cells in the CIK culture. Thus the CIK culture at the end consisted in large part of T cells, but NK, which are $CD3^-/CD56^+$, and NKT cells were not a major component.

In all other cultures the amount of $CD3^+$ cells (~94%) was lower than in the CIK culture and the amount of $CD56^+$ cells (22%-24%) was slightly higher. This showed the presence of NK cells in these cultures, even though the amounts were only 5%-10% and still in low levels. The KRN7000 culture achieved a reasonable amount of $Vα24^+$ cells; almost 25% of the culture consisted of these cells. But not all of these cells were type I NKT cells. About 15% of the culture was $CD3^+/CD56^+$, about 14% was $CD3^+/Vα24^+$ but only 4% was $CD56^+/Vα24^+$. Because Vα24 is part of a TCR it is clear that all $Vα24^+$ cells were also $CD3^+$. Thus only 4% of the KRN7000 stimulated cells had all properties which identify them as type I NKT cells: they were $CD3^+$, $Vα24^+$ and $CD56^+$. Thus the remaining 11% $CD3^+/CD56^+$ cells and the remaining 10% $CD3^+/Vα24^+$ cells were no type I NKT cells. The 10% of the cells which were $CD3^+/Vα24^+$ seem to have been T cells. Because they did not express CD56 they were no NKT cells but because they expressed Vα24 they were also no common T cells but invariant T cells which are able to recognize glycosphingolipids. The 11% $CD3^+/CD56^+$ cells were CIK cells. Another difference can be seen in the percentages of CD4 and CD8. The percentage of CD4 (64%-68%) was higher than in the CIK culture and the CD8 percentage (53%) lower. This may be due to the effects of the IFN-γ and the IL-2 given to the cultures. The IFN-γ inhibits the growth of T_H2 cells, while IL-2 stimulates the growth of all T cells. Because IFN-γ was not given to the other cultures, the IL-2 could stimulate the growth of all T cells in the cultures. This gave rise to the difference in the $CD4^+$ percentage of these cultures with the CIK culture. The

Gb3 culture was almost completely $CD3^+$ (~95%). A fraction of NK cells and NKT cells ($CD3^+CD56^+$) was also present in this culture while these populations were smaller than in the KRN7000 culture. This culture was negative for Vα24 and thus did not include any type I NKT cells.

Conclusions

In conclusion, without the expression of Vα24 on CIK cells, these cells can not be assumed to be normal NKT cells, in this context type I NKT cells. But it may be possible that the CIK cells belong to the type II NKT cells which possess different properties than the type I NKT cells. This has to be investigated based on the CD1d reactivity of the CIK cells, because both types of NKT cells are reactive to the antigen presenting molecule CD1d [8], [9].

Notes

Conflicts of interest

None declared.

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