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The Role of Natural Killer cells in the apoptosis of cancerous cells and its potential use in immunotherapy

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The Role of Natural Killer cells in the apoptosis of cancerous cells and its potential use in immunotherapy

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Abstract

An essay exploring the mechanisms by which NK cells can destroy any non-self cell, primarily cancer and the resulting selection pressure that is applied. By looking at how NK cells can be expanded to clinically relevant levels, a potential clinical trial is proposed. This is then used to test the theoretical benefits of NK cell-based immunotherapy compared to standard cancer treatments such as chemo-therapy and IL-2 therapy.

Natural killer cells, known more commonly as NK cells, are the body's defence mechanism against foreign and mutated cells that could potentially cause harm to the body. One of the key functions of NK cells is to protect the body from mutated cells which rapidly and uncontrollably divide, known as cancer cells. When a cancerous tumour has formed in the body it can metastasise[1]; this is because cancer weakens the lining of the blood vessels (Martin et al., 2013). NK cells are extremely important in stopping metastasis as they one of the key mechanisms through which cancerous cells can be killed via apoptosis (programmed cell death) while travelling through the circulatory or lymphatic system. By recognising and then destroying the mutated cells, NK cells reduce the chance of successful metastasis occurring, thereby increasing the chance of survival. The overall significance of NK cells in the body's natural defences is therefore incredibly important as it has the potential to stop the spread of cancer.

NK cells also have the ability to attack tumours which make them a prime candidate to shrink the size of tumours or remove them from the body altogether. This means NK cells have the potential to be an alternative treatment for cancer to chemotherapy and IL-2. Therefore as well as exploring the mechanisms by which NK cells lyse (kill) cancerous cells, we will be exploring the methods of expanding NK cell culture sizes and purifying samples to provide an effective treatment for people with various forms of cancer.

[1] Metastasis is when part of the tumor breaks away, travels through the blood stream or lymphatic system and forms a secondary tumor elsewhere in the body.

How NK cells identify the infected or mutated cell

NK cells identify cancer cells by using the receptors on their plasma membrane. There are two main types of receptors involved in the inhibition of the NK cell response, the C-type lectin-like receptors and the Killer-cell immunoglobulin-like receptors (KIR's) (Cheent and Khakoo, 2009).

The C-type lectin-like receptors are in the form of a CD94 protein covalently bonded to an NKG2 protein forming a CD94/NKG2 complex. The NKG2 part of the complex contains either an inhibitory NKG2A or NKG2B, or an activating NKG2C, NKG2D, NKG2E or NKG2F receptor. In normal cells, the HLA receptor[2] will bind with the A, C or E variation of the NKG2 complex and send signals inhibiting or activating the NK cell depending on the variation bound. This shows that either the cell is healthy and therefore should not be destroyed, or damaged resulting in the cell being lysed. It is as of yet not fully understood why the NKG2 protein has 3 complexes which can bind to the HLA-E, two activating and one inhibiting, as this appears to be counterintuitive to the mechanism of how NK cells identify if a target cell should be lysed as we will discuss later. We do know though that if the HLA-E contains an antigen (Kochan et al., 2013), or if the HLA-E has been altered due to a mutation, the activating response from the receptors is sufficient to begin the process of the target cell undergoing lysis. The NKG2D is another crucial protein complex that is expressed by NK cells. It binds to the ligands on the cell, that indicate whether the cell is under stress, such as when a cell becomes cancerous. If this happens, it results in the triggering of a strong cytotoxic response leading to the immediate lysis of the target cells. The CD94: NKG2D complex is as a result crucial to the identification and destruction of cancer.

Receptors that are part of the KIR family also play a significant role in the apoptosis of cancerous cells. KIR receptors have many different forms which are evident from Table 1 and play a major role in the regulation of NK cell immune tolerance. The 'missing-self' hypothesis theorises that the NK cell must express at least one type of KIR which is receptive to the MHC class I type molecule produced by the HLA gene complex expressed by healthy tissue. This allows for the NK cells that develop to be able to differentiate between non-self and self-cells to ensure that they do not damage healthy tissue. The hypothesis has recently been refined to suggest that approximately 9% of NK cells do not have inhibitory receptors that can bind to the MHC class I type complex (Yawata et al., 2008). Despite this NK cells do still preferentially activate on cells lacking this self-protein. The process is still not fully understood as not all the ligands and receptors that are involved have been identified and studied, but the presence or absence of the MHC class I type protein still plays a significant role in lysis caused by NK cells.

[2] The HLA gene complex codes for the HLA proteins that are part of the MHC class I type protein. These are the ligands that bind to the CD94: NKG2 complex.

		Receptor	Ligand
Activating	Natural cytotoxicity receptors	NKp30	BAT-3
		NKp44	Viral haemagglutinin
		NKp46	Viral haemagglutinin
	C-type lectin-like receptors	CD94: NKG2C	HLA-E
		CD94: NKG2E	HLA-E
		NKG2D	MIC-A/B, ULBPs
	Others	CD244 (2B4)	CD48
		CD16	IgG
		CD226 (DNAM-1)	CD112, CD155
Inhibitory	KIR family	2DL1	Group 2 HLA-C
		2DL2/3	Group 1 HLA-C
		2DL5	Unknown
		3DL1	BW4+HLA-B
		3DL2	HLA-A3/A11
	C-type lectin-like receptors	CD94: NKG2A	HLA-E
		NKR-P1A	LLT1

Table 1: A table of each receptor and the ligand on the target cell plasma membrane that it checks (Cheent & Khakoo, 2009)

As shown in Table 1 each receptor will perform a check on a ligand to ensure that the cell is healthy and should therefore not be destroyed. NK cells do not contain one dominant receptor unlike most lymphocytes which rely on a primary receptor to identify diseased cells; instead, they likely rely on a mixture of activating and inhibitory signals to identify if the cell that is being checked is a self-cell. For lysis to occur the signal produced by the activating receptors must exceed the signal produced by the inhibitory receptors. This means the cell relies on coactivating receptors (Lanier, 2008), hence the HLA-E protein complex binding to the CD94: NKG2A/C/E variations of the C-type lectin-like receptors appears to oppose this idea. It may suggest that the CD94: NKG2A/C/E complex does not, in fact, mediate cell lysis but instead has another function which we have not yet discovered.

The mechanism by which NK cells destroy cancerous cells

NK cells can destroy the target cell by releasing cytotoxins stored within specialised organelles adapted to release them via exocytosis.

The primary chemicals present within NK cells which mediate cell lysis are perforin and granzyme B (GzmB). To deliver the GzmB and perforin into the target cell, the perforin is used to temporarily damage the cell surface membrane and increase the flow of Ca^{2+}ions into the cell. In response the target cell repairs the damage to the membrane and in the process forms an endosomal sack around the GzmB and perforin and endocytoses it (Thiery et al., 2011). The perforin then acts by forming a pore that is approximately 10-20nm in diameter in the membrane of the endosomal sack storing the GzmB, causing it to be released until eventually the sack completely breaks down. This is thought as part of the perforin amino acid chain is partially homologous to the C9 protein, a pore forming protein found in bacteria cell surface membranes (Stanley et al., 1985). Therefore it is likely that these two share similar properties and utilise a similar mechanism for membrane penetration (Pipkin & Lieberman, 2007). Once the GzmB is inside the cell, apoptosis then occurs.

The GzmB triggers what is known as the caspase apoptosis pathway. It reacts with caspase 3 which then cleaves the proteins ICAD and bid in the cytoplasm. The cleaving of bid triggers the mitochondrial apoptotic pathway (Sutton et al., 2000) as seen in Figure 1. The cleaving of ICAD results in CAD endonuclease being activated causing the caspase apoptotic pathway to occur. In this pathway, caspase 3, found inside the cell, simultaneously cleaves the DFF45 protein causing DFF40 to begin the process of fragmenting the DNA (Sharif-Askari, 2001). The CAD endonuclease can then proceed to break down the DNA fragments, resulting in the cell dying.

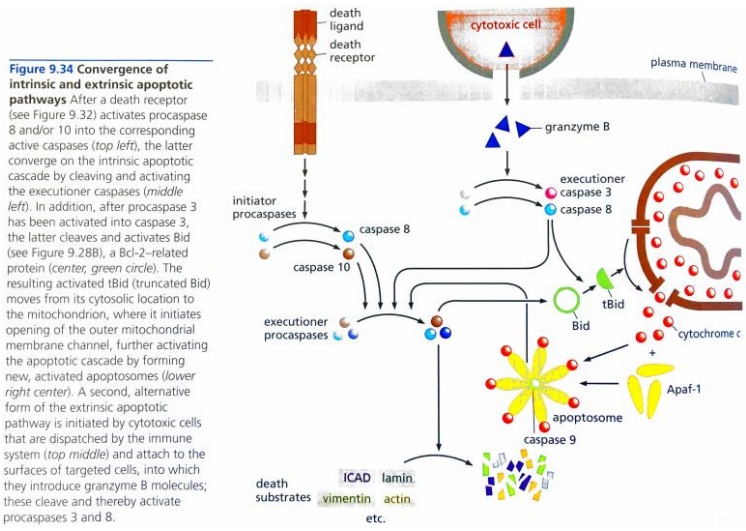

Figure 9.34 Convergence of intrinsic and extrinsic apoptotic pathways After a death receptor (see Figure 9.32) activates procaspase 8 and/or 10 into the corresponding active caspases (*top left*), the latter converge on the intrinsic apoptotic cascade by cleaving and activating the executioner caspases (*middle left*). In addition, after procaspase 3 has been activated into caspase 3, the latter cleaves and activates Bid (see Figure 9.28B), a Bcl-2–related protein (*center, green circle*). The resulting activated tBid (truncated Bid) moves from its cytosolic location to the mitochondrion, where it initiates opening of the outer mitochondrial membrane channel, further activating the apoptotic cascade by forming new, activated apoptosomes (*lower right center*). A second, alternative form of the extrinsic apoptotic pathway is initiated by cytotoxic cells that are dispatched by the immune system (*top middle*) and attach to the surfaces of targeted cells, into which they introduce granzyme B molecules; these cleave and thereby activate procaspases 3 and 8.

Figure 1: A diagram showing the mitochondrial apoptotic pathway that is also triggered by the granzyme B released by the NK cell (Weinberg, 2014a, pp375,762).

The problems that cancerous cells pose to any potential treatment

The issue faced with cancer treatments is similar to antibiotic resistance in the sense that the harsh selection pressure that a vast number of NK cells places on a group of cancerous cells results in very fast adaptation.

For example, due to the survival of the fittest, only the cancerous cells that have developed ways to hide or avoid NK cells are left alive, the cells that survive then proceed to expand quickly and result in the growth of a tumour which has gained a resistance to NK cell lysis. The primary defence mechanisms involved in preventing apoptosis in cancerous cells are shown in Table 2. All these mutations reduce the chance of interactions between NK cells and cancerous cells resulting in apoptosis.

Strategy	**Mechanism**
Hide stress	Repress NKG2D ligands
Inactivate immunocytes	Destroy immunocyte receptors Saturate immunocytes with adenosine
Neutralise intracellular toxins	Enzymatic detoxification of H_2O_2 prostaglandin E2

Table 2: A table showing key mutations that commonly occur in cancerous cells which help cancer avoid detection by lymphocytes, including NK cells, or prevent apoptosis from occurring (Weinberg, 2014b, pp.762).

One of the key challenges in finding a reliable solution to cancer using immunotherapy is trying to overcome these mutations to ensure that lymphocytes, specifically NK cells, can retain their lethality towards cancer. To overcome the problems of expression of receptors, lymphocytes can be genetically engineered to produce complementary antigens to the receptors on the tumour cells that can also bind to NK cells. Hence it would result in a response being triggered by the NK cell when it binds to the antigen, killing the target cell despite it originally being undetectable. This is known as Antigen-dependent cellular cytotoxicity[3] (Klingemann, 2015) but faces the issue that by genetically engineering lymphocytes, graft-vs-host disease may occur. Therefore lymphocytes which may attack the newly implemented, genetically engineered lymphocytes must be removed which further complicates the procedure in patients with this adaptation of their cancer. This could be overcome by introducing specialised treatment for individual patients, using their lymphocytes as the starting cell but this could be an incredibly expensive, time-consuming and impractical process involving large numbers of patients.

Lots of cancers develop a way of downregulating the MHC class I type protein to avoid detection by T-lymphocytes, which causes a response by the NK cells due to the lack of an MHC class I ligand providing an inhibitory response to the NK cells. Some cancers, therefore, develop defence mechanisms even further to defend against attacks by NK cells.

[3] Cytotoxicity is a measure of how toxic a substance or cell is to another cell.

They do this by shedding away the ligands, that indicate that a cell is under stress, into the intercellular fluid around them. These proteins then act as 'decoy' ligands, continually binding to the receptors on NK cells and wearing them away. Eventually, once the NK cell reaches the cancer cell, the CD94: NKG2D protein is no longer responsive to the cell stress proteins. This once again hides the cancer but this time to the NK cell.

There is another key alteration which poses a threat to NK cell cancer treatments which is that when cancerous cells become immune to cytostatic drugs[4] there is a cross-resistance to NK cells preventing them from lysing these cells. It was found that HLA proteins were upregulated in cancer that had become resistant to cytostatic drugs (Classen et al., 2003), resulting in the increase of the inhibitory response that NK cells receive from the cancerous cell. Therefore the cancer is 'disguised' by surpassing the effect of the activating receptors with the inhibitory effect.

Finally, in patients with leukaemia NK cell therapy could also pose a potential problem. This is because the cancer cells will be growing on the bone marrow and as a result, can affect the growth of new NK cells and cause them to be defective (Costello et al., 2002). The leukaemia cells are therefore protected and this poses a potential dilemma for NK cell in vivo[5] expansion.

NK Cells ability to treat cancer

NK cells have an incredibly precise and lethal killing mechanism, but require specific cell signals to initiate this process. These signals are based primarily around the C-type lectins and the KIRs which provide both activating and inhibitory responses as explained previously. The potential that NK cells have to shrink tumours in the brain or on other major organs to make them operable and safer to treat is vast as most cancers do lack MHC class I expression. By using NK cell-based immunotherapy, the risk of metastasis will decrease drastically as well. This is because any cancerous cells in the lymphatic or circulatory system are more likely to encounter an NK cell and trigger a response. Therefore this improves the ease at which the cancer can be treated and increases the survival rate of patients as the cancer cannot metastasise.

Preparing, stimulating and purifying NK cell cultures

Recent research (Oyer et al., 2016) has suggested that using PM21 particles[6] can cause the expansion of a culture of NK cells by an unprecedented amount, producing a sample that is big enough to be clinically relevant. This finally opens the door for using NK cells as an immunotherapy because large samples can be created for testing.

The results from the experiment done by Oyer et al. (2016) showed that NK cell expansion was increased by 82500% on average after 14 days ex vivo, and in vivo the number of NK cells increased by 300% after 12 days. At this point, the NK cells were found in the brain, bone marrow, liver, lungs and spleen, all of which suggests that the NK cells could be used as a treatment for tumours almost anywhere in the body, instead of just in a specific area.

[4] Cytostatic drugs are often used as part of chemotherapy.
[5] In vivo referring to methods of expanding NK cells within the body.
[6] PM21 particles produced by a type of genetically modified immortal leukemia cell line which expresses both 41-BBL and membrane bound interleukin-21

The benefits of a particle-based expansion method can also not be understated. By using PM21 particles the need for cancerous feeder cells which were to be used to expand culture sizes originally is now gone. This is crucial as there is no longer a danger associated with introducing a cancerous cell into the body and risking the formation of a tumour. The PM21 particles can also be used as an in vivo treatment as PM21 maintains high numbers of NK cells for extended periods of time which ensures that patients have a low risk of metastasis during a potential treatment plan. In the experiment, it was shown that patients who had stimulation via PM21 particles had over 400 NK cells per microliter of blood, in contrast to the less than 20 that could previously be achieved after day 14. Finally, it was also found that by using PM21 particles to stimulate the growth of NK cells they appeared to become more receptive to the HLA-E protein and had increased cytotoxicity (Oyer et al., 2016).

Interleukin-2 (IL-2) is one of the most important methods for increasing the cytotoxicity of NK cells. It was found that by introducing recombinant interleukin-2 into a culture of NK cells, the activity of NK cells is enhanced (Trinchieri, 1984) which happened via gamma interferon (IFN Gamma), the production of which is stimulated by the increase in IL-2 level. The IFN-gamma then is what stimulates and regulates the NK cell cytotoxicity (Weigent et al., 1983). IFN-Gamma is produced by NK cells and results in a cell signalling cascade (Schroder, 2003) which finally leads to the increase in cytotoxicity. IL-2 treatment is an FDA approved treatment for cancer, specifically for adults with metastatic melanoma or metastatic kidney cancer (Stanford Health Library, 2017) and is commonly known as one of the most effective cancer treatments. The effect of IL-2 on the cytotoxicity of NK cells increases if the patient has melanoma cancer, therefore likely indicating a correlation between the enhanced effects of IL-2 on NK cell cytotoxicity and the effectiveness of Il-2 therapy in melanoma cancer. Unfortunately, there are many side effects to taking IL-2 treatment, such as diarrhoea, tachycardia and generalised aches and pains but also has the potential to cause respiratory congestion and in extreme cases capillary leak syndrome[7] (Chemocare, 2017). This results in many people not taking the full course of IL-2 treatment due to the side effects that come with the treatment.

To calculate the concentration and volume that would be required to stimulate the maximum response by NK cells, the following must be done to analyse the data from Weigent et al. (1983). The equation shown below must be used, where C is the concentration of the product, A is the number of molecules that react per unit volume of the solution, m is the mass of the solute used and v is the volume of solvent used. By rearranging and substituting in the 60 Units/ml, 10^5 Units per gramme given by the experiment, the ratio of NK cell volume to IL-2 volume, which provides the maximum response by NK cells, can be calculated:

$$C = A \times \frac{m}{v}$$

$$\frac{C}{A} = \frac{m}{v}$$

$$\frac{60}{10^5} = 0.0006 g ml^{-1} (0.6 mg ml^{-1})$$

[7] Capillary leak syndrome is when blood exits the capillaries into the body fluid.

The dose of IL-2 provided in NK cell-based immunotherapy would therefore not be over the FDA approved standard dose given in current IL-2 therapy. The FDA approved standard is 0.037 mg kg^{-1}, so an average person of 81 kg would take a dose of 3 mg during one session. 5ml of NK cells would be required to deliver the same dose of IL-2 as a single session of IL-2 therapy. In an ideal world, assuming an effector-target ratio of 1:1[8], a $2cm^3$ large (approximately 7.8mm diameter) volume of the tumour is attacked by NK cells per session. In NK cell therapy the treatment will be more flexible, as the PM21 particles and NK cell supplements can be taken without the IL-2 if the side effects are too great. Due to the increased cytotoxicity and increased NK cell count due to the PM21 particles, IL-2 will not be essential. The use of IL-2 would also be used only to provide an initial increase in cytotoxicity while the PM21 particles are stimulating in vivo expansion of cells with naturally higher cytotoxicity. To improve the potency further, Weigent provides evidence which shows that the cytotoxicity of the NK cell more than triples over an 18 hour time period after taking IL-2. Therefore the timeframe between each dose will be approximately 9 hours to maintain maximum effectiveness, which is the same interval as with current IL-2 treatment.

There is also an issue with trying to purify a sample of NK cells. Currently, the cost of the purification equipment ranges between £500 - £1000 to produce a 90% pure sample that would have the potential to destroy a 1cm tumour. This is a cheaper alternative to chemotherapy which costs approximately £3,100 and so can contend with current popular therapies, especially in countries where patients have to pay for their own treatment and medication.

Potential Clinical Trial

A clinical trial can be used to test the potential of an NK cell-based immunotherapy for cancer in comparison to current methods for cancer treatment such as chemotherapy.

The NK cell supplement should be created via initial ex vivo expansion by PM21 particles after the purification step has taken place to ensure that the highest percentage of NK cells in the solution is achieved. The volume of NK cell solution which should be used will be based on the earlier discussed effector-target ratio of 1:1. With this assumption, 5 ml of NK cells will be given per dose and the number of doses given will be dependent on the size of the tumour, assuming that the tumour will shrink by approximately $2cm^3$ each time. Then 3 mg of IL-2 will be added as calculated above to increase the cytotoxicity of the NK cells and to maximise the speed and intensity of the treatment. The entire process will be supplemented by 1.6ml of PM21 particles as suggested by Oyer who used 600 micrograms per millilitre of NK cells. The mixture would then be given to patients every 9 hours to maintain the highest possible levels of cytotoxicity. By not drawing out the treatment, the risk of potentially causing immune-escape (which is the adaptation of cancer as was discussed earlier) is minimised. Although this process of adaptation does generally take a long time, there is still a risk of it occurring and therefore for the safety of the patients, this risk should be minimised. The results can then be used to better identify if a large pure sample of NK cells, stimulated by IL-2 and PM21 would provide a substantial increase in effectiveness when compared to the current IL-2 therapy and chemotherapy.

[8] This is usually accepted as the standard effector-target ratio when discussing NK and tumour cells.

The ideal patients to test the new therapy would be those who have cancers which express a lack of the MHC class I expression and an overexpression of the stress ligands receptive to the CD94: NKG2D receptor (Jacobs et al., 2012). The patients should also be young enough to withstand the potential discomfort and strain that a treatment similar to IL-2 could put on the body. This would be the ideal scenario to begin testing a potential NK cell therapy as it would present the best-case scenario for tumour attack by the NK cells. The patients physical and mental condition will be monitored each day to maximise patient care and ensure that the PM21 did drastically increase NK cell concentration and has no adverse effects on the body. The primary things that will be checked are the NK cell count and the change in tumour size; this is to assess the effectiveness of the treatment. These results could then be plotted on a graph to show if there is a statistically significant correlation between the two.

The drug will be delivered directly into the bloodstream intravenously. This is to ensure that the NK cells would spend the maximum time possible within the patient's circulatory system and to reduce the number of cells that die during the delivery. Intravenous delivery is also a less invasive procedure than similar methods that ensure that the majority of NK cells are kept alive during delivery. The patients will then be compared to both an IL-2 and chemotherapy sample of similar patients, with the same form of cancer at a similar stage. This is to compare the effectiveness of each treatment and therefore identify if NK cell-based immunotherapy is a better alternative to current methods.

The program for drug administration should preferably be as follows. In the week leading up to the NK cell immunotherapy program, the patient should take 1.6ml of PM21 and 5ml of NK cells to begin the expansion of NK cells intravenously as it usually takes one week for there to be a noticeable change in NK cell concentration. Then an initial dose of 8ml with the ratio of NK cell to IL-2 being 5:3 should be delivered to the patient, this would be supplemented by the continuation of 1.6ml of PM21 twice weekly as Oyer used in his experiments. The NK cell IL-2 combination will then be taken every 9 hours intravenously and should continue for either 5 days, or for the number of sessions that would theoretically supply a sufficient dose to remove the tumour plus two, i.e. the size of the tumour in cm^3 divided by 2 all plus 2. The extra two sessions are to ensure that even if not all the NK cells interacted with at least one cancerous cell or some die on delivery, the tumour will be killed. The reason the treatment is stopped after 5 days is for two reasons. The first being that the high dose of IL-2 will often result in patients not receiving the full treatment due to side effects. Because of this the duration of the treatment cannot be for too long as it may be discouraging for patients and result in them opting to stop mid-treatment. Secondly, it takes approximately 5 days for the number of NK cells to reach its maximum level, at which point the cancer will be attacked by the body without the need to increase the cytotoxicity further by using IL-2.

Conclusion

NK cells have a precise and lethal killing mechanism which therefore presents a potential method for an immunotherapy cancer treatment. This lethality is controlled by utilising the various activating and inhibitory receptors on the cell surface membrane. It works by using perforin to disrupt the plasma cell membrane and the endosomal sack to release the granzyme B inside the cell which initiates both the mitochondrial and the caspase apoptotic pathway.

The speed and precision of NK cells make them a prime candidate to act as a treatment for cancer. With recent breakthroughs in, in and ex vivo expansion of NK cells using PM21 particles, the opportunity is opened up to finally use clinically relevant quantities of NK cells to create a new immunotherapy for cancer. This is an exciting time for the development of cancer treatments and I believe that NK cells will be at the forefront of new and innovative cancer therapies in the future.

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