Engineering designer dendritic cells to activate natural killer cells

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Referat

In der Immuntherapie gibt es zahlreiche vielversprechende Ansätze im Kampf gegen Krebs, wie z.B. die therapeutische Impfung gegen Krebs mittels dendritischer Zellen (DCs). Dabei werden DCs aus Patientenblut generiert und üblicherweise mit Hilfe eines standardisierten Zytokincocktails, bestehend aus Tumornekrosefaktor (TNF) α, Prostaglandin E2 (PGE_2), Interleukin (IL)-1β und IL-6, gereift. Anschließend werden die aktivierten DCs mit einem tumorspezifischen Antigen beladen, um schließlich wieder dem Patienten verimpft zu werden. Das primäre Ziel dahinter ist die antigenspezifische Aktivierung von zytotoxischen T-Zellen (CTLs), welche die Tumorzellen angreifen und töten können. Um die Antitumorwirkung noch weiter zu unterstützen, ist die Aktivierung des angeborenen Immunsystems, wie der natürlichen Killerzellen (NK-Zellen), von großem Nutzen. NK-Zellen sind nicht nur in der Lage, die Stimulation weiterer T-Lymphozyten zu induzieren, sondern können Tumorzellen auch direkt zerstören. Jedoch können die üblicherweise verwendeten DCs die für die NK-Zellaktivierung wegweisenden Zytokine IL-12 und IL-15 nicht sezernieren. Eine zentrale Rolle in Verbesserungsansätzen für die therapeutische Impfung gegen Krebs spielt die Aktivierung des nuclear factor kappa-light-chain-enhancer of activated B-cells-(NF-κB)-Signalweges der DCs. Dieser kann durch Elektroporation der DCs mit messenger (m)RNA getriggert werden, die eine konstitutiv aktive inhibitor of nuclear factor kappa-B kinase subunit beta (caIKKβ) kodiert. Es wurde bereits gezeigt, dass caIKKβ-DCs dadurch die Fähigkeit bekamen, IL-12 zu produzieren und T-Zellen deutlich effektiver aktivieren konnten. In dieser Arbeit wurde gezeigt, dass caIKKβ-DCs auch NK-Zellen effizient aktivieren können. Dies wurde durch eine erhöhte Expression von CD54, CD69 und CD25 auf NK-Zellen, deren Fähigkeit IFNγ zu produzieren sowie hoher lytischer Aktivität nachgewiesen. Da caIKKβ-DCs jedoch weiterhin kein IL-15 sezernieren konnten, wurde, um die Aktivierung der NK-Zellen weiter zu optimieren, neben der caIKKβ-RNA ein chimäres IL-15/IL-15Rα-Konstrukt in die DCs transfiziert. Dadurch konnten NK-Zellen mit einem noch deutlich erhöhten Aktivitätsniveau generiert werden. Die Fähigkeit von caIKKβ-DCs, sowohl die adaptive als auch die angeborene Immunantwort zu aktivieren, zeigt ein hohes Potenzial für die klinische Wirksamkeit von Impfungen mit DCs.

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Abstract

Immunotherapy offers several encouraging methods in the fight against cancer, including therapeutic vaccination using dendritic cells (DCs). DCs for vaccination are generated from a patient's blood and typically matured using a standard cytokine cocktail consisting of tumor necrosis factor (TNF) α, prostaglandin E2 (PGE2), interleukin (IL)-1β, and IL-6. The matured DCs are loaded with a tumor-specific antigen and are reinjected into the patient. The primary goal is the antigen-specific activation of cytotoxic T cells (CTLs), which in turn attack and kill tumor cells. To further support the antitumor effect, the activation of the innate immune system, such as natural killer (NK) cells, is of great benefit. NK cells are not only able to induce the stimulation of additional CTLs but can also directly attack tumor cells. However, DCs matured with the standard protocol cannot secrete IL-12 and IL-15, which are key cytokines for NK cell activation. The activation of the nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) signaling pathway of DCs plays a central role in improving therapeutic vaccination against cancer. The NF-κB signaling pathway can be activated by electroporation of DCs with messenger (m)RNA encoding a constitutively active inhibitor of nuclear factor kappa-B kinase subunit beta (caIKKβ). It has already been shown that caIKKβ-DCs were able to produce IL-12 and significantly activate T cells. In this study, it was shown that caIKKβ-DCs can also efficiently activate NK cells. This was demonstrated by an increased expression of CD54, CD69, and CD25 on NK cells, their ability to produce IFNγ, and their high lytic activity against target cells. However, since caIKKβ-DCs still could not secrete IL-15, a chimeric IL-15/IL-15Rα construct was transfected into caIKKβ-DCs to further optimize NK cell activation. As a result, NK cells with even higher levels of activity were generated. The ability of caIKKβ-DCs to activate both the adaptive and innate immune response demonstrates a high potential for the clinical effectiveness of DC vaccination.

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1 Introduction and aim

Cancer is still one of the main causes of death in Europe [1]. Especially in cases where the tumour has metastasised, a systemic therapy is necessary to eradicate the tumour cells. For most cancer entities, chemotherapy or radiation is the standard treatment option when tumour cells have metastasised. This, however, does not always lead to satisfactory results. Another approach to a systemic attack on tumour cells is immunotherapy. Initially, the immune system can detect and put up a fight against malignant cells, however, over time tumour cells develop different strategies in order to evade or silence immune responses [2]. The aim of immunotherapy is to make the immune system aware of the malignant tissue so that the immune cells can find and destroy the tumour [3]. Different approaches have been developed to use immune cells against the malignant cells, such as immune checkpoint inhibition and chimeric antigen receptor (CAR)-T cell therapy [4]. Another prominent approach is vaccination with dendritic cells (DCs), which is subject of this dissertation.

1.1 Dendritic cells and the immune system

As antigen presenting cells (APCs), DCs surveil the body to detect pathogenic agents and present them to naïve T cells [5]. DCs can be activated by several danger signals, such as inflammatory cytokines, e.g. tumour necrosis factor alpha (TNFα), identification of pathogen-associated molecular patterns (PAMPS) or lipopolysaccharides (LPS), by toll-like receptors (TLRs), and by sensors of cell death [6,7]. Upon triggering, usually involving the nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) pathway, the DCs are matured and shift into an activated state [8]. This leads to the enhanced processing of antigens; the secretion of pro-inflammatory cytokines, such as interleukin (IL-) 6, IL-8, IL-12, TNFα, and the chemokine CXCL8; the upregulation of MHC-molecules and the upregulation of further co-stimulatory-molecules, such as cluster of differentiation (CD) 80 and the chemokine receptor type 7 (CCR7) [7,9]. Once the DC has recognised a pathogen, they take up the pathogenic agents [10]. The antigens are then processed and loaded onto major histocompatibility complex (MHC) molecules to be presented to effector cells. Not only extracellular antigens are recognised by DCs, but also antigens, e.g. virus-particles, which have infiltrated the DC's cytosol directly,

are presented [11]. Whilst the uptake of extracellular antigens primarily leads to the presentation on MHC-class-II-molecules, antigens detected in the cytosol are transferred onto MHC-class-I-molecules [11]. Meanwhile the DCs migrate towards lymphatic tissues in order to present the antigens to effector cells. The upregulation of CCR7 plays a central role in this process as it makes the DCs more sensitive to the chemokine ligand 21 (CCL21) and 19 (CCL19), which lead the DCs through the lymphatic system into secondary lymphatic tissues [9,12]. Once the DCs have migrated towards lymphatic tissues they can finally activate antigen specific $CD4^+$ T-helper cells (Th cells), which have immune regulatory effects, *via* MHC-class-II presentation, or through MHC-class-I presentation, they activate cytotoxic $CD8^+$ T cells (CTLs) to directly kill target cells [6,11]. Through upregulation of co-stimulatory molecules and especially through secretion of pro-inflammatory cytokines, the reaction of the adaptive immune response is enhanced, but in addition to this the innate immune system is stimulated and ready to help eradicate the intruder, as NK cells are especially triggered through IL-12 secretion [11,13]. Regarding the activation of NK cells by DCs, the secretion of various pro-inflammatory cytokines plays an essential role [14]. IL-12 is the most relevant cytokine for NK-cell activation, but IL-15 is also a pivotal cytokine for NK-cell proliferation [14,15]. It has been shown that the secretion of IL-12 is essential for the induction of IFNγ production by NK cells, whereas studies have suggested IL-15 triggers NK-cell proliferation [15–17]. Further cytokines, such as e.g. IL-18, which has a synergizing effect with IL-12 to induce IFNγ secretion and also leads to enhanced NK cell cytotoxicity, or type I interferons have been shown to contribute in NK-cell and DC crosstalk [13,14]. However, direct cell-cell contacts between DCs and NK cells seem to be essential for optimal NK-cell activation, especially for communication *via* IL-12 [17].

1.2 Immune escape by cancer cells

Cancer cells can develop mechanisms in order to evade the immune responses [3]. The stages of immune escape by cancer cells can be described as the "three E's of cancer immunoediting": Elimination, Equilibrium, and Escape [2] (Figure 1).

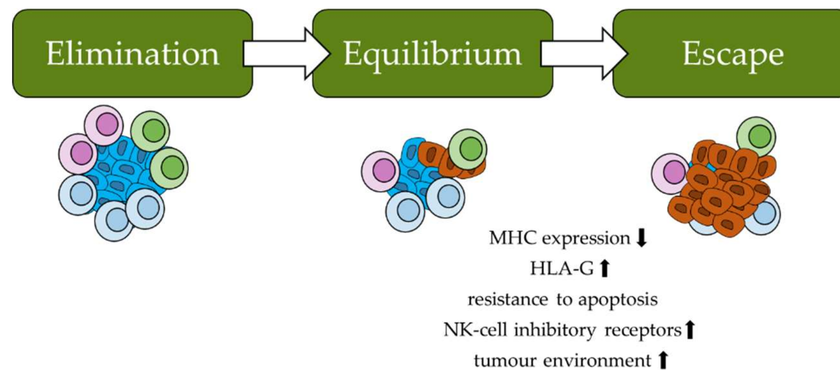

Figure 1: The three E's of cancer immunoediting"
Elimination: Different immune cells (exemplified as green, light blue and purple cells) recognize and eliminate transformed cells (blue cells).
Equilibrium: Some selective tumour cells transform and acquire tactics to escape recognition by the immune system (brown cells)
Escape: The more of these evasive mechanisms the tumour cells acquire, the more difficult it becomes for the immune system to detect tumour cells, leading to a complete tumour escape.
Adapted and modified from Dunn, Lloyd and Schreiber (2004)

Initially, the immune system recognizes transformed cells, for instance through neoantigen presentation and can therefore eliminate these oncogenic cells [2,3,18]. As a result of genomic instability some selective tumour cells transform and acquire tactics to escape recognition by the immune system, e.g. by expressing self-antigens, to which T cells have been tolerized; by downregulation or loss of MHC-class-I-antigen expression; the upregulation of the non-classical class-I HLA-G antigens, which prevent NK-cell and T-cell responses; resistance to apoptosis; expression of NK-cell inhibitory receptors, or a tumour environment that inhibits T-cell infiltration [2,3,18] (Figure 1). The more of these evasive mechanisms the tumour cells acquire, the more difficult it becomes for the immune system to keep up the fight against the tumour, eventually leading to a complete tumour escape [2,18] (Figure 1). Therefore, the re-activation of the immune response is a very promising approach to fighting cancer cells.

1.3 Dendritic cells for vaccination

As DCs build the bridge between the adaptive and innate immune response, they are ideal candidates for vaccination against tumour cells. DC-based vaccines are tested on a wide variety of tumours, melanoma being an ideal candidate as its immunogenicity is well investigated, but also leukaemia, prostate cancer, renal cell carcinoma, and several others are being investigated in clinical trials [19,20]. For DC vaccination, the standard protocol generates monocyte-derived DCs (moDCs), a subset of DCs which differentiate from monocyte progenitors during inflammatory reactions [21] (Figure 2a). To generate moDCs for vaccination, monocytes are first isolated from the patient's blood, and are then differentiated into immature DCs (iDCs) through incubation with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 over a period of up to 8 days [20,22,23] (Figure 2b). For DC maturation, a standard cytokine-cocktail, consisting of TNFα, prostaglandin E2 (PGE_2), IL-1β, and IL-6 is usually used [24] (Figure 2c). The matured DCs (mDCs) are then loaded with tumour-specific antigens to be then reinjected into the patient [25] (Figure 2e,f). An elegant way to load the DCs with antigens is the transfection of messenger RNA (mRNA) encoding tumour associated antigens (TAAs), which leads to an MHC-class-I restricted presentation [26]. The tumour antigens used for presentation can be directly taken from biopsies or tissue taken from surgery on the patient's tumour [19] (Figure 2d). The mRNA can then be isolated and amplified, or commonly known tumour antigens can be used [19]. An easy method to transfer the mRNA molecules into the DCs is electroporation [27]. With this technique, small pores in the cell membrane temporarily open as a result of an electrical pulse, which allows the mRNA to enter into the cytoplasm. The transfected mRNA can then be translated and the resulting protein can be processed in an MHC-class-I context [28] The matured and loaded DCs are then reinjected into the patient, either into the veins (i.v.), into the skin, either sub-cutaneous (s.c.), or intradermal (i.d.), or directly into the lymph nodes (i.n.) [29] (Figure 2f). The primary goal is to then activate CTLs (Figure 2g) to enable antigen-specific tumour killing [30] (Figure 2h).

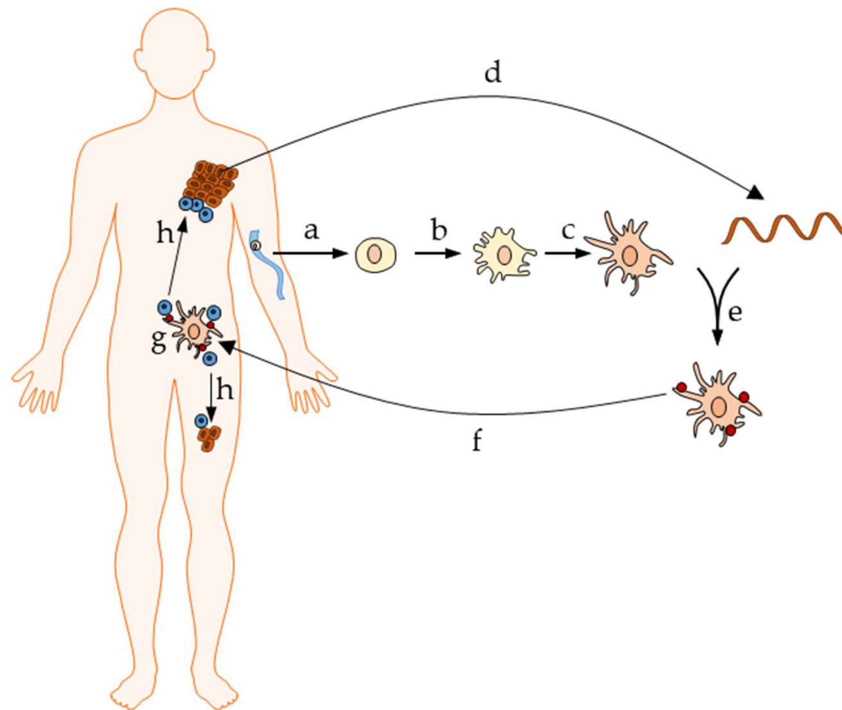

Figure 2: Example of generation of DCs for DC-based vaccination.
(a) The commonly used standard protocol for DC-based vaccination generates DCs out of monocytes. **(b)** The monocytes are then differentiated into immature DCs. **(c)** iDCs are matured with the standard cytokine-cocktail. **(d+e)** mDCs are loaded with mRNA encoding tumour-specific antigens. **(f)** The matured and loaded DCs are then reinjected into the patient. **(g)** The DCs should then present the tumour-specific epitopes to CTLs, which, once activated, can then **(h)** attack the tumour. For details see text above. (The Motifolio Scientific Illustration Toolkit was used.)
Adapted and modified from Dörrie *et al.* (2020).

However, even though an immune response can be induced with this method, the clinical responses do not meet expectations, and different strategies for improving this protocol are under investigation. Most approaches focus on increasing the immunogenicity of the DCs, in order to generate an enhanced and more long-lasting CTL response [26]. Unfortunately, the ability of DCs matured with the standard cytokine cocktail to additionally activate the innate immune system i.e. NK cells is limited [26,31]. The main disadvantage of the standard protocol is the limited secretion of IL-12 by cytokine-cocktail-matured DCs (cmDCs) [32], a cytokine which is most relevant for both the activation of CTLs [33] and that of NK cells [13].

The NF-κB pathway plays a central role in the activation of DCs, and its enhanced activation forms the focus of many methods for improving DC vaccination [32,34–39].

1.3.1 The role of the NF-κB pathway

NF-κB is a complex transcription factor which coordinates the immune response in almost all cell types [40], and is therefore essential for the maturation of DCs [41,42]. It can be activated by various danger signals, such as by pro-inflammatory cytokines, activation of TLRs, or simply stress-signals, and eventually leads to the upregulation of co-stimulatory molecules and the secretion of pro-inflammatory cytokines, such as IL-12 [40,43]. NF-κB mostly occurs as a heterodimer consisting of different proteins such as p50 bound with RelA or cRel [40,44] (Figure 3). In inactive cells, NF-κB is bound by IκB, which inhibits NF-κB from translocating into the nucleus [44] (Figure 3). NF-κB can be regulated by two pathways: the NF-κB essential modulator (NEMO) (the canonical pathway) and the NEMO-independent pathway (the non-canonical pathway) [40]. In the canonical pathway, NEMO is complexed with two IκB-kinases, IKKα and IKKβ [40,43] (Figure 3). Due to various inflammatory stimuli, the IκB-kinase is triggered and phosphorylates IκB which is bound to NF-κB. IκB is then ubiquitinated and degraded within the proteasome [45] (Figure 3). NF-κB is now free to translocate into the nucleus and binds to the DNA which activates certain target genes [44] (Figure 3).

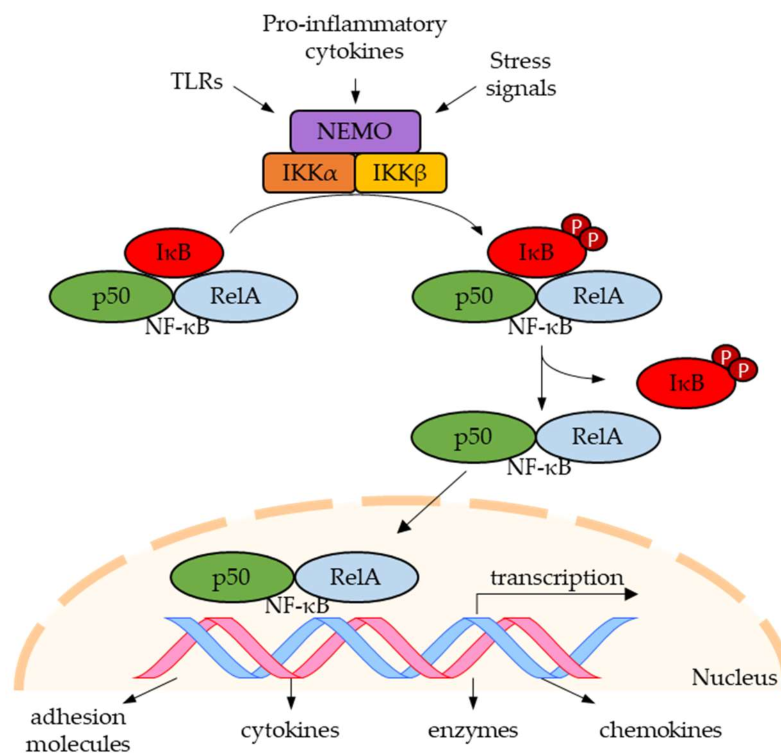

Figure 3: The canonical pathway.
Activation of the NF-κB pathway through activation of NEMO, as described in detail in the text above. (The Motifolio Scientific Illustration Toolkit was used.) Adapted and modified from Bonizzi *et al.* (2004) and Strnad and Burke (2007).

It must be pointed out that when DCs are matured with the standard cytokine cocktail, the NF-κB pathway is activated [42], but due to the lack of alarm signals, the activation does not achieve its full potential. By using different stimuli to enhance the activation of NF-κB, for instance by using different TLR agonists, an enhanced activation of DCs could be demonstrated [34–36,38]. Likewise, the usage of IFNγ together with monophosphoryl lipid A (MPLA), which triggers TLRs, as an alternative maturation cocktail, as tested by Massa *et al.*, led to the creation DCs which were able to secrete IL-12 [32].

The RNA-based Immunotherapy research group at the Department of Dermatology at the Universitätsklinikum Erlangen, Germany, has established a method to enhance the activity of dendritic cells through transfection of a constitutively active stabilized IKKβ (caIKKβ) mutant into DCs to activate the NF-κB pathway [37].

In order to explicitly activate the NF-κB pathway, a stabilised mutant of IKKβ was designed by exchanging the amino acids serines with glutamic acids in the active centres, which mimics phosphorylation [37]. To enable a prolonged expression of this mutant in order to use it for mRNA electroporation, all degradation-related serines and threonines were exchanged with alanines in the putative destabilising PEST sequences of the proteins [37]. Pfeiffer and co-workers could demonstrate that through transfection of caIKKβ in cmDCs, several activation markers, such as CD70, CD86, CD83, and others, were significantly upregulated and that the DCs gained the ability to secrete inflammatory cytokines, such as IL-12, IL-8, IL-6, and TNFα [37]. The ability of DCs to migrate towards lymphatic tissue is highly relevant, and the electroporation of caIKKβ-RNA did not significantly reduce the DCs' capability to migrate towards CCL19 [37]. Furthermore, Pfeiffer *et al.* could show that caIKKβ-transfected DCs were able to repetitively expand antigen-specific $CD8^+$ T cells with a better killer function [37].

1.3.2 Natural killer cells as effector cells

Several studies have shown that not only is an adaptive immune response essential for an anti-tumour effect, but that the innate immune system, especially NK cells, is also of utmost importance in the fight against the malignant tissue [9,46,47]. NK cells are characterised as $CD3^-/CD56^+$ lymphocytes [48]. A major advantage of the usage of NK cells for immunotherapy is that once activated, NK cells are immediately ready to do their job of killing target cells and do not need to undergo long activation procedures [48]. Two types of receptors are distinguished which regulate the activation of NK cells: the inhibitory receptors, which prevent NK cells from targeting healthy autologous cells, and activating receptors [49]. NK cells are only triggered when the activating signals exceed the inhibiting ones [49]. Through MHC-class-I specific inhibitory receptors, NK cells can be controlled so that they do not attack MHC-class-I expressing healthy cells [50] and can also detect tumour cells which have downregulated MHC expression [51]. Therefore tumour entities which have used MHC loss as a means of immune escape would be ideal targets for NK-cell-based immunotherapy [52].

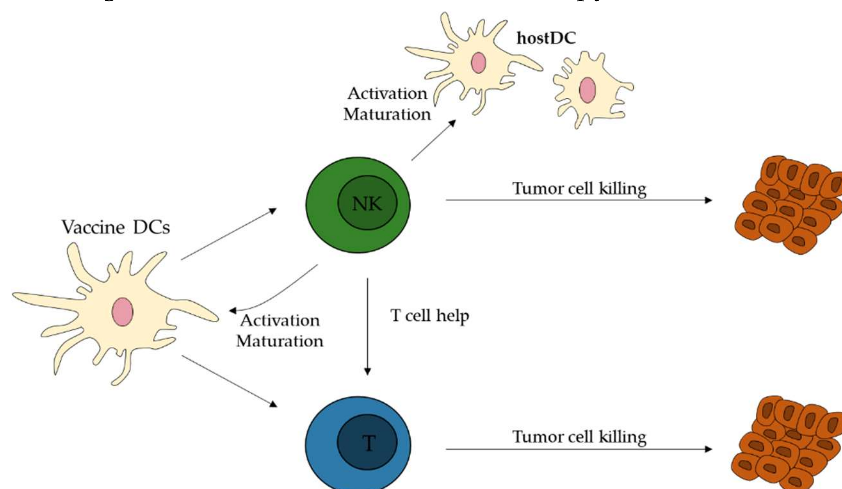

Figure 4: NK cells as effector cells
Activated NK cells can lyse target cells directly, promote the activation and maturation of further DCs, leading to an enhanced activation of CTLs. And through the secretion of IFNγ can stimulate CTLs directly.
Adapted and modified from Eva Lion *et al.* (2012).

Upon activation, NK cells secrete IFNγ and TNFα and are able to lyse target cells directly, e.g. by degranulation and death receptor ligation (Figure 4) [14,53,54]. Activated NK cells in turn promote the maturation of DCs [55], which then leads to a superior activation of CTLs without the help of Th cells [56] (Figure 4). Furthermore, NK cells can stimulate T-cell activation directly through the secretion of IFNγ [14,57] (Figure 4).

1.3.3 Interleukin 15 and the IL-15 receptor

As mentioned above, another prominent cytokine for NK-cell activation is IL-15. IL-15 plays a significant role in the development of the innate and adaptive immune response as it plays a role in the proliferation, development, homeostasis, and activation of CTLs and NK cells [58–61]. It is produced by several cell types, such as DCs, macrophages, and monocytes but also by fibroblasts and epithelial cells from numerous tissues [59]. IL-15 can either be secreted in its soluble form or is presented in complex with the IL-15 receptor α chain (IL-15Rα) [62] (Figure 5). Neighbouring cells, like T cells or NK cells, can detect IL-15 which is presented with the IL-15Rα *in trans* with their IL-2/IL-15Rβ and -γ chains [58,62] (Figure 5).

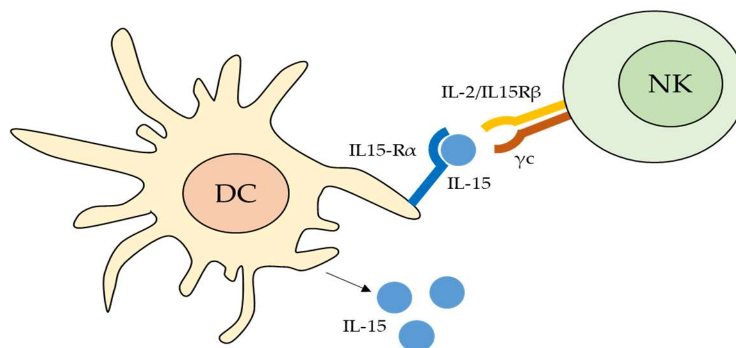

Figure 5: Presentation of IL-15.
IL-15 can either be secreted by DCs as soluble IL-15 or can be trans-presented with the IL-15Rα which can be recognized by NK cells with their IL-2/IL-15Rβ and -γ chains.
Adapted and modified from Ali *et al.* (2015) and Patidar *et al.* (2016).

Therefore, IL-15, next to IL-12, could strongly support the activation of NK cells. However, even though DCs transfected with caIKKβ were now able to secrete IL-12, the secretion of IL-15 was still not increased [37]. As simply applying soluble IL-15 for immunotherapy could lead to transient toxicity [63], the presentation of IL-15 in complex with IL-15Rα seems more promising. Several groups have shown that the presentation of IL-15 complexed with IL-15Rα through APCs could lead to a significant increase in NK-cell and CTL activation [64–66].

1.4 Aims of this dissertation

In view of the many advantageous features of NK cells, the activation of NK cells with DC vaccines is extremely beneficial. As described above, DCs matured with the standard cytokine cocktail are not able to secrete IL-12, and are therefore not able to activate NK cells sufficiently [31,32]. The activation of the NF-κB pathway through electroporation of caIKKβ-RNA gave these DCs the ability to secrete IL-12 and improved their capability to activate and expand CTLs [37]. As part of my research, tests were conducted to ascertain whether caIKKβ-transfected DCs were able to improve the activation of NK cells *via* measurement of the activation markers on NK cells and their secretion of proinflammatory cytokines. Furthermore, it was analysed if these DCs were able to activate NK cells sufficiently enough to enable a lytic capacity towards target cells. As the activation of NK cells and CTLs could be in competition with one another, further experiments were performed to determine if caIKKβ-transfected DCs could activate both cell types simultaneously.

As described above, adding IL-15 in complex with IL-15Rα could lead to an increased activation of NK cells *via* DC-vaccines. Hence, a specifically designed chimeric protein consisting of IL-15 and the IL-15Rα chain (chIL15) was used to be expressed on the DCs' surface *via* mRNA electroporation, in order to further improve the effectiveness of DC vaccines in addition to activating the NF-κB pathway. A chimeric protein was chosen, as using one complex instead of two proteins would simplify the DC transfection process for clinical application. By using a covalent link, a dissociation of the complex could be prevented [52]. To address whether the surface expression of the IL-15 complex could support NF-κB activated DCs' capability to activate NK cells, mRNA encoding caIKKβ was co-transfected with mRNA encoding chIL-15 into DCs which had been matured with the standard cytokine cocktail. The activation of NK cells and their ability to lyse target cells was then determined [52].

2 Discussion

2.1 Advantages of mRNA electroporation in dendritic cells

The use of mRNA as a vaccine against SARS-CoV-2 has emphasized the great potential of mRNA technology [67]. mRNA can be used for a variety of immunological therapeutic approaches, including the optimisation of immunotherapy against tumour cells [67]. mRNA is also well established in the generation of DC vaccines [19].

Transfection of mRNA encoding TAAs into DCs is an elegant way to establish an MHC-class-I restricted presentation, which is requisite for activating CTLs [26]. Several ways to transfect DCs with mRNA exist, e.g. simple co-incubation of mRNA with DCs, or mRNA electroporation [19]. Electroporation of mRNA is often used, as its transfection rates can reach up to 90% and as it requires no additional chemicals it is a safe method which can easily be performed under good manufacturing practice (GMP) conditions [19,26,68]. The major advantages of inserting mRNA *ex vivo* into DCs to then use these DCs as vaccines, in contrast to simply using mRNA as direct vaccines in order to achieve an activation of DCs *in vivo*, is the considerably better controllability, the possibility to mature the DCs before electroporation with mRNA by using different cytokine cocktails [24,32,35,38], and the possibility to modify the DCs even further, to enable an even better activation status and stimulation capacity towards effector cells. Since the use of mRNA electroporation for the loading of DCs with TAAs has already been tested for clinical use [26,68], further modification of DCs by electroporation with mRNA can easily be incorporated into the standard protocol [26]. Using caIKKβ-RNA is a simple and safe method to activate the NF-κB pathway and requires no major extra steps in the development of DC vaccines. The level of NF-κB activation can be optimally controlled by using caIKK-RNA electroporation, as the expression levels of the transfected proteins can be adjusted, and an over-activation of the NF-κB pathway can be prevented [68]. This also offers promising and convenient possibilities to take further steps to manipulate DCs, such as co-transfection with chIL15. However, the use of DCs produced with the standardised protocol is quite costly, labour intensive and time consuming [32]. To address this problem, several groups have focused on creating fast protocols for DC maturation [32,35,69].

2.2 Improving dendritic-cell vaccines

2.2.1 Activating the NF-κB pathway

The activation of the NF-κB pathway is an excellent way to improve DC-based vaccination, as the effect of DCs currently used for tumour vaccination as monotherapy is not satisfactory [26]. The major disadvantage of DCs which are matured with the standard cytokine cocktail is the lack of IL-12 secretion, a cytokine which is essential for the activation of both the adaptive and innate immune response [9,32]. It is important to optimize the current protocol not only to gain an enhanced CTL response, but also to achieve further immunostimulatory functions by activating the innate immune response [9,46,47]. As the NF-κB pathway is the key player for an optimal DC maturation, triggering this pathway is the focus of many approaches to improve DC-based vaccination [32,34–36,38]. The approach used by the RNA-based Immunotherapy research group at the Department of Dermatology in Erlangen for additional activation of the NF-κB pathway was to transfect moDCs, matured with the standard cytokine cocktail with caIKKβ [37]. As a result, the NF-κB pathway could be fully activated and the moDCs gained the ability to secrete IL-12 and shifted into an enhanced activation status [37]. Furthermore these DCs were able to activate CTLs with a superior lytic capacity and a memory-like phenotype [37]. As part of my research, I was now also able to demonstrate that the activation of the NF-κB pathway through transfection of caIKKβ was efficient enough to additionally stimulate NK cells with a high lytic capacity and the ability to secrete IFNγ as well as small quantities of TNFα [31]. As in theory the simultaneous activation of NK cells and CTLs by DCs could lead to competition, it was demonstrated that the stimulation of NK cells did not diminish the activation of tumour specific CTLs [31]. Moreover, it was illustrated that in this setting, direct interaction between DCs and NK cells was necessary and no bystander cells were needed for an optimal NK-cell activation [31]. In this context, an interesting side effect could be seen: although NK cells had to have initial cell-cell contact with the DCs in order to be activated, we observed that NK cells that had no contact with DCs were now also slightly activated [31]. This could indicate a positive feedback mechanism induced by activated NK cells, probably due to the secretion of IFNγ together with other pro-inflammatory cytokines, as reviewed by Boehm *et al* [70]. In line with these results, other groups have

used different strategies to improve DC vaccination, all leading to the activation of the NF-κB pathway [32,35,38]. Many used altered maturation cocktails in their improved protocol which contained different TLR agonists, e.g. R-848, a TLR-7/8 agonist [35]; MLPA, a TLR-4 agonist [32], or poly-I:C, a TLR-3 agonist [38]. Anguille *et al.* generated so-called IL-15 DCs by first replacing IL-4 with IL-15 during DC differentiation and then using a maturation cocktail consisting of TNFα, IFNγ, PGE_2, and R-848 [35]. αDC1s, created by Maillad *et al.*, were generated by using a maturation mixture consisting of IFNα, IFNγ, TNFα, IL-1β, and poly-I:C [38], whereas Massa *et al.* used a maturation cocktail containing IFNγ and MLPA [32]. DCs matured with all these alternative maturation cocktails were, as with the usage of caIKKβ-DCs, able to effectively activate NK cells [31,32,35,38]. As mentioned above, the secretion of IL-12 by DCs is one of the most favourable features and αDC1s and DCs matured with MPLA and IFNγ, as well as caIKKβ-DCs were able to secrete IL-12, whereas IL-15 DCs were only able to secrete IL-12 when co-cultured with further CD40L-transfected mouse fibroblasts, which replace the DCs' interaction with Th cells and activate NF-κB. [31,32,35,38].

2.2.2 Improved activation of NK cells

Through the additional activation of the NF-κB pathway by electroporation of caIKKβ-RNA the immunogenicity of DCs could be enhanced, enabling them to activate NK cells as well as achieving an improved CTL response [31,37]. The activation of NK cells is highly beneficial, as they can support the anti-tumour effect by further activating DCs, increasing CTL activation and also directly attacking and killing tumour cells [55,56,71]. However, the activation of NK cells should not be considered as only playing a supporting role in an already established CTL response. The activation of NK cells is especially relevant for an anti-tumour response against tumour cells which have used MHC loss as a means of immune escape, as NK cells can recognize tumour cells with downregulated MHC expression, in contrast to CTLs, to whom the tumour cells would be invisible [51]. Therefore, an optimized activation of NK cells for tumour entities, such as cervical cancer, colorectal cancer, gastric cancer, and oesophageal squamous cell carcinoma, which regularly display loss of MHC-class-I, would be of great importance for an optimized anti-tumour response [72].

In order to improve NK-cell activation further, the focus was now on IL-15. As mentioned above, IL-15, next to IL-12, is a cytokine which supports NK-cell activation by DCs [14]. The use of NF-κB-activated DCs by electroporation of caIKKβ-RNA, however, only led to the secretion of IL-12, and the secretion of IL-15 was only very limited [37]. As it had been demonstrated that the presentation of IL-15 *in trans* leads to optimal NK-cell activation [15,64–66], we created a chimeric IL-15/IL-15Rα molecule to be transfected into DCs. Electroporation of caIKKβ-RNA together with chIL15-RNA led to an improved activation status of NK cells, an enhanced secretion of IFNγ, and higher lytic capacity towards the MHC-negative cell line K562 [52]. DCs which were transfected with chIL15 alone were not able to effectively increase NK-cell activation [52]. It seems that in this setting, IL-15 alone was not sufficient to activate NK cells without the additional activation of the NF-κB pathway [52]. The fusion of IL-15 to the IL-15Rα, in contrast to simply using the mRNA of each factor separately, has many advantages: the production and transfection of one RNA vs. two separate ones is easier, cheaper and bears less variability. As IL-15 shows high transient toxicity when applied systematically [63], the fusion of IL-15 to the IL-15Rα, which was achieved by a covalent link, could prevent IL-15 from causing systemic effects [52].

2.3 Conclusions and outlook

Taken together, electroporation of caIKKβ-RNA is an easy and safe method to activate CTLs and NK cells with high lytic capacities, which is pivotal for tumour elimination. Generating DCs, which can enhance the activation of NK cells, provides a possibility to further individualize cancer immunotherapy, especially for tumour entities with downregulated MHC expression, in order to specifically respond to tumour immune evasion mechanisms [52]. A clinical phase I trial has begun at the Department of Dermatology at the Universitätsklinikum Erlangen to test the efficacy of caIKKβ-transfected DCs loaded with tumour-RNA against metastatic uveal melanoma usually together with a standard immune checkpoint blockade therapy (NCT04335890). This study measures safety, tolerability and toxicity as primary outcome [73]. Regarding toxicity, local reactions at the DC injection sites, flu-like symptoms and fatigue have been reported [19]. Cytokine release syndrome (CRS), which is cause by T-cell activation und the resulting increased secretion of pro-inflammatory cytokines, and severe autoimmune side effects are commonly known effects of immunotherapy, nevertheless the overall safety profile of DC vaccination is very good compared to other immunotherapeutic approaches, as reviewed by Dörrie *et al* [19].

The additional electroporation of chIL15 seems to be a promising method to further improve caIKKβ-transfected DCs to increase NK-cell activation. However, the studies with chIL15 are still at an early stage and further experiments focusing on the full effect of chIL15 need to be carried out before its usage in clinical trials.

3 References

1. Eurostat. Causes of death statistics; 2021. https://ec.europa.eu/eurostat/statistics-explained/index.php?title=Causes_of_death_statistics#Main_findings [accessed September 29, 2021].
2. Dunn GP, Old LJ, Schreiber RD. 2004. The three Es of cancer immunoediting. Annu Rev Immunol. 22. 22. p. 329–60. DOI: 10.1146/annurev.immunol.22.012703.104803.
3. Seliger B. 2005. Strategies of tumor immune evasion. BioDrugs. 19. 196. p. 347–54. DOI: 10.2165/00063030-200519060-00002.
4. van den Bulk J, Verdegaal EM, Miranda NF de. 2018. Cancer immunotherapy: broadening the scope of targetable tumours. Open Biol. 8. 86. DOI: 10.1098/rsob.180037.
5. Steinman RM. 1991. The dendritic cell system and its role in immunogenicity. Annu Rev Immunol. 9. 9. p. 271–96. DOI: 10.1146/annurev.iy.09.040191.001415.
6. Guermonprez P, Valladeau J, Zitvogel L, Théry C, Amigorena S. 2002. Antigen presentation and T cell stimulation by dendritic cells. Annu Rev Immunol. 20. 20. p. 621–67. DOI: 10.1146/annurev.immunol.20.100301.064828.
7. Clark GJ, Angel N, Kato M, et al. 2000. The role of dendritic cells in the innate immune system. Microbes and Infection. 2. 23. p. 257–72. DOI: 10.1016/S1286-4579(00)00302-6.
8. Rescigno M, Martino M, Sutherland CL, Gold MR, Ricciardi-Castagnoli P. 1998. Dendritic cell survival and maturation are regulated by different signaling pathways. J Exp Med. 188. 18811. p. 2175–80. DOI: 10.1084/jem.188.11.2175.
9. Moretta A. 2002. Natural killer cells and dendritic cells: rendezvous in abused tissues. Nat Rev Immunol. 2. 212. p. 957–64. DOI: 10.1038/nri956.
10. Reis e Sousa C. 2001. Dendritic Cells as Sensors of Infection. Immunity. 14. 145. p. 495–8. DOI: 10.1016/S1074-7613(01)00136-4.
11. Banchereau J, Steinman RM. 1998. Dendritic cells and the control of immunity. Nature. 392. 3926673. p. 245–52. DOI: 10.1038/32588.
12. Scandella E, Men Y, Gillessen S, Förster R, Groettrup M. 2002. Prostaglandin E2 is a key factor for CCR7 surface expression and migration of monocyte-derived dendritic cells. Blood. 100. 1004. p. 1354–61. DOI: 10.1182/blood-2001-11-0017.
13. Yu Y, Hagihara M, Ando K, et al. 2001. Enhancement of human cord blood CD34+ cell-derived NK cell cytotoxicity by dendritic cells. J Immunol. 166. 1663. p. 1590–600. DOI: 10.4049/jimmunol.166.3.1590.
14. Walzer T, Dalod M, Robbins SH, Zitvogel L, Vivier E. 2005. Natural-killer cells and dendritic cells: "l'union fait la force". Blood. 106. 1067. p. 2252–8. DOI: 10.1182/blood-2005-03-1154.
15. Ferlazzo G, Pack M, Thomas D, et al. 2004. Distinct roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs. Proc Natl Acad Sci U S A. 101. 10147. p. 16606–11. DOI: 10.1073/pnas.0407522101.

16. Andrews DM, Scalzo AA, Yokoyama WM, Smyth MJ, Degli-Esposti MA. 2003. Functional interactions between dendritic cells and NK cells during viral infection. Nat Immunol. 4. 42. p. 175–81. DOI: 10.1038/ni880.

17. Borg C, Jalil A, Laderach D, et al. 2004. NK cell activation by dendritic cells (DCs) requires the formation of a synapse leading to IL-12 polarization in DCs. Blood. 104. 10410. p. 3267–75. DOI: 10.1182/blood-2004-01-0380.

18. Prendergast GC. 2008. Immune escape as a fundamental trait of cancer: focus on IDO. Oncogene. 27. 2728. p. 3889–900. DOI: 10.1038/onc.2008.35.

19. Dörrie J, Schaft N, Schuler G, Schuler-Thurner B. 2020. Therapeutic Cancer Vaccination with Ex Vivo RNA-Transfected Dendritic Cells-An Update. Pharmaceutics. 12. 122. DOI: 10.3390/pharmaceutics12020092.

20. Palucka K, Banchereau J. 2012. Cancer immunotherapy via dendritic cells. Nat Rev Cancer. 12. 124. p. 265–77. DOI: 10.1038/nrc3258.

21. Lee C, Lee M, Rhee I. 2018. Distinct features of dendritic cell-based immunotherapy as cancer vaccines. Clin Exp Vaccine Res. 7. 71. p. 16–23. DOI: 10.7774/cevr.2018.7.1.16.

22. Bender A, Sapp M, Schuler G, Steinman RM, Bhardwaj N. 1996. Improved methods for the generation of dendritic cells from nonproliferating progenitors in human blood. Journal of Immunological Methods. 196. 1962. p. 121–35. DOI: 10.1016/0022-1759(96)00079-8.

23. Sallusto F, Lanzavecchia A. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J Exp Med. 179. 1794. p. 1109–18. DOI: 10.1084/jem.179.4.1109.

24. Jonuleit H, Kühn U, Müller G, et al. 1997. Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. Eur J Immunol. 27. 2712. p. 3135–42. DOI: 10.1002/eji.1830271209.

25. Constantino J, Gomes C, Falcão A, Neves BM, Cruz MT. 2017. Dendritic cell-based immunotherapy: a basic review and recent advances. Immunol Res. 65. 654. p. 798–810. DOI: 10.1007/s12026-017-8931-1.

26. Schuler G. 2010. Dendritic cells in cancer immunotherapy. Eur J Immunol. 40. 408. p. 2123–30. DOI: 10.1002/eji.201040630.

27. Gilboa E, Vieweg J. 2004. Cancer immunotherapy with mRNA-transfected dendritic cells. Immunol Rev. 199. 199. p. 251–63. DOI: 10.1111/j.0105-2896.2004.00139.x.

28. Gerer KF, Hoyer S, Dörrie J, Schaft N. 2017. Electroporation of mRNA as Universal Technology Platform to Transfect a Variety of Primary Cells with Antigens and Functional Proteins. Methods Mol Biol. 1499. 1499. p. 165–78. DOI: 10.1007/978-1-4939-6481-9_10.

29. Butterfield LH. 2013. Dendritic cells in cancer immunotherapy clinical trials: are we making progress? Front Immunol. 4. 4. p. 454. DOI: 10.3389/fimmu.2013.00454.

30. Palucka K, Banchereau J. 2013. Dendritic-cell-based therapeutic cancer vaccines. Immunity. 39. 391. p. 38–48. DOI: 10.1016/j.immuni.2013.07.004.
31. Bosch NC, Voll RE, Voskens CJ, et al. 2019. NF-κB activation triggers NK-cell stimulation by monocyte-derived dendritic cells. Ther Adv Med Oncol. 11. 11. p. 1758835919891622. DOI: 10.1177/1758835919891622.
32. Massa C, Seliger B. 2013. Fast dendritic cells stimulated with alternative maturation mixtures induce polyfunctional and long-lasting activation of innate and adaptive effector cells with tumor-killing capabilities. J Immunol. 190. 1907. p. 3328–37. DOI: 10.4049/jimmunol.1202024.
33. Colombo MP, Trinchieri G. 2002. Interleukin-12 in anti-tumor immunity and immunotherapy. Cytokine & Growth Factor Reviews. 13. 132. p. 155–68. DOI: 10.1016/s1359-6101(01)00032-6.
34. Boullart ACI, Aarntzen EHJG, Verdijk P, et al. 2008. Maturation of monocyte-derived dendritic cells with Toll-like receptor 3 and 7/8 ligands combined with prostaglandin E2 results in high interleukin-12 production and cell migration. Cancer Immunol Immunother. 57. 5711. p. 1589–97. DOI: 10.1007/s00262-008-0489-2.
35. Anguille S, Smits ELJM, Cools N, Goossens H, Berneman ZN, van Tendeloo VFI. 2009. Short-term cultured, interleukin-15 differentiated dendritic cells have potent immunostimulatory properties. J Transl Med. 7. 7. p. 109. DOI: 10.1186/1479-5876-7-109.
36. Bonehill A, Tuyaerts S, van Nuffel AMT, et al. 2008. Enhancing the T-cell stimulatory capacity of human dendritic cells by co-electroporation with CD40L, CD70 and constitutively active TLR4 encoding mRNA. Mol Ther. 16. 166. p. 1170–80. DOI: 10.1038/mt.2008.77.
37. Pfeiffer IA, Hoyer S, Gerer KF, et al. 2014. Triggering of NF-κB in cytokine-matured human DCs generates superior DCs for T-cell priming in cancer immunotherapy. Eur J Immunol. 44. 4411. p. 3413–28. DOI: 10.1002/eji.201344417.
38. Mailliard RB, Wankowicz-Kalinska A, Cai Q, et al. 2004. alpha-type-1 polarized dendritic cells: a novel immunization tool with optimized CTL-inducing activity. Cancer Res. 64. 6417. p. 5934–7. DOI: 10.1158/0008-5472.CAN-04-1261.
39. Calderhead DM, DeBenedette MA, Ketteringham H, et al. 2008. Cytokine maturation followed by CD40L mRNA electroporation results in a clinically relevant dendritic cell product capable of inducing a potent proinflammatory CTL response. J Immunother. 31. 318. p. 731–41. DOI: 10.1097/CJI.0b013e318183db02.
40. Mitchell S, Vargas J, Hoffmann A. 2016. Signaling via the NFκB system. Wiley Interdiscip Rev Syst Biol Med. 8. 83. p. 227–41. DOI: 10.1002/wsbm.1331.
41. Hernandez A, Burger M, Blomberg BB, et al. 2007. Inhibition of NF-kappa B during human dendritic cell differentiation generates anergy and regulatory T-cell activity for one but not two human leukocyte antigen DR mismatches. Hum Immunol. 68. 689. p. 715–29. DOI: 10.1016/j.humimm.2007.05.010.

42. Tas SW, Jong EC de, Hajji N, et al. 2005. Selective inhibition of NF-kappaB in dendritic cells by the NEMO-binding domain peptide blocks maturation and prevents T cell proliferation and polarization. Eur J Immunol. 35. 354. p. 1164–74. DOI: 10.1002/eji.200425956.

43. Strnad J, Burke JR. 2007. IkappaB kinase inhibitors for treating autoimmune and inflammatory disorders: potential and challenges. Trends Pharmacol Sci. 28. 283. p. 142–8. DOI: 10.1016/j.tips.2007.01.005.

44. Bonizzi G, Karin M. 2004. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. Trends Immunol. 25. 256. p. 280–8. DOI: 10.1016/j.it.2004.03.008.

45. Li ZW, Chu W, Hu Y, et al. 1999. The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. J Exp Med. 189. 18911. p. 1839–45. DOI: 10.1084/jem.189.11.1839.

46. Lion E, Smits ELJM, Berneman ZN, van Tendeloo VFI. 2012. NK cells: key to success of DC-based cancer vaccines? Oncologist. 17. 1710. p. 1256–70. DOI: 10.1634/theoncologist.2011-0122.

47. Minetto P, Guolo F, Pesce S, et al. 2019. Harnessing NK Cells for Cancer Treatment. Front Immunol. 10. 10. p. 2836. DOI: 10.3389/fimmu.2019.02836.

48. Morvan MG, Lanier LL. 2016. NK cells and cancer: you can teach innate cells new tricks. Nat Rev Cancer. 16. 161. p. 7–19. DOI: 10.1038/nrc.2015.5.

49. Lanier LL. 2008. Up on the tightrope: natural killer cell activation and inhibition. Nat Immunol. 9. 95. p. 495–502. DOI: 10.1038/ni1581.

50. Kumar S. 2018. Natural killer cell cytotoxicity and its regulation by inhibitory receptors. Immunology. 154. 1543. p. 383–93. DOI: 10.1111/imm.12921.

51. Cornel AM, Mimpen IL, Nierkens S. 2020. MHC Class I Downregulation in Cancer: Underlying Mechanisms and Potential Targets for Cancer Immunotherapy. Cancers (Basel). 12. 127. DOI: 10.3390/cancers12071760.

52. Bosch NC, Martin L-M, Voskens CJ, et al. 2021. A Chimeric IL-15/IL-15Rα Molecule Expressed on NFκB-Activated Dendritic Cells Supports Their Capability to Activate Natural Killer Cells. IJMS. 22. 2219. p. 10227. DOI: 10.3390/ijms221910227.

53. Abel AM, Yang C, Thakar MS, Malarkannan S. 2018. Natural Killer Cells: Development, Maturation, and Clinical Utilization. Front Immunol. 9. 9. p. 1869. DOI: 10.3389/fimmu.2018.01869.

54. Caligiuri MA. 2008. Human natural killer cells. Blood. 112. 1123. p. 461–9. DOI: 10.1182/blood-2007-09-077438.

55. Gerosa F, Baldani-Guerra B, Nisii C, Marchesini V, Carra G, Trinchieri G. 2002. Reciprocal activating interaction between natural killer cells and dendritic cells. J Exp Med. 195. 1953. p. 327–33. DOI: 10.1084/jem.20010938.

56. Adam C, King S, Allgeier T, et al. 2005. DC-NK cell cross talk as a novel CD4+ T-cell-independent pathway for antitumor CTL induction. Blood. 106. 1061. p. 338–44. DOI: 10.1182/blood-2004-09-3775.

57. Martín-Fontecha A, Thomsen LL, Brett S, et al. 2004. Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. Nat Immunol. 5. 512. p. 1260–5. DOI: 10.1038/ni1138.

58. Ali AK, Nandagopal N, Lee S-H. 2015. IL-15-PI3K-AKT-mTOR: A Critical Pathway in the Life Journey of Natural Killer Cells. Front Immunol. 6. 6. p. 355. DOI: 10.3389/fimmu.2015.00355.

59. Fehniger TA, Caligiuri MA. 2001. Interleukin 15: biology and relevance to human disease. Blood. 97. 971. p. 14–32. DOI: 10.1182/blood.v97.1.14.

60. Kennedy MK, Glaccum M, Brown SN, et al. 2000. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. J Exp Med. 191. 1915. p. 771–80. DOI: 10.1084/jem.191.5.771.

61. Zhang X, Sun S, Hwang I, Tough DF, Sprent J. 1998. Potent and Selective Stimulation of Memory-Phenotype CD8+ T Cells In Vivo by IL-15. Immunity. 8. 85. p. 591–9. DOI: 10.1016/s1074-7613(00)80564-6.

62. Patidar M, Yadav N, Dalai SK. 2016. Interleukin 15: A key cytokine for immunotherapy. Cytokine & Growth Factor Reviews. 31. 31. p. 49–59. DOI: 10.1016/j.cytogfr.2016.06.001.

63. Berger C, Berger M, Hackman RC, et al. 2009. Safety and immunologic effects of IL-15 administration in nonhuman primates. Blood. 114. 11412. p. 2417–26. DOI: 10.1182/blood-2008-12-189266.

64. Hasan AN, Selvakumar A, Shabrova E, et al. 2016. Soluble and membrane-bound interleukin (IL)-15 Rα/IL-15 complexes mediate proliferation of high-avidity central memory CD8+ T cells for adoptive immunotherapy of cancer and infections. Clin Exp Immunol. 186. 1862. p. 249–65. DOI: 10.1111/cei.12816.

65. Stoklasek TA, Schluns KS, Lefrançois L. 2006. Combined IL-15/IL-15Ralpha immunotherapy maximizes IL-15 activity in vivo. J Immunol. 177. 1779. p. 6072–80. DOI: 10.4049/jimmunol.177.9.6072.

66. van den Bergh J, Willemen Y, Lion E, et al. 2015. Transpresentation of interleukin-15 by IL-15/IL-15Rα mRNA-engineered human dendritic cells boosts antitumoral natural killer cell activity. Oncotarget. 6. 642. p. 44123–33. DOI: 10.18632/oncotarget.6536.

67. Beck JD, Reidenbach D, Salomon N, et al. 2021. mRNA therapeutics in cancer immunotherapy. Mol Cancer. 20. 201. p. 69. DOI: 10.1186/s12943-021-01348-0.

68. Schaft N, Dörrie J, Thumann P, et al. 2005. Generation of an optimized polyvalent monocyte-derived dendritic cell vaccine by transfecting defined RNAs after rather than before maturation. J Immunol. 174. 1745. p. 3087–97. DOI: 10.4049/jimmunol.174.5.3087.

69. Dauer M, Obermaier B, Herten J, et al. 2003. Mature dendritic cells derived from human monocytes within 48 hours: a novel strategy for dendritic cell differentiation from blood precursors. J Immunol. 170. 1708. p. 4069–76. DOI: 10.4049/jimmunol.170.8.4069.

70. Boehm U, Klamp T, Groot M, Howard JC. 1997. Cellular responses to interferon-gamma. Annu Rev Immunol. 15. 15. p. 749–95. DOI: 10.1146/annurev.immunol.15.1.749.

71. Mocikat R, Braumüller H, Gumy A, et al. 2003. Natural Killer Cells Activated by MHC Class ILow Targets Prime Dendritic Cells to Induce Protective CD8 T Cell Responses. Immunity. 19. 194. p. 561–9. DOI: 10.1016/s1074-7613(03)00264-4.

72. Hazini A, Fisher K, Seymour L. 2021. Deregulation of HLA-I in cancer and its central importance for immunotherapy. J Immunother Cancer. 9. 98. DOI: 10.1136/jitc-2021-002899.

73. Koch EAT, Schaft N, Kummer M, et al. 2022. A One-Armed Phase I Dose Escalation Trial Design: Personalized Vaccination with IKKβ-Matured, RNA-Loaded Dendritic Cells for Metastatic Uveal Melanoma. Front Immunol. 13. 13. p. 785231. DOI: 10.3389/fimmu.2022.785231.

4 Thesis

In this dissertation, the following hypotheses were made and addressed

1. NF-κB activated dendritic cells are able to secrete IL-12.

2. DCs electroporated with caIKKβ-RNA can activate natural killer cells.

3. Natural killer cells activated with NF-κB-dendritic cells secrete pro-inflammatory cytokines, such as TNFα and IFNγ and have a lytic capacity towards target cells.

4. For optimal activation of natural killer cells, cell-cell interaction between caIKKβ-dendritic cells and natural killer cells is required.

5. Natural killer cells and $CD8^+$ T cells can both be activated *via* caIKKβ-dendritic cells without competitive effects.

6. A chimeric IL-15 / IL-15Rα can be expressed on the surface of dendritic cells *via* mRNA electroporation.

7. IL-15 signalling can improve NK-cell activation by NF-κB-dendritic cells.

Publications

NF-κB activation triggers NK-cell stimulation by monocyte-derived dendritic cells

by

Naomi C. Bosch, Reinhard E. Voll, Caroline J. Voskens, Stefanie Gross, Barbara Seliger, Gerold Schuler, Niels Schaft, and Jan Dörrie

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NF-κB activation triggers NK-cell stimulation by monocyte-derived dendritic cells

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Abstract

Background: In therapeutic cancer vaccination, monocyte-derived dendritic cells (moDCs) efficiently activate specific T-cell responses; however, optimizing the activation of innate immune cells could support and improve the antitumor effects. A major disadvantage of moDCs matured with the standard cytokine cocktail (consisting of IL-1β, IL-6, TNFα, and PGE_2) is their inability to secrete IL-12p70. IL-12 prominently activates natural killer (NK) cells, which are crucial in innate antitumor immunity, as they act as helper cells for the induction of a cytotoxic T lymphocyte (CTL) response and are also able to directly kill the tumor.
Methods: Previously we have shown that triggering the NF-κB pathway in moDCs by transfection of mRNA encoding constitutively active IKKβ (caIKKβ) led to IL-12p70 secretion and improved the dendritic cells' capability to activate and expand CTLs with a memory-like phenotype. In this study, we examined whether such dendritic cells could activate autologous NK cells.
Results: moDCs matured with the standard cytokine cocktail followed by transfection with the caIKKβ-RNA were able to activate autologous NK cells, detected by the upregulation of CD54, CD69, and CD25 on the NK cells, their ability to secrete IFNγ, and their high lytic activity. Moreover, the ability of NK-cell activation was not diminished by simultaneous T-cell activation.
Conclusion: The capacity of caIKKβ-DCs to activate both the adaptive and innate immune response indicates an enhanced potential for clinical efficacy.

Keywords: adoptive cellular immunotherapy, dendritic cells, interleukin-12, natural killer cells, NF-κB

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Introduction

Dendritic cells (DCs) play a vital role in the immune system. They build the bridge between the adaptive and innate immune system because they can activate both T cells *via* major histocompatibility complex (MHC) presentation of antigens in conjunction with co-stimulatory signals[1] and the innate immune system such as NK cells.[2] Therefore, DCs have been used for therapeutic tumor vaccination with the primary goal of activating cytotoxic T lymphocytes (CTLs) to enable elimination of tumor cells.[3] Recently, evidence emerged that not only adaptive immune responses, but also the activation of the innate immune system is important to fight against the malignant tissue.[4,5] NK cells activated by vaccine DCs can: (a) induce the maturation of further DCs,[6,7] which in turn leads to additional activation of CTLs in a $CD4^+$ T cell-independent manner,[8] (b) directly activate additional naïve T cells through IFNγ secretion,[9] and (c) attack and directly kill tumor cells,[10] which can then lead to a T-cell cross-presentation of released tumor material by DCs.[11]

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The standard protocol for cancer vaccination generates DCs from monocytes by incubation with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 over 6 days.[12,13] These immature DCs are usually matured using a standard cytokine cocktail consisting of TNFα, prostaglandin E2 (PGE_2), IL-1β, and IL-6.[14] However, so far the efficacy of tumor vaccination with these DCs, like other cancer vaccines, is limited and behind expectations when used as monotherapy.[15] Therefore, different strategies for improvement are currently under investigation including combinations with checkpoint inhibitors, use of optimal tumor antigens, and increase of the immunostimulatory capacity of moDCs.

We[16] and others[17,18] have already observed, that a limitation of the standard maturation protocol is that the generated DCs spontaneously secrete only low concentrations of IL-12p70. This cytokine plays a pivotal role in the induction of T cell-mediated immune responses[19] and also in the activation of NK cells.[20] Consequently, additional factors either apart from or in addition to the standard maturation cocktail, are needed to more efficiently activate DCs.

A key player in the process of DC activation is the transcription factor NF-κB, which can be activated through the classical and the alternative pathways. The classical NF-κB pathway is induced through different danger signals, for example, *via* pro-inflammatory cytokines or activation of Toll-like receptors (TLRs),[21] which then results in the activation of specific target genes. After receiving the activation signal, the IκB kinase (IKK) complex (IKKα, IKKβ, and IKKγ, the latter also called NEMO) phosphorylates IκB, which then releases NF-κB (consisting of RelA and p50).[22] NF-κB then translocates into the nucleus to activate its target genes,[23] such as for example, IL-12.

The standard maturation cocktail already activates the NF-κB pathway in DCs,[24] but not to its full potential. Regarding the different strategies to improve DC vaccination, the NF-κB pathway is regularly involved, for example, through transfection of CD40 ligand[25] or the use of different TLR agonists,[26–29] the latter employing a combination of CD40 ligand, CD70 and constitutively active (ca)TLR4 (TriMix). Massa and co-authors used IFNγ together with monophosphoryl lipid A (MPLA) as an alternative maturation cocktail, which activates NF-κB, and led to DCs with the ability to secrete IL-12p70 and also to activate both innate and adaptive immune responses.[17]

We used a stabilized and constitutively active mutant of IKKβ as a direct and supplementary activation signal for the NF-κB pathway. To this end, we transfected caIKKβ-encoding mRNA by electroporation into DCs matured with the standard cytokine cocktail.[16,30] This procedure resulted in DCs with an increased activation status and the ability to secrete IL-12p70. Moreover, these DCs activated T cells with a higher lytic capacity and a memory-like phenotype.[16] In this study, we investigated whether the activation of the NF-κB pathway creates DCs that can also more effectively activate NK cells.

Materials and methods

Cells

Blood was obtained from healthy donors following informed consent and approval by the institutional review board (Ethikkommission der Friedrich-Alexander-Universität Erlangen Nürnberg, Ref. no. 4158), and peripheral blood mononuclear cells (PBMCs) were isolated using density centrifugation with Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) as described previously.[31] To generate moDCs, monocytes were separated first from the nonadherent fraction (NAF) by plastic adherence and differentiated to immature DCs over 6 days in DC medium consisting of RPMI 1640 (Lonza, Verviers, Belgium) supplemented with 1% nonautologous human plasma (Sigma-Aldrich, St. Louis, United States), 2 mM L-glutamine (Lonza), and 20 mg/l gentamycin (Lonza), adding fresh DC medium with GM-CSF (800 IU/ml; Miltenyi Biotec, Bergisch Gladbach, Germany) and IL-4 (250 IU/mL; Miltenyi Biotec) on days 1, 3, and 5, as described previously.[31] On day 6, DCs were matured using the standard cytokine cocktail consisting of 200 IU/ml IL-1β (CellGenix, Freiburg, Germany), 1000 IU/ml IL-6 (Miltenyi Biotec), 10 ng/ml TNFα (Beromun, Boehringer Ingelheim Pharma, Germany), and 1 μg/ml PGE_2 (Pfizer, Zurich, Switzerland). DCs were electroporated after 24 h of maturation.

NK cells were isolated from autologous PBMCs *via* negative selection using the Human NK cell Enrichment Set-DM (BD Biosciences, Heidelberg, Germany) according to the manufacturer's description.

Cells were incubated at 37°C with 5% CO_2 unless stated otherwise.

In vitro RNA transcription and electroporation of DCs

In vitro transcription of mRNA was carried out using the mMESSAGE mMACHINE™ T7 ULTRA Transcription Kit (Life Technologies, Carlsbad, CA, USA) and purified with an RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturers' protocols. The RNA used for electroporation encoded a constitutively active mutant of IKKβ,[16] which activates the classical NF-κB pathway. In a total volume of 100 µl, 6×10^6 DCs were electroporated with 30 µg caIKK-RNA or 5 µg EGFP-RNA or as a control mock electroporated using a square-wave pulse, 1 ms, and 1250 V/cm as recently described in detail.[32]

Co-cultures

Transfected DCs were harvested 2–4 h after electroporation and were either directly used for co-culture experiments or were pulsed with 10 µg/ml MelanA EAAGIGILTV-peptide (GenScript, Leiden, Netherlands) for 1 h and then used for co-culture. Donors employed for peptide pulsing were haplo-typed HLA-A0201.

DCs were co-cultured with fresh autologous PBMCs or purified NK cells at the indicated ratios and incubated in MLPC medium consisting of RPMI 1640 (Lonza), 10% nonautologous human serum (Sigma-Aldrich), 2 mM L-glutamine (Lonza), 20 mg/l gentamycin (Lonza), 10 mM HEPES (PAA Laboratories, GE Healthcare Life Sciences, Pasching/Linz, Austria), 1 mM sodium pyruvate (Lonza), and 1% nonessential amino acid (100×; Lonza), whereas PBMCs and NK cells only cultured in the respective medium served as control. For co-incubations at ratios of 1:2, 1×10^6 DCs/ml and 2×10^6 PBMCs/ml were seeded in a 24- or 48-well, depending on cell numbers, while for co-incubations at a ratio of 1:10 the final concentrations were 2×10^5 DCs/ml and 2×10^6 PBMCs/ml. DCs and NK cells were co-cultured at ratios of 5:1 and 1:1. For co-incubations at a ratio of 5:1, the final concentrations were 1×10^6 DCs/ml and 2×10^5 NK cells/ml. For co-incubations at a ratio of 1:1, the final concentrations were 1×10^6 DCs/ml and 1×10^6 NK cells/ml. PBMCs or NK cells cultured alone served as control. Cells were harvested after 24 h, 48 h, and after 1 week of co-incubation, whereas supernatants were taken after 24 h and 48 h. Co-cultures over 1 week were split and fresh medium was added depending on their expansion rate.

Transwell analysis

To separate the cell populations from each other while allowing the transfer of soluble factors, transwell polycarbonate membrane cell culture inserts (Corning Incorporated, New York, United States) were used. Transfected DCs and freshly isolated PBMCs were counted and resuspended in 1×10^6 cells/ml and 2×10^6 cells/ml in MLPC medium, respectively. Of these suspensions, 350 µl DCs were seeded in a 24-well plate, either alone, adding 250 µl MLPC medium, or together with 250 µl PBMCs. After adding the membrane (pore size: 0.4 µm) 100 µl PBMCs were seeded in the upper compartment. We harvested cells after 48 h from both the upper and lower compartment and supernatant was taken.

Cell surface marker analysis

Cells were harvested after 24 h, 48 h, and 1 week. The expression of surface markers was analyzed by flow cytometry using anti-CD80-FITC, anti-CD70-PE, anti-CD40-PE and their corresponding isotype controls, and anti-CD56-FITC, anti-CD3-APC-Cy7 or anti-CD3-V500, anti-CD69-PE, anti-CD25-BV421 or anti-CD25-PE, and anti-CD54-APC or anti-CD54-PE (all from BD Biosciences) as recently described.[33] Immunofluorescence was measured using a FACS Canto II (BD Biosciences), data were acquired with FACSDiva software (BD Biosciences) and evaluated with FCS Express software, version 5 (DeNovo Software). An average of approximately 6500 NK cells per measurement was acquired, with a minimum of 500 and a maximum of 23,000 cells.

MHC-tetramer staining

Co-cultures containing peptide-pulsed DCs and PBMCs at a ratio of 1:10 (final concentrations 2×10^5 DCs/ml and 2×10^6 PBMCs/ml) were harvested after 1 week. Cultures with DCs that had not been peptide-pulsed served as controls. T cells specific for the MelanA peptide were detected with HLA-A0201-PE ELAGIGILTV tetramer (produced in house according to Rodenko *et al.*[34]).

Harvested cells were stained with the tetramer for 15 min, then anti-CD3-APC-H7, anti-CD4-AlexaFluor700, anti-CD8-PE-Cy7, anti-CD56-BV421, anti-CD16-FITC, anti-CD69-APC, and anti-CD27-BUV395 (all from BD Bioscience) were added and incubated for another 20 min. After washing twice with phosphate buffered saline, cells were acquired on a FACS Fortessa (BD Bioscience). $CD8^+$ T cells and NK cells were distinguished and characterized *via* expression of CD3, CD56, CD8, and CD69. The gating strategy is depicted in Supplemental Figure S8.

Cytokine secretion analysis

The supernatants of the co-cultures were taken after 24 h and 48 h of incubation. Cytokine concentrations were determined using the Human Th1/Th2 Cytometric Bead Array Kit II (BD Biosciences) or the Human Inflammatory Cytometric Bead Array Kit (BD Biosciences) following the manufacturer's instructions. Immunofluorescence was measured using a FACS Canto II (BD Biosciences), data were acquired with FACSDiva software (BD Biosciences) and evaluated with FCS Express software, version 5 (DeNovo Software).

To illustrate cytokine secretion on a per cell level, we normalized each cytokine concentration to cell number. We calculated the IL-12p70 secretion per 10^6 DCs as follows: the cytokine concentration of IL-12p70 was multiplied by 2 for the conditions Mock only, Mock 1:2, caIKKβ only, and caIKKβ 1:2, or multiplied by 10 for the conditions Mock 1:10 and caIKKβ 1:10. The TNFα and IFNγ secretion per 10^6 NK cells was calculated as follows: the average cytokine concentration of IFNγ and TNFα was multiplied by 2 for the conditions NK only, Mock 1:1, and caIKKβ 1:1, or multiplied by 10 for the conditions Mock 5:1 and caIKKβ 5:1.

Cytotoxicity assay

The cytolytic capacity of NK cells was determined after 1 week of co-incubation with DCs in a standard 4–6 h ^{51}Cr release assay as described previously.[35] Briefly, the target cell line K562 was labeled with 100 μCi of $Na_2{}^{51}CrO_4/10^6$ cells. Target cells were washed and subsequently cultured in 96-well plates (Thermo Fisher, Waltham, MA, USA) at 1000 cells/well. The labelled cells were then incubated with titrated amounts of effector cells (E:T ratios of 20:1, 6:1, 2:1, and 0.6:1) for 4–6 h, followed by collection of supernatants for measurement of released chromium concentrations using the Wallac 1450 MicroBeta plus Scintillation Counter (Wallac, Turku, Finland). The percentage of lysis was calculated using the following formula:

$$\left[(\text{measured release} - \text{background release})\right] / \left[(\text{maximum release} - \text{background release})\right] \times 100\%.$$

Statistical analysis

For the creation of graphs and statistical analysis GraphPad Prism, version 7 (GraphPad Software, La Jolla, USA) was employed. p values were determined comparing the respective conditions (for DC and PBMC co-cultures: Mock 1:2 + caIKKβ 1:2 and Mock 1:10 + caIKKβ 1:10; for DC and NK co-cultures: Mock 5:1 + caIKKβ 5:1, Mock 1:1 + caIKKβ 1:1) using the paired Student's t test assuming a Gaussian distribution. It should be mentioned that not all formal requirements for the paired Student's t test are fulfilled here: owing to our limited sample sizes, normal distribution cannot be tested. On the other hand, when we performed very similar experiments with more donors in the past, we usually observed a Gaussian distribution. In addition, Student's t test is rather robust, even if this criterion is mildly violated.[36]

Results

Stimulation with caIKKβ-transfected mature DCs leads to the upregulation of activation markers on NK cells

Electroporation of caIKKβ mRNA in DCs matured with the standard cytokine cocktail leads to efficient activation of the classical NF-κB pathway, resulting in an enhanced activation state of mature DCs[16] accompanied by a more long-lasting and higher stimulation capacity towards T cells.[16] Transfection efficiency of DCs with mRNA is generally very robust with over 90% positive cells[37] (Supplemental Figure S1A). The activation of the NF-κB pathway through caIKKβ-mRNA transfection is demonstrated by the upregulation of several activation markers such as CD70, CD80, and CD40 in the whole population of the DCs (Supplemental Figure S1B).

To analyze whether DCs transfected with caIKKβ could also trigger the activation of NK cells,

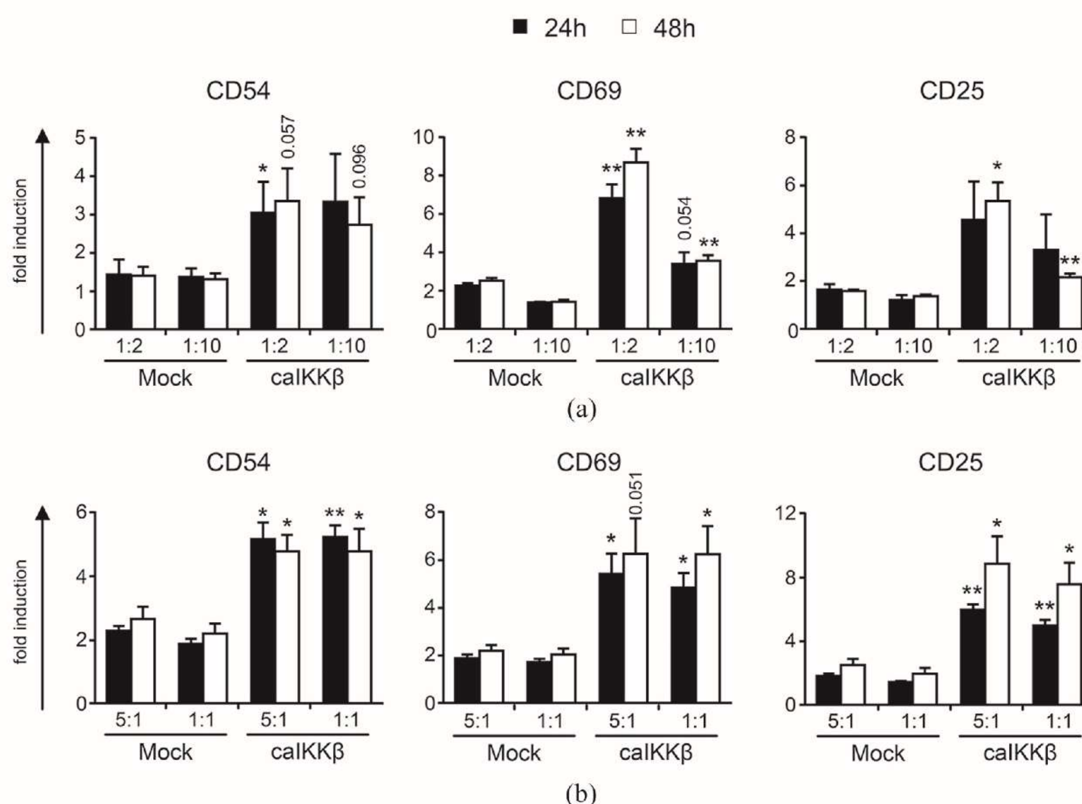

Figure 1. Stimulation with caIKKβ-transfected mature dendritic cells (DCs) results in the upregulation of activation markers on NK cells.

Cytokine-matured DCs were electroporated either with caIKKβ-RNA or as a control were mock electroporated. (a) Transfected DCs were co-cultured with fresh autologous peripheral blood mononuclear cells (PBMCs) 2–4 h after electroporation at a ratio of 1:2 (final concentrations: 1×10^6 DCs/ml and 2×10^6 PBMCs/ml) or 1:10 (final concentrations: 2×10^5 DCs/ml and 2×10^6 PBMCs/ml). To determine background levels, PBMCs were cultured alone. Cells were harvested after 24 h or 48 h and the expression of the surface markers CD54, CD69, and CD25 was determined *via* flow cytometry (using the gating strategy shown in Supplemental Figure S2). All values show the upregulation of each surface marker, calculated relative to the mean fluorescence intensity (MFI) of PBMCs alone. The average fold induction of four different donors with the SEM is shown; for original data, see Supplemental Table S1. Each donor was analyzed in independent experiments. (b) DCs were co-cultured with fresh autologous NK cells at a ratio of 5:1 (final concentrations: 1×10^6 DCs/ml and 2×10^5 NK cells/ml) or 1:1 (final concentrations: 1×10^6 DCs/ml and 1×10^6 NK cells/ml). To determine background levels, NK cells were cultured alone. Cells were analyzed as described in (a). Average fold induction (relative to MFI of NK cells alone) is shown from four different donors with SEM; for original data see Supplemental Table S2. *p* values were calculated to the respective mock condition with the paired Student's *t* test using the specific MFI values, $^{**}p \leq 0.01$, $^{*}p \leq 0.05$, numbers indicate *p* value of $0.05 \leq p \leq 0.1$.

caIKKβ-transfected DCs or mock-transfected DCs were co-cultured with PBMCs at a cell ratio of 1:2 and 1:10, for 24h, 48h, and 1week. PMBCs cultured in the absence of DCs served as control. At the indicated time points cells were stained with antibodies directed against CD56 and CD3 to define CD3⁻CD56⁺ NK-cells as indicated in Supplemental Figure S2. NK-cell activation was assessed by measuring the expression of CD25, CD54, and CD69 by flow cytometry. These markers are well known to be upregulated on activated NK cells.[38,39]

On NK cells stimulated with caIKKβ-DCs, the activation markers CD54, CD69, and CD25 were upregulated significantly, in comparison with NK cells stimulated with mock-electroporated DCs (Figure 1(a) and Supplemental Figure S3). The expression of CD54 on NK cells stimulated with caIKKβ-transfected DCs was about twice as high as on NK cells stimulated with mock-electroporated DCs at both cell ratios of 1:2 and 1:10, reaching significance after 24h at a DC/PBMC ratio of 1:2 (Figure 1(a)). In conditions with a ratio of 1:2, the expression of CD25 was also significantly higher on NK cells stimulated with caIKKβ-DCs after 48h. The strongest effect was observed for CD69 with up to eightfold increased expression levels on NK cells co-incubated with caIKKβ-transfected DCs at a DC/PBMC ratio of

1:2. In contrast, the CD69 expression on NK cells co-cultured with mock-DCs only increased up to twofold (Figure 1(a)) when compared with the background mean fluorescence intensity (MFI) of PBMCs that were cultured alone. The expression of CD69 and CD25 on NK cells stimulated with caIKKβ-DCs at a cell ratio of 1:10 was upregulated to a lesser extent, and reached significance after 48 h (Figure 1(a)).

After showing that caIKKβ-DCs activated NK cells in co-culture with PBMCs, we studied whether this activation was also possible with purified NK cells, or if bystander cells were necessary. For this, transfected DCs were co-cultured with purified NK cells at a cell ratio of 5:1 and 1:1 or, to measure background expression levels, NK cells were cultured alone. Indeed, again all three activation markers on the NK cells were highly and significantly upregulated by stimulation through caIKKβ-DCs (Figure 1(b) and Supplemental Figure S4).

The upregulation of the activation markers CD54, CD69, and CD25 on NK cells was preserved up to 1 week of co-incubation with caIKKβ-DCs, however only reaching significance for CD54 at a ratio of 1:1 (Supplemental Figure S5A). After 1 week of incubation, NK cells incubated alone did not sufficiently survive, whereas NK cells co-cultured with mock or caIKKβ-DCs were able to persist during this period. After 1 week of co-culture, NK cells stimulated with caIKKβ-DCs changed their morphology and increased in size (Supplemental Figure S5B). In addition, the intensity of the CD56 expression increased (Supplemental Figure S5C) indicating a superior activation state of these NK cells, compared with the controls. Thus, caIKKβ-DCs were well capable of activating NK cells and this activation occurred independently of bystander cells.

The presented data support the hypothesis that caIKKβ-DC enhance NK-cell activation, based on CD25, CD54, and CD69 expression, but further research is necessary to determine the direct effects of caIKKβ-DCs on the expression CD16 and natural cytotoxicity receptors, including NKG2D.

Stimulation of NK cells with caIKKβ-DCs leads to the secretion of pro-inflammatory cytokines

To determine which cytokines are involved in the stimulation of NK cells by cytokine-matured DCs, in which the NF-κB pathway was activated, caIKKβ-electroporated DCs or mock-electroporated DCs were either cultured alone, or co-cultured with PBMCs at a DC/PBMC ratio of 1:2 and 1:10. As an additional control, PBMCs were cultured in the absence of DCs. Supernatants were harvested after 24 h or 48 h and the secretion of different cytokines was determined *via* a cytometric bead array.

We[16] and others[17] have previously shown that DCs matured with the standard cytokine cocktail do not sufficiently secrete IL-12p70. However, activation of the NF-κB pathway in such DCs enabled the secretion of this cytokine was shown by our group[16] and also in Figure 2(a) and (b). As IL-12p70 is an important cytokine for the activation of NK cells,[17] we studied whether caIKKβ-DCs have a positive effect on NK-cell activation. A reason for the lower IL-12p70 concentration in the DC/PBMC co-cultures at a cell ratio of 1:10 compared with the DC/PBMC co-cultures at a cell ratio of 1:2 could simply be the presence of fewer DCs in the former. To demonstrate this, the IL-12p70 production was normalized to DC numbers which showed a constant production of approximately 1 ng IL-12p70 per 10^6 DCs (Supplemental Figure S6A).

As IFNγ and TNFα are strongly secreted by activated NK cells, it was analyzed whether both cytokines were secreted after NK cell stimulation with caIKKβ-DCs, known to produce intermediate amounts of TNFα, but not IFNγ.[16,30] Both cytokines were barely secreted in co-cultures with mock-transfected DCs, whereas high and significant quantities were detected in co-cultures with caIKKβ-DCs (Figure 2(a)). IFNγ was hardly secreted by caIKKβ-DCs alone, or by PBMCs alone, but was highly secreted in co-cultures stimulated with caIKKβ-DCs after 24 h and 48 h, especially at a DC/PBMC ratio of 1:2, barely missing the level of significance (Figure 2(a)). After 24 h, a low concentration of TNFα was detected in co-cultures with mock-transfected DCs, caIKKβ-DCs, and PBMCs alone, but it was strongly and significantly secreted in co-cultures with caIKKβ-electroporated DCs, at both DC/PBMC ratios of 1:2 and 1:10 (Figure 2(a)). After 48 h TNFα secretion decreased (Figure 2(a)). IL-10 was only secreted in marginal amounts in each condition (data not shown).

To address whether bystander cells produced the IFNγ and TNFα, transfected DCs were

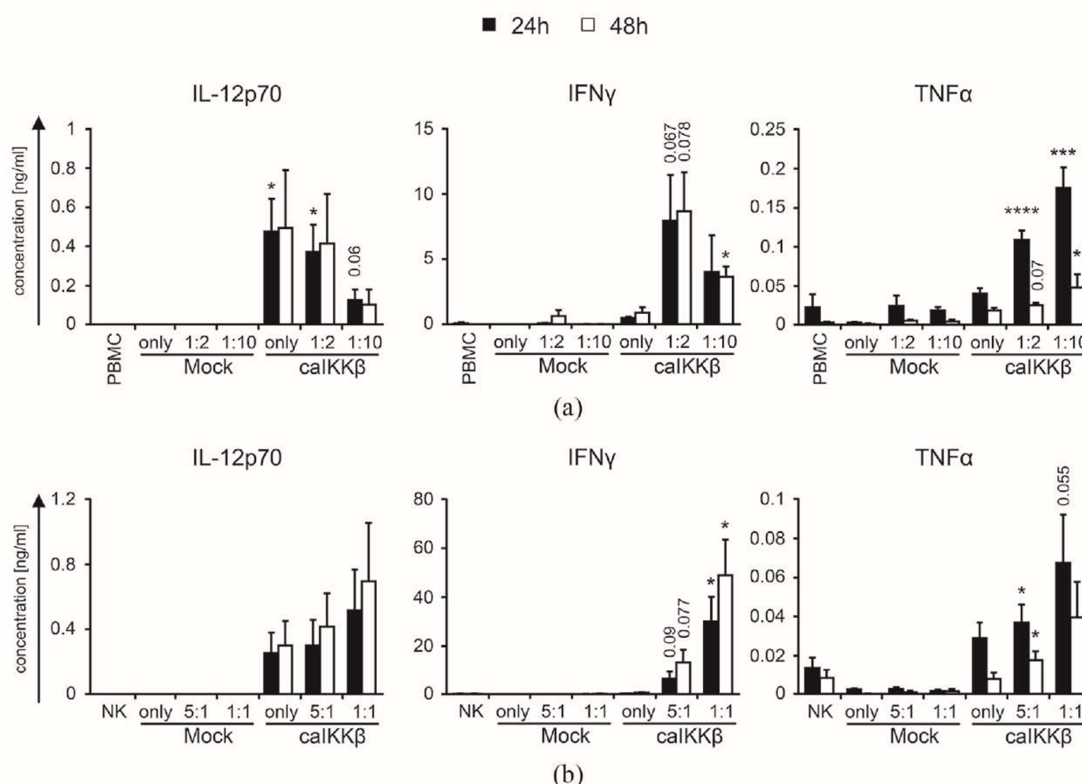

Figure 2. caIKKβ-DCs induce NK cells to secrete pro-inflammatory cytokines.
Cytokine-matured dendritic cells (DCs) were either electroporated with RNA encoding caIKKβ or as a control were mock electroporated. (a) Transfected DCs were co-cultured 2–4 h after electroporation with fresh autologous peripheral blood mononuclear cells (PBMCs) at a ratio of 1:2 (final concentrations: 1×10^6 DCs/ml and 2×10^6 PBMCs/ml) or 1:10 (final concentrations: 2×10^5 DCs/ml and 2×10^6 PBMCs/ml). As controls, PBMCs and DCs were cultured alone. Secretion of IL-12p70, TNFα, and IFNγ was measured in the supernatant by Cytometric Bead Array after 24 h and 48 h of co-incubation. Average cytokine concentrations with SEM are shown from 7 (24 h) or 4 (48 h) different donors; for original data see Supplemental Table S3. (b) Transfected DCs were co-cultured with fresh autologous NK cells at a ratio of 5:1 (final concentrations: 1×10^6 DCs/ml and 2×10^5 NK cells/ml) or 1:1 (final concentrations: 1×10^6 DCs/ml and 1×10^6 NK cells/ml). As controls, NK cells and DCs were cultured alone. Cytokine secretion was measured as described in (a). Average cytokine concentrations are shown from 5 (24 h) or 4 (48 h) different donors; for original data see Supplemental Table S4. *p* values were calculated to the respective mock condition with the paired Student's *t* test, ****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$, numbers indicate *p* values of $0.05 \leq p \leq 0.1$.

co-cultured with purified NK cells at a DC/NK cell ratio of 5:1 and 1:1 and cytokine concentrations were determined in the supernatant. In co-cultures with purified NK cells and caIKKβ-electroporated DCs, IFNγ was still very efficiently and significantly secreted at a cell ratio of 1:1 (Figure 2(b)). TNFα was still secreted at higher concentrations in co-cultures of purified NK cells with caIKKβ-DCs (barely missing significance at a ratio of 1:1 after 24 h) compared with NK cells with mock DCs, but in lower concentrations compared with co-incubations of PMBCs and caIKKβ-DCs (Figure 2(b)). The large difference in cytokine secretion levels between DC/NK co-cultures at a cell ratio of 5:1 and 1:1 could possibly be a result of fewer NK cells present at a cell ratio of 5:1. Therefore, the cytokine production was normalized to NK cell numbers, demonstrating that IFNγ was indeed constantly secreted at a concentration of approximately 60 ng (24 h) to 100 ng (48 h) per 10^6 NK cells (Supplemental Figure S6B). It is noteworthy that the secretion of TNFα per 10^6 NK cells was clearly higher if more DCs were seeded together with the same amount of NK cells (Supplemental Figure S6B). These data indicate that the NK cells are probably the main sources for IFNγ secretion, but we cannot say for sure which cells are primarily responsible for TNFα secretion. Another possibility is that through a DC/NK cell crosstalk, the activated NK cells trigger additional secretion of pro-inflammatory cytokines in the DCs. In summary, the interaction of NK cells with caIKKβ-transfected DCs triggered the production of large quantities of IFNγ, and also small, but significant quantities of TNFα, proving an active interaction of the caIKKβ-DCs with the NK cells.

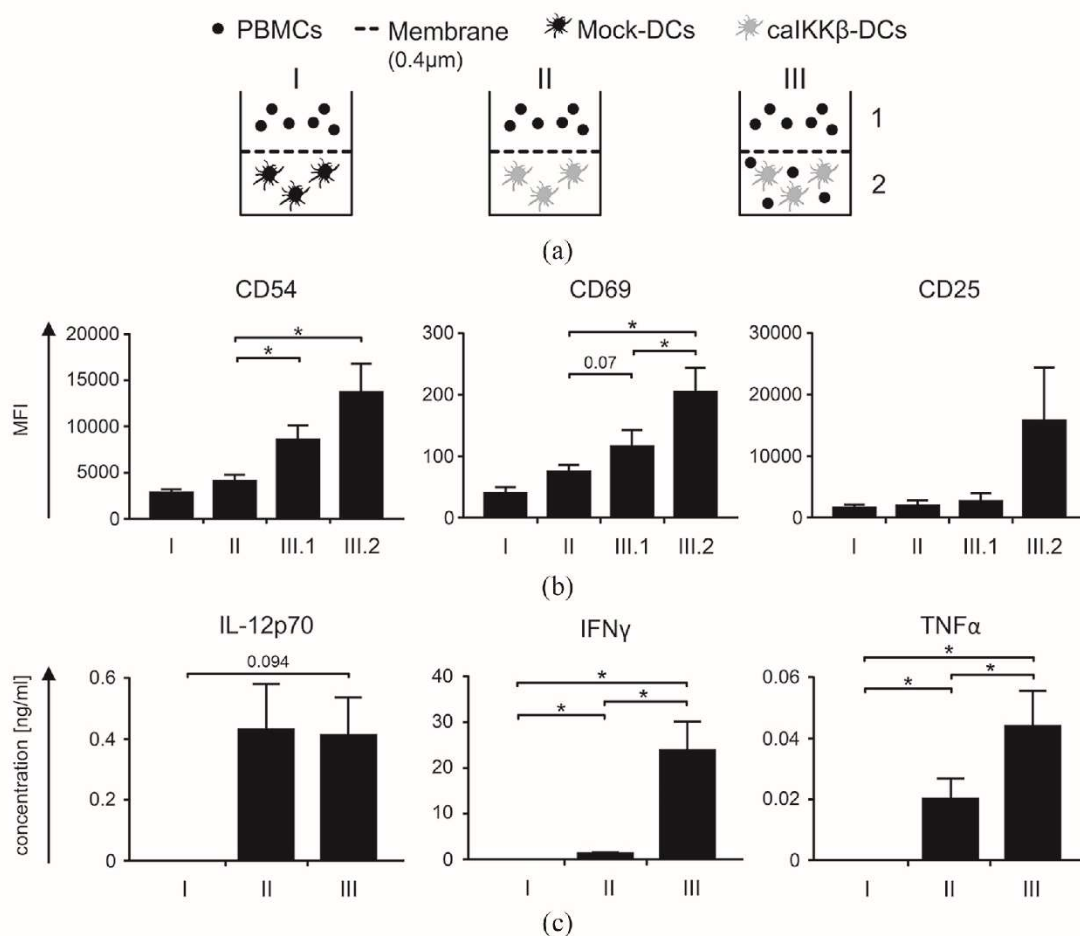

Figure 3. Cell–cell interaction is necessary for best NK-cell activation by caIKKβ-DCs.
(a) Cytokine-matured dendritic cells (DCs) were electroporated with caIKKβ-RNA or, as a negative control, were mock electroporated. A transwell assay was carried out, to analyze whether cell–cell interaction between DCs and NK cells was required, using a membrane allowing transfer of soluble factors while separating cell populations, 2–4 h after electroporation. DCs and fresh autologous peripheral blood mononuclear cells (PBMCs) were either completely separated through a 0.4 μm pore sized membrane (I = mock DCs, II = caIKKβ-DCs) or were co-cultured in the lower compartment (III.2) and separated from further PBMCs in the upper (III.1). Each condition was incubated for 48 h. (b) Surface marker expressions (CD54, CD69, and CD25) on NK cells (using the gating strategy shown in Supplemental Figure S2) were determined for each condition as described in (a) by flow cytometry. Average values of 4 (I) or 5 (II and III) different donors with SEM are shown; for original data, see Supplemental Table S5. (c) The concentrations of IL-12p70, TNFα, and IFNγ in the supernatants from each condition were measured by Cytometric Bead Array. Average values of 4 (I) or 5 (II and III) different donors with SEM are shown; for original data, see Supplemental Table S6. All donors were analyzed in independent experiments. *p* values were evaluated using paired Student's *t* test, $^{*}p \leq 0.05$, numbers indicate *p* values of $0.05 \leq p \leq 0.1$ in (b) and (c).

The cell–cell interaction between caIKKβ-transfected DCs and NK cells is necessary for optimal NK-cell activation

In addition to IL-12p70 a variety of other cytokines are induced by NF-κB-activation of caIKKβ-transfected DCs.[16,30] Therefore, it was investigated whether the secreted soluble molecules by the caIKKβ-DCs were sufficient to activate the NK cells or if direct cell–cell interaction is needed. Mock-transfected and caIKKβ-transfected DCs were subjected to a transwell assay, which prevents cell contact of PBMCs and DCs, but allows soluble factors to pass (Figure 3(a)). DCs and PBMCs were either completely separated from each other (Figure 3(a); I + II), or DCs and PBMCs were co-cultured and separated from further PBMCs (Figure 3(a); III). To measure NK-cell activation, the expression of CD54, CD25, and CD69 was determined after 48 h of incubation (using the gating strategy shown in Supplemental Figure S2, to gate on NK cells). The expression of all three surface markers was the highest when DCs and PBMCs had direct cell–cell contact (Figure 3(b); III.2 and Supplemental Figure S7). For CD54 and

CD69, the differences to NK cells separated from pure caIKKβ-DC were significant. Interestingly, CD54 and also slightly CD69 expression was upregulated on PMBCs that were separated from the PBMC/caIKKβ-DC co-culture (Figure 3(b); III.1), although not as high when PBMCs and DCs were in direct contact. This result indicates that a DC/NK interaction resulted in the release of soluble factors with some NK-cell activation capacity.

Secretion of IFNγ was only sufficiently and significantly detectable when DCs and PBMCs were allowed to interact directly (Figure 3(c)). TNFα was strongly secreted when caIKKβ-DCs and PBMCs were co-cultured, intermediately when caIKKβ-DCs and PBMCs were separated and not at all in the mock condition (Figure 3(c)). These data show that caIKKβ-DCs and NK cells require direct cell–cell interaction for improved NK-cell activation. Activated NK cells seem to induce the activation of further NK cells, independently of direct cell contact.

caIKKβ-DCs can simultaneously activate both CD8⁺ T cells and NK cells

The classical function of DCs in therapeutic tumor vaccination is the activation of tumor-specific T cells that attack the tumor. Hence, it is essential that the DCs' ability to activate $CD8^+$ T cells is not diminished. On the other hand, it may be possible that the T cells that are stimulated by the DCs compete with the NK cells for the DC-mediated activation. To investigate this, we analyzed whether NK cells and T cells were in competition with one another or if they could both be activated simultaneously in a caIKKβ-DC/PBMC co-culture. Therefore, caIKKβ-RNA-electroporated and mock-electroporated DCs were loaded with a $CD8^+$ T-cell epitope from the melanoma antigen MelanA, or were left untreated as a control. These DCs were co-cultured with autologous PBMCs at a cell ratio of 1:10. After 1 week of stimulation, cells were stained with antibodies directed against CD56, CD3, and CD8 to distinguish between $CD3^-/CD56^+$ NK cells and $CD8^+/CD3^+/CD56^-$ T cells (using the gating strategy in Supplemental Figure S8). caIKKβ-DCs loaded with the MelanA peptide were able to expand MelanA-specific $CD8^+$ T cells on average to 0.75% of all $CD8^+$, whereas mock-electroporated DCs were able to yield an average of 0.16% MelanA-specific $CD8^+$ T cells. A representative donor out of four is shown in Figure 4A; data from all donors are depicted in Supplemental Table S7. To display NK-cell activation the expression of CD69 was determined (Figure 4B). The expression of CD69 on NK cells stimulated by caIKKβ-DCs with MelanA peptide was almost exactly as high as on NK cells stimulated by caIKKβ-DCs without a peptide (Figure 4B). These data indicate that at least in this model system, caIKKβ-DCs were able so specifically activate $CD8^+$ T cells, while simultaneously interacting with NK cells. In addition, NK-cell activation was not diminished in the presence of a tumor antigen-derived T-cell epitope, indicating no competitive effects between T-cell and NK-cell activation.

caIKKβ-electroporated mature DCs induce NK cells that can lyse K562 target cells

One of the most desirable properties of DCs for their use in tumor vaccination is their ability to activate effector cells to initiate tumor killing. We could previously show that $CD8^+$ T cells stimulated with caIKKβ-DCs were activated with a superior lytic capacity towards tumor cells compared with DCs matured with the standard protocol.[16] As NK cells could also eliminate tumor cells, a standard cytotoxicity assay was performed to determine whether caIKKβ-DCs could also stimulate NK cells to lyse tumor cells. Therefore, caIKKβ- or mock-transfected DCs were co-cultured with autologous PMBCs at a cell ratio of 1:2 and 1:10 (Figure 5(a)) or with autologous purified NK cells at a cell ratio of 5:1 and 1:1 (Figure 5(b)) for 1 week. The resulting cell population was then used in a cytotoxicity assay against K562 cells with a target-to-effector ratios of 1:20, 1:6, 1:2, and 3:2.

Mock-electroporated DCs could not sufficiently activate NK cells as they were not able to lyse the target cells (Figure 5A, B). In contrast, the caIKKβ-DCs were able to stimulate PBMCs and also purified NK cells, resulting in NK cells that efficiently lysed the K562 cell line (Figure 5). Stimulated PMBCs were able to lyse K562 cells at a target-to-effector ratio of up to 1:2, reaching significance at a target-to-effector ratio of 1:20, when DCs and PMBCs had been co-cultured at a cell ratio of 1:10 (Figure 5(a)). caIKKβ-DC/PBMC co-cultures at a cell ratio of 1:2 also led to a cell population with an enhanced ability to lyse K562 cells, however, without reaching significance. Regarding purified NK cells, both caIKKβ/NK cell ratios of 5:1 and 1:1 were able to equip these NK cells with the ability to significantly lyse

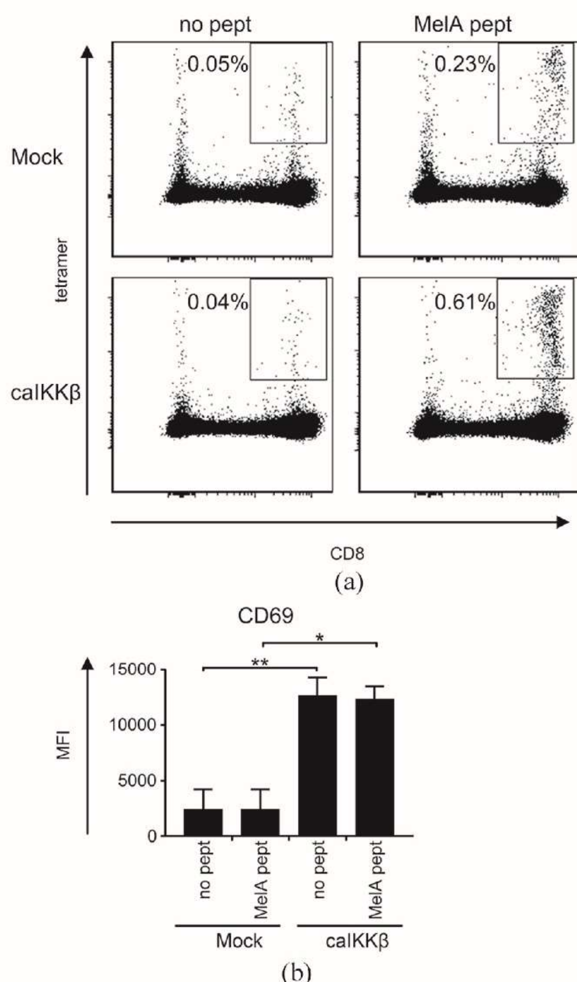

Figure 4. Stimulation of peripheral blood mononuclear cells (PBMCs) with caIKKβ-DCs leads to activation of both NK cells and CD8− T cells. Cytokine-matured dendritic cells (DCs) were electroporated either with caIKKβ-RNA or, as a control, were mock electroporated. Transfected DCs were then either loaded with a CD8+ T-cell epitope from the melanoma antigen MelanA (MelA pept) or were left untreated (no pept). These DCs were co-cultured with fresh autologous PMBCs at a ratio of 1:10 (final concentrations: 2×10^5 DCs/ml and 2×10^6 PBMC/ml) and incubated for 1 week. (a) MelanA-specific CD8+ T cells were measured by peptide-HLA-tetramer staining. To identify CD8+ T cells the gating strategy shown in Supplemental Figure S8A–E was used. The percentage of MelanA-specific CD8+ T cells out of all CD8+ T cells was calculated. Dot plots from a representative donor out of four individual donors is shown; for all original data, see Supplemental Table S7. (b) The expression of CD69 on NK cells (using the gating strategy shown in Supplemental Figure S8A–D to identify NK cells) was determined for each condition *via* flow cytometry. The average MFI of four different donors with the SEM is shown; for original data, see Supplemental Table S8. *p* values were calculated to the respective mock condition with paired Student's *t* test. $^{**}p\leq0.01$, $^{*}p\leq0.05$.

K562 cells at a target-to-effector ratio of 1:20. Purified NK cells that were stimulated with caIKKβ DCs were able to lyse K562 cells at a target-to-effector ratio of up to 1:2, when co-incubated at a ratio of 5:1 and even up to 1:0.6 when co-incubated at a ratio of 1:1.

Discussion

The DCs currently used for tumor vaccination were mainly optimized for induction of potent tumor-specific T cells, but the clinical efficacy observed after treatment with DCs as monotherapy suggested that an improvement of this approach is required. Therefore, next to new combinatorial approaches, it is of great importance to generate DCs with immunostimulatory functions beyond CTL induction.

Our group has established a method to enhance the activation of monocyte-derived DCs matured with the standard cytokine cocktail through subsequent transfection with a caIKKβ in order to additionally activate the NF-κB pathway. This strategy generated DCs with several advantageous features: (i) the activation of NF-κB led to an increased activation status of DCs by upregulation of several activation markers, while their ability to migrate towards lymphatic tissue remained intact; (ii) they spontaneously and continuously secreted IL-12p70; and, thus, (iii) activate CD8+ T cells that displayed a memory-like phenotype characterized by an upregulation of CD27 with a superior lytic capacity.[16] DCs matured with only the standard cocktail required CD4+ T cell help to secrete IL-12p70 and to induce CD8+ T cells with similar features.[40]

In the study described here, we show that caIKKβ-DCs strongly activate NK cells in contrast to DCs generated with the standard protocol. Following contact with caIKKβ-DCs, activated NK cells were able to secrete high amounts of IFNγ and also some TNFα (Figure 2), which can promote further activation of DCs,[9] and naïve T cells[6] for induction of robust cytotoxic T-cell responses. Indeed, a clearly increased expansion of tumor antigen-specific CD8+ T cells by the caIKKβ-DC was found when compared with conventional DCs in presence of NK-cells (Figure 4). Nevertheless, in theory NK cells and T cells might compete for the DCs, thus resulting in a lower NK activation when the DCs had to stimulate both types of effector cells simultaneously. However, the fact that loading caIKKβ-DCs with an antigen resulted in the generation of specific CD8+ T cells and that this did not influence NK-cell activation (Figure 4) indicated that

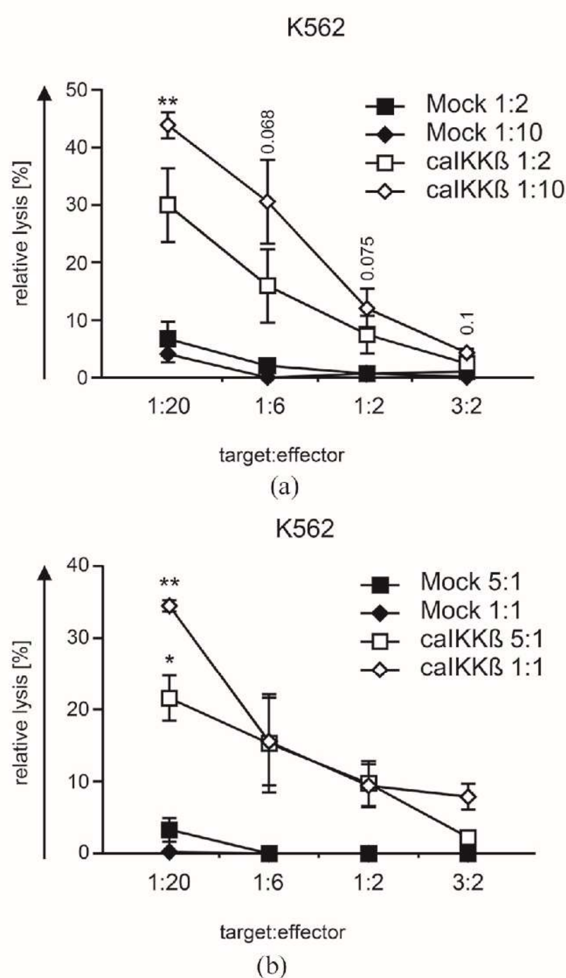

Figure 5. NK cells stimulated with caIKKβ-DCs can kill K562 cells.
Cytokine-matured DCs were electroporated either with caIKKβ-RNA or, as a control, were mock electroporated. (a) Transfected dendritic cells (DCs) were co-cultured with fresh autologous peripheral blood mononuclear cells (PBMCs) at a ratio of 1:2 (final concentrations: 1×10^6 DCs/ml and 2×10^6 PBMCs/ml) or 1:10 (final concentrations: 2×10^5 DCs/ml and 2×10^6 PBMCs/ml) and incubated for 1 week. The cytolytic capacity of the resulting cell population was determined in a ^{51}chromium release assay. The K562 cell line was used as target at the indicated effector to target ratios. Average values ± SEM of three independent donors, each analyzed in triplicates, are shown; for original data see Supplemental Table S9. (b) Transfected DCs were co-cultured with fresh autologous NK cells at a ratio of 5:1 (final concentrations: 1×10^6 DCs/ml and 2×10^5 NK cells/ml) and 1:1 (final concentrations: 1×10^6 DCs/ml and 1×10^6 NK cells/ml) and incubated for 1 week. The lytic capacity of the resulting NK cells was determined as depicted in (a). Average values ± SEM of three independent donors, each analyzed in triplicates, are shown; for original data, see Supplemental Table S10. *p* values were calculated to the respective mock condition using the paired Student's *t* test, $^{**}p\leq0.01$, $^{*}p\leq0.05$, numbers indicate *p* values of $0.05\leq p\leq0.1$.

no competition between T-cell and NK-cell activation occurred in the utilized model system.

In addition, the DC-activated NK cells by themselves were able to effectively lyse the classical HLA-negative NK-cell target K562 (Figure 5). Hence, the simultaneous activation of CTL and NK cells would allow an attack on the tumor *via* tumor antigens presented in HLA class I and efficiently preempt the immune escape mechanism of HLA class I loss. The mechanisms by which the NK cells exert this killing remains to be further investigated; so far, we excluded degranulation as well as production of IFNγ and TNFα *via* CD107a and intracellular staining (data not shown) suggesting that cell-surface interaction might to play a role here.

The observation that even immature moDCs are in principle able to activate NK cells was made many years ago, but the classical maturation cocktail did not increase this ability.[41] Therefore, other groups have focused on creating improved protocols using alternative maturation mixtures, mostly containing different TLR agonists,[17,27,28] inducing DCs to more efficiently activate effector cells. Anguille *et al.* used so-called IL-15-DCs by replacing IL-4 with IL-15 during the differentiation of DCs and then using TNFα, IFNγ, PGE_2, and R-848 (a TLR-7/8 agonist) for maturation.[27] The maturation cocktail used by Massa *et al.* contained IFNγ and MLPA,[17] which is a ligand for TLR-4, whereas Mailliard *et al.* used a maturation mixture consisting of IFNα, IFNγ, TNFα, IL-1β, and a TLR-3 agonist (p-I:C), creating so-called α-type-1-polarized DCs (αDC1).[28]

The caIKKβ-DCs, like the IL-15-DCs, αDC1, and DCs matured with MLPA and IFNγ were all able to effectively activate NK cells as shown in the upregulation of CD69 (Figure 1),[17,42,43] CD25 (Figure 1),[17,43] CD54 (Figure 1), and further activation markers.[43] caIKKβ DCs were able to activate both NK cells in co-cultures with PBMCs and also with purified NK cells showing that bystander cells were not necessary for NK-cell activation. NK cells activated through caIKKβ-DCs or MPLA and IFNγ matured DCs were both able to secrete IFNγ (Figure 2).[17] Both these DCs and also IL-15-DCs were able to induce NK cells to effectively kill certain tumor cell lines (Figure 5).[17,43] Cytotoxicity of NK cells activated by αDC1s was not analyzed.[42] Regarding IFNγ production, IL-15-DCs alone were already able to secrete IFNγ themselves, whereas in IL-15-DC/NK co-culture the secretion of IFNγ did not increase significantly.[43] αDC1 were able to induce IFNγ production by NK cells, but only

when αDC1 were co-cultured with PBMCs (or together with CD40L stimulation). In αDC1/NK cell co-cultures, neither IFNγ secretion was detectable, nor was an upregulation of CD69 seen, showing that co-factors (such as CD40L) are needed for NK-cell activation with αDC1.[42] In this context, it is noteworthy that CD40L is a *bona fide* activator of the NF-κB pathway.

The standard maturation cocktail contains PGE_2 as it has been shown that it is important for the DCs' ability to migrate to the lymph nodes (LNs).[44,45] However, PGE_2 interferes with the IL-12p70 secretion by DCs.[46,47] Through electroporation of caIKKβ-RNA in DCs matured with the standard maturation cocktail, this problem could be overcome, as these DCs still could migrate towards the LN, but had the ability to secrete high amounts of IL-12p70.[16] For IL-15-DCs PGE_2 was contained in their maturation cocktail, indeed creating DCs that could migrate towards the LN. However, these DCs were not able to secrete IL-12p70 when left alone, only gaining this ability when co-cultured with CD40L-transfected 3T3 mouse fibroblasts, representing the CD40–CD40L interaction between DCs and helper T cells.[27] Even though PGE_2 was not included in the maturation cocktail to create αDC1s, these DCs were still able to migrate towards the corresponding chemokine, although not quite as well as DCs matured with the standard protocol.[28] Despite strong CCR7 expression on DCs matured with MPLA and IFNγ, these DCs did not show efficient migratory capacity towards CCL21, indicating a low potency to migrate towards the LN.[48] Both αDC1 and DCs matured with MPLA and IFNγ were able to secrete IL-12p70.[17,28]

The ability to secrete IL-12p70 is one of the most favorable features for vaccine DCs. IL-12p70 plays a crucial role in the development of a $CD8^+$ T-cell memory,[49] and it is also important for a Th1 response.[50] Massa *et al.* showed that NK cells are highly dependent on IL-12p70 for the production of IFNγ, whereas IL-12p70 does not play a central role in the cytotoxicity of NK cells.[17] In line with others,[2,20] we observed that the soluble factors secreted by the caIKKβ-DC, including IL-12p70, did not induce NK cells to secrete IFNγ, but that direct cell–cell interaction was required. An interesting observation was that once NK cells had become activated *via* direct interaction with caIKKβ-DCs, further NK cells that could not directly interact with these DCs were also slightly activated, as indicated by upregulation of CD54 and slightly CD69, but not CD25 (Figure 3). It is possible that IFNγ in concert with other cytokines produced by activated NK cells, led to the stimulation of further NK cells (as reviewed by Boehm *et al.*[51]). This may indicate a positive feedback mechanism for NK cell recruitment but this process as well as the induced activation program within those NK cells requires further investigations.

In conclusion, caIKKβ-DCs meet many features for an optimal vaccination: they can migrate towards lymphatic tissue, secrete IL-12p70 for more than 2 days, activate CTL with a memory-like phenotype and NK cells. The possibility to activate the NF-κB pathway by mRNA electroporation is another advantage as this is a safe method approved and tested for clinical use.[15,31] Therefore, we believe that caIKKβ-DCs are a powerful tool for anticancer vaccination and we are about to start testing these DCs in a phase I clinical trial.

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Conflict of interest statement

The authors declare the following potential conflict of interest: REV, GS, NS, and JD are named as inventors on a patent on caIKK-RNA-electroporated DCs (WO/2012/055551).

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Supplemental material

Supplemental material for this article is available online.

References

1. Guermonprez P, Valladeau J, Zitvogel L, *et al.* Antigen presentation and T cell stimulation by dendritic cells. *Annu Rev Immunol* 2002; 20: 621–667.
2. Fernandez NC, Lozier A, Flament C, *et al.* Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo. *Nat Med* 1999; 5: 405–411.
3. Palucka K and Banchereau J. Dendritic-cell-based therapeutic cancer vaccines. *Immunity* 2013; 39: 38–48.
4. Moretta A. Natural killer cells and dendritic cells: rendezvous in abused tissues. *Nat Rev Immunol* 2002; 2: 957–964.
5. Lion E, Smits EL, Berneman ZN, *et al.* NK cells: key to success of DC-based cancer vaccines? *Oncologist* 2012; 17: 1256–1270.
6. Gerosa F, Baldani-Guerra B, Nisii C, *et al.* Reciprocal activating interaction between natural killer cells and dendritic cells. *J Exp Med* 2002; 195: 327–333.
7. Mocikat R, Braumuller H, Gumy A, *et al.* Natural killer cells activated by MHC class I(low) targets prime dendritic cells to induce protective CD8 T cell responses. *Immunity* 2003; 19: 561–569.
8. Adam C, King S, Allgeier T, *et al.* DC-NK cell cross talk as a novel $CD4^+$ T-cell-independent pathway for antitumor CTL induction. *Blood* 2005; 106: 338–344.
9. Martin-Fontecha A, Thomsen LL, Brett S, *et al.* Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. *Nat Immunol* 2004; 5: 1260–1265.
10. Caligiuri MA. Human natural killer cells. *Blood* 2008; 112: 461–469.
11. Krebs P, Barnes MJ, Lampe K, *et al.* NK-cell-mediated killing of target cells triggers robust antigen-specific T-cell-mediated and humoral responses. *Blood* 2009; 113: 6593–6602.
12. Romani N, Gruner S, Brang D, *et al.* Proliferating dendritic cell progenitors in human blood. *J Exp Med* 1994; 180: 83–93.
13. Sallusto F and Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med* 1994; 179: 1109–1118.
14. Jonuleit H, Kuhn U, Muller G, *et al.* Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *Eur J Immunol* 1997; 27: 3135–3142.
15. Schuler G. Dendritic cells in cancer immunotherapy. *Eur J Immunol* 2010; 40: 2123–2130.
16. Pfeiffer IA, Hoyer S, Gerer KF, *et al.* Triggering of NF-kappaB in cytokine-matured human DCs generates superior DCs for T-cell priming in cancer immunotherapy. *Eur J Immunol* 2014; 44: 3413–3428.
17. Massa C and Seliger B. Fast dendritic cells stimulated with alternative maturation mixtures induce polyfunctional and long-lasting activation of innate and adaptive effector cells with tumor-killing capabilities. *J Immunol* 2013; 190: 3328–3337.
18. Pedersen AE, Thorn M, Gad M, *et al.* Phenotypic and functional characterization of clinical grade dendritic cells generated from patients with advanced breast cancer for therapeutic vaccination. *Scand J Immunol* 2005; 61: 147–156.
19. Colombo MP and Trinchieri G. Interleukin-12 in anti-tumor immunity and immunotherapy. *Cytokine Growth Factor Rev* 2002; 13: 155–168.
20. Yu Y, Hagihara M, Ando K, *et al.* Enhancement of human cord blood $CD34^+$ cell-derived NK cell cytotoxicity by dendritic cells. *J Immunol* 2001; 166: 1590–1600.
21. Luo JL, Kamata H and Karin M. IKK/NF-kappaB signaling: balancing life and death–a new approach to cancer therapy. *J Clin Invest* 2005; 115: 2625–2632.
22. Li ZW, Chu W, Hu Y, *et al.* The IKKβ subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. *J Exp Med* 1999; 189: 1839–1845.
23. Bonizzi G and Karin M. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol* 2004; 25: 280–288.
24. Tas SW, de Jong EC, Hajji N, *et al.* Selective inhibition of NF-kappaB in dendritic cells by the NEMO-binding domain peptide blocks

maturation and prevents T cell proliferation and polarization. *Eur J Immunol* 2005; 35: 1164–1174.

25. Calderhead DM, DeBenedette MA, Ketteringham H, *et al.* Cytokine maturation followed by CD40L mRNA electroporation results in a clinically relevant dendritic cell product capable of inducing a potent proinflammatory CTL response. *J Immunother* 2008; 31: 731–741.
26. Boullart AC, Aarntzen EH, Verdijk P, *et al.* Maturation of monocyte-derived dendritic cells with Toll-like receptor 3 and 7/8 ligands combined with prostaglandin E2 results in high interleukin-12 production and cell migration. *Cancer Immunol Immunother* 2008; 57: 1589–1597.
27. Anguille S, Smits EL, Cools N, *et al.* Short-term cultured, interleukin-15 differentiated dendritic cells have potent immunostimulatory properties. *J Transl Med* 2009; 7: 109.
28. Mailliard RB, Wankowicz-Kalinska A, Cai Q, *et al.* alpha-type-1 polarized dendritic cells: a novel immunization tool with optimized CTL-inducing activity. *Cancer Res* 2004; 64: 5934–5937.
29. Bonehill A, Tuyaerts S, Van Nuffel AM, *et al.* Enhancing the T-cell stimulatory capacity of human dendritic cells by co-electroporation with CD40L, CD70 and constitutively active TLR4 encoding mRNA. *Mol Ther* 2008; 16: 1170–1180.
30. Gerer KF, Erdmann M, Hadrup SR, *et al.* Preclinical evaluation of NF-κB-triggered dendritic cells expressing the viral oncogenic driver of Merkel cell carcinoma for therapeutic vaccination. *Ther Adv Med Oncol* 2017; 9: 451–464.
31. Schaft N, Dorrie J, Thumann P, *et al.* Generation of an optimized polyvalent monocyte-derived dendritic cell vaccine by transfecting defined RNAs after rather than before maturation. *J Immunol* 2005; 174: 3087–3097.
32. Gerer KF, Hoyer S, Dorrie J, *et al.* Electroporation of mRNA as universal technology platform to transfect a variety of primary cells with antigens and functional proteins. *Methods Mol Biol* 2017; 1499: 165–178.
33. Schaft N, Dorrie J, Muller I, *et al.* A new way to generate cytolytic tumor-specific T cells: electroporation of RNA coding for a T cell receptor into T lymphocytes. *Cancer Immunol Immunother* 2006; 55: 1132–1141.
34. Rodenko B, Toebes M, Hadrup SR, *et al.* Generation of peptide-MHC class I complexes through UV-mediated ligand exchange. *Nat Protoc* 2006; 1: 1120–1132.
35. Hofflin S, Prommersberger S, Uslu U, *et al.* Generation of CD8^{+} T cells expressing two additional T-cell receptors (TETARs) for personalised melanoma therapy. *Cancer Biol Ther* 2015; 16: 1323–1331.
36. Salkind N. *Encyclopedia of research design.* Los Angeles: Sage, 2010.
37. Schaft N, Wellner V, Wohn C, *et al.* CD8^{+} T-cell priming and boosting: more antigen-presenting DC, or more antigen per DC? *Cancer Immunol Immunother* 2013; 62: 1769–1780.
38. Clausen J, Vergeiner B, Enk M, *et al.* Functional significance of the activation-associated receptors CD25 and CD69 on human NK-cells and NK-like T-cells. *Immunobiology* 2003; 207: 85–93.
39. Robertson MJ, Caligiuri MA, Manley TJ, *et al.* Human natural killer cell adhesion molecules. Differential expression after activation and participation in cytolysis. *J Immunol* 1990; 145: 3194–3201.
40. Hoyer S, Prommersberger S, Pfeiffer IA, *et al.* Concurrent interaction of DCs with CD4^{+} and CD8^{+} T cells improves secondary CTL expansion: it takes three to tango. *Eur J Immunol* 2014; 44: 3543–3559.
41. Ferlazzo G, Tsang ML, Moretta L, *et al.* Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells. *J Exp Med* 2002; 195: 343–351.
42. Gustafsson K, Ingelsten M, Bergqvist L, *et al.* Recruitment and activation of natural killer cells in vitro by a human dendritic cell vaccine. *Cancer Res* 2008; 68: 5965–5971.
43. Anguille S, Van Acker HH, Van den Bergh J, *et al.* Interleukin-15 dendritic cells harness NK cell cytotoxic effector function in a contact- and IL-15-dependent manner. *PLoS One* 2015; 10: e0123340.
44. Luft T, Jefford M, Luetjens P, *et al.* Functionally distinct dendritic cell (DC) populations induced by physiologic stimuli: prostaglandin E(2) regulates the migratory capacity of specific DC subsets. *Blood* 2002; 100: 1362–1372.
45. Scandella E, Men Y, Gillessen S, *et al.* Prostaglandin E2 is a key factor for CCR7 surface expression and migration of monocyte-derived dendritic cells. *Blood* 2002; 100: 1354–1361.
46. Kalinski P, Schuitemaker JH, Hilkens CM, *et al.* Prostaglandin E2 induces the final maturation of IL-12-deficient CD1a^{+}CD83^{+} dendritic cells: the levels of IL-12 are determined during the final dendritic cell maturation and are resistant

to further modulation. *J Immunol* 1998; 161: 2804–2809.

47. Kalinski P, Hilkens CM, Snijders A, *et al.* IL-12-deficient dendritic cells, generated in the presence of prostaglandin E2, promote type 2 cytokine production in maturing human naive T helper cells. *J Immunol* 1997; 159: 28–35.

48. Massa C, Thomas C, Wang E, *et al.* Different maturation cocktails provide dendritic cells with different chemoattractive properties. *J Transl Med* 2015; 13: 175.

49. Pearce EL and Shen H. Generation of CD8 T cell memory is regulated by IL-12. *J Immunol* 2007; 179: 2074–2081.

50. Kalinski P, Hilkens CM, Wierenga EA, *et al.* T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. *Immunol Today* 1999; 20: 561–567.

51. Boehm U, Klamp T, Groot M, *et al.* Cellular responses to interferon-gamma. *Annu Rev Immunol* 1997; 15: 749–795.

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by

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A Chimeric IL-15/IL-15Rα Molecule Expressed on NFκB-Activated Dendritic Cells Supports Their Capability to Activate Natural Killer Cells †

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Abstract: Natural killer (NK) cells, members of the innate immune system, play an important role in the rejection of HLA class I negative tumor cells. Hence, a therapeutic vaccine, which can activate NK cells in addition to cells of the adaptive immune system might induce a more comprehensive cellular response, which could lead to increased tumor elimination. Dendritic cells (DCs) are capable of activating and expanding NK cells, especially when the NFκB pathway is activated in the DCs thereby leading to the secretion of the cytokine IL-12. Another prominent NK cell activator is IL-15, which can be bound by the IL-15 receptor alpha-chain (IL-15Rα) to be transpresented to the NK cells. However, monocyte-derived DCs do neither secrete IL-15, nor express the IL-15Rα. Hence, we designed a chimeric protein consisting of IL-15 and the IL-15Rα. Upon mRNA electroporation, the fusion protein was detectable on the surface of the DCs, and increased the potential of NFκB-activated, IL-12-producing DC to activate NK cells in an autologous cell culture system with ex vivo-generated cells from healthy donors. These data show that a chimeric IL-15/IL-15Rα molecule can be expressed by monocyte-derived DCs, is trafficked to the cell surface, and is functional regarding the activation of NK cells. These data represent an initial proof-of-concept for an additional possibility of further improving cellular DC-based immunotherapies of cancer.

Keywords: adoptive cellular immunotherapy; IL-15; natural killer cell; dendritic cell; NF-κB

1. Introduction

In the fight against cancer, therapeutic vaccination with dendritic cells (DCs) shows great potential. When patients' DCs are loaded with tumor antigens, these are able to activate tumor-specific $CD8^+$ cytotoxic T cells (CTLs), which then may eradicate the tumor [1]. Studies have shown that an additional innate immune response is highly beneficial when fighting the malignant tissue [2–4]. The standard protocol for cancer vaccination generates DCs from monocytes [5,6], which are subsequently matured using the standard cytokine cocktail consisting of TNFα, prostaglandin E2 (PGE_2), IL-1β, and IL-6 [7]. However, this procedure alone is not sufficient to achieve the desired anti-tumor effect [1] for various reasons, which are still under debate. These include that DCs, used as a vaccine, do not

reach the immunogenicity of a pathogen-driven inflammation, are often limited in IL-12 production, and do not properly interact with the innate immune system [8,9]. Additional activation of the NFκB pathway through electroporation with RNA encoding constitutively active (ca)IKKβ after maturation may give DCs the missing features [10,11]. Our group could already show that electroporation with caIKKβ leads to an upregulation of several activation markers, such as CD70, CD80 and CD40 and triggers the DCs' ability to secrete bioactive IL-12 [10], a characteristic that merely cytokine cocktail-matured DCs lacked, but which is one of the most essential cytokines for the activation of the adaptive [12] and innate [13] immune response, especially against tumors. We found that NFκB-activated DCs displayed an enhanced ability to induce CTLs with a high lytic capacity and a memory-like phenotype [10] and to activate NK cells with the ability to lyse tumor cells [14]. Another pivotal cytokine for the activation of NK cells is IL-15 [15]. IL-15 itself is found in different functional forms either soluble or complexed with the IL-15 receptor α chain (IL-15Rα). This complex can provide IL-15 signaling *in trans* to adjacent cells [16].

The intracellular trafficking and processing of IL-15 is complex. Two premature isoforms are translated, which contain different signaling peptides, but are otherwise similar in their protein sequence. Nevertheless, processing and secretion of the isoforms follow different paths, which are individually regulated. Nevertheless, both require the presence of the IL-15Rα to reach the cell surface [17,18]. Hence, the secretion of IL-15 does not directly correlate with its transcription.

Van den Bergh and his group showed that transfection with a combination of mRNA encoding IL-15Rα and mRNA encoding IL-15 resulted in a significantly increased NK-cell activation when compared to IL-15 alone [19]. Furthermore, the same stimulatory effect was observed when T cells were stimulated with either soluble IL-15/IL-15Rα complexes or with membrane bound IL-15/IL-15Rα presented on APCs [20]. Stoklasek et al. observed a robust proliferation of memory $CD8^+$ T cells, NK cells and T cells in mice treated with soluble IL-15/IL-15Rα complexes [21]. On the other hand, systemic application of IL-15 in therapeutic applications is limited due to its high toxicity [22].

In continuation of the work of Van den Bergh et al. [19], we created a chimeric protein consisting of IL-15 and the IL-15Rα chain (chIL15), specially designed for efficient surface expression on DCs, which are intended for clinical application. The use of one chimeric protein instead of the two original proteins would simplify the DC-transfection process and the covalent link prevents the complex from dissociation. As initial proof-of-concept, we examine here whether expression of such a chimeric construct is feasible on DCs and if it could boost the potential of IL-12-producing DCs to activate NK cells.

2. Results

We recently showed that cytokine cocktail-matured DCs (cmDC), electroporated with caIKKβ-encoding RNA, in order to activate the NFκB-pathway, were able to activate NK cells effectively [14], endowing them with the ability to secrete IFNγ and a lytic capacity towards target cells. Another relevant cytokine for the activation of NK cells is IL-15 [23], especially when IL-15 was trans-presented with the IL-15Rα [19]. We, therefore, designed a chimeric IL-15/IL-15Rα (chIL15) surface molecule to be transfected into DCs in addition to caIKKβ-RNA, and tested, whether this led to an enhanced NK-cell activation.

2.1. chIL15 Is Expressed on chIL15-mRNA-electroporated DCs

As it has been shown that the complex formation of IL-15 with its receptor is important for its efficacy [19,20], we designed a fusion protein of IL-15 and the IL-15Rα genetically fused via a flexible linker. Initially, we used a fusion protein consisting of the full-length IL-15 transcript 1 with its own signal peptide sequence linked to the mature IL-15Rα (chIL15old) (Figure 1A). After transfection of chIL15old into mDCs however, little IL-15Rα was detectable by surface marker staining with an IL-15Rα-specific antibody (Figure 1B, red line) and Western blot (Supplementary Figure S1). We, therefore, postulated that the endogenous signal sequences are not suitable to mediate sufficient expression, processing,

or trafficking [24] in DCs and therefore the chimeric protein was not properly expressed and shuttled to the cell surface. To overcome this we replaced the complete IL-15 signal peptide including the pro-peptide with the CD25 signal peptide (Figure 1A). CD25 was chosen for the following reasons: it is a surface molecule and not a soluble factor, it is a close homolog of the IL-15Rα, and it is efficiently expressed on the surface of mature DCs. To investigate whether this new chimeric IL-15/IL-15Rα (chIL15) leads to better surface expression, the protein was detected by surface staining with an IL-15Rα-specific antibody. Therefore, moDCs were matured with the standard cytokine cocktail and transfected with chIL15, or, as a control, were electroporated without adding any mRNA (mock). The chIL15 surface expression was determined via flow cytometry 2, 4, 6, and 24 h after electroporation. After 24 h over 50% of the cells stained positive (Figure 1B, blue line), and already after 2 h, a visible chIL15 expression was detected with an increase over 4 and 6 h until 24 h after electroporation (Figure 1C). The presence and expression of chIL15 could also be verified by Western blot analysis (Supplementary Figure S2). We were, therefore, able to express the fusion protein efficiently on the DC's surface.

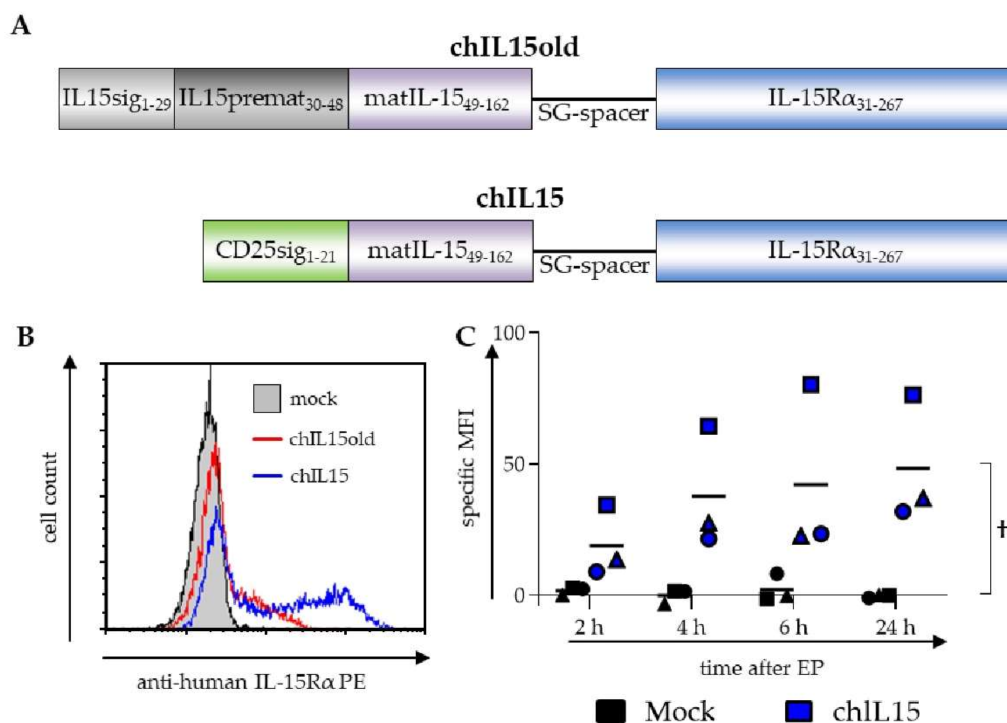

Figure 1. Composition and expression of the chimeric IL15 constructs. (**A**) Originally, the protein sequence of the complete IL-15 transcript variant 1 was fused to that of the IL-15Rα via a flexible SG-linker ($SG_3(SG_4)_3SG_3S$). This construct is referred to as chIL15old. To improve the construct, the first 48 AA of the IL-15 transcript variant 1 that constitute the signal peptide and the pro-peptide were replaced with the signal sequence of CD25. The signal sequence of the IL-15Rα was removed so that the linker was directly fused to the first sushi-domain. This altered construct is referred to as chIL15. (**B**) To measure the expression of the chIL15old and the chIL15 construct on the surface of DCs, cytokine matured DCs were electroporated with mRNA encoding the chIL15old (red line) or chIL15 (blue line), or as a negative control were mock electroporated (grey histogram). The cells were stained with an IL-15Rα-specific antibody and analyzed by flow cytometry. A histogram of DCs electroporated with chIL15old, chIL15, or without RNA (mock) and stained for IL-15Rα 24 h after electroporation is shown ($n = 3$ for chIL15, $n = 1$ for chIL15old). (**C**) The expression kinetics of chIL15 was determined 2, 4, 6, and 24 h after electroporation. The specific mean fluorescence intensity (MFI) was calculated by subtracting the isotype control MFI from the MFI measured with the IL-15Rα-specific antibody (different symbols represent the three individual experiments). The interaction p-value between the mock and the chIL15 condition was calculated by two-way ANOVA, † $p \leq 0.05$.

2.2. Stimulation with DCs Transfected with a Combination of caIKKβ and chIL15 Leads to an Enhanced Activation of NK Cells

To analyze whether the surface expression of chIL15 can increase the ability of caIKKβ-transfected cmDCs to activate NK cells, cmDCs were either electroporated with chIL15 RNA or caIKKβ RNA or with the combination of both. DCs electroporated without RNA (mock) were used as negative controls. The electroporated DCs were co-cultured for 48 h with autologous peripheral blood cells (PBMCs) at a ratio of 1:10 and, as additional controls, each cell type was cultured alone.

To measure NK-cell activation, the cells were stained for CD3, CD56 and the well-established NK-cell activation markers CD69, CD25, and CD54 [25,26]. Cells were analyzed by flow cytometry and NK cells were identified within the mixed population of PBMCs by gating on $CD3^{-}/CD56^{+}$ cells, as this population represents the majority of *bona fide* NK cells [27] (see Supplemental Figure S3 for details). The mean fluorescence intensity (MFI) of each activation marker was calculated relative to the MFI of PBMCs alone. As we could already show previously [14], all three activation markers on NK cells were upregulated between 2- to 4-fold when PBMCs were co-cultured with caIKKβ-transfected DCs, reaching significance with regard to CD69 and CD25 (Figure 2A,B). In co-cultures, in which the DCs were co-transfected with chIL15 and caIKKβ, the expression of all three NK-cell activation markers was even higher (significant for CD69 and CD25), rising up to almost 5-fold (Figure 2A,B). Comparing co-cultures with mock and chIL15 DCs only, a slight increase in each activation marker was observed, which was statistically not significant. These data indicate that the activation of the main population of NK cells ($CD3^{-}/CD56^{+}$) by caIKKβ-transfected DCs can be further improved by additional IL-15 signaling.

As NK cells secrete large quantities of IFNγ upon activation [2], the concentration of this cytokine was quantified in the supernatants of the co-cultures. As expected, the secretion of IFNγ was significantly increased when PBMCs were co-cultured with caIKKβ-transfected DCs (Figure 2C). Co-cultures with DCs transfected with both caIKKβ and chIL15 secreted almost twice as much IFNγ (Figure 2C). DCs cultured alone and transfected with caIKKβ alone and those that were transfected with chIL15 and caIKKβ, also secreted lower quantities of IFNγ, while DCs electroporated only with chIL15-RNA lacked IFNγ secretion (Figure 2C).

We previously demonstrated that both isolated NK cells and PBMCs co-cultured with caIKKβ-transfected DCs for one week had a superior lytic capacity against HLA-negative targets [14]. Since cytotoxicity is the most desirable property for an anti-tumor effect, we examined whether the co-electroporation of chIL15-RNA together with the caIKKβ-RNA would further increase the lytic capacity of the cells that had been co-cultured with such DCs against the classical HLA-negative NK-target cell line K562. This was tested in a standard ^{51}chromium release assay with target-to-effector ratios of 1:6, 1:2, and 1:0.6. Cells from co-cultures with DCs electroporated with both chIL15- and caIKKβ-RNA were able to significantly lyse K562 cells in all target-to-effector ratios, and cells from co-cultures with DCs, electroporated with caIKKβ mRNA alone only marginally missed the formal *p*-value threshold (Figure 2D). Again, we observed an increase of lytic capacity against K562 cells when PBMCs were co-cultured with DCs that were electroporated with both chIL15- and caIKKβ-RNA compared to co-cultures with only caIKKβ-DCs, reaching the highest lytic capacity of 35% at a target-to-effector cell ratio of 1:6 (Figure 2D). The lytic capacity was even twice as high in co-cultures with chIL15- and caIKKβ-transfected DCs at the target-to-effector ratios of 1:2 and 1:0.6, reaching significance at a ratio of 1:0.6 (Figure 2D). As a control, PBMCs were cultured alone, but no difference to PBMCs co-cultured with mock-electroporated DCs was found (data not shown).

Taken together, the activation of NK cells by caIKKβ-transfected DCs can be further improved by additional IL-15 signaling resulting in an even higher upregulation of the activation markers CD69, CD25, and CD54 (Figure 2A,B), an enhanced secretion of IFNγ (Figure 2C), and a higher lytic capacity towards HLA-negative target cells (Figure 2D), albeit other cells in the co-culture may contribute to the latter two observations.

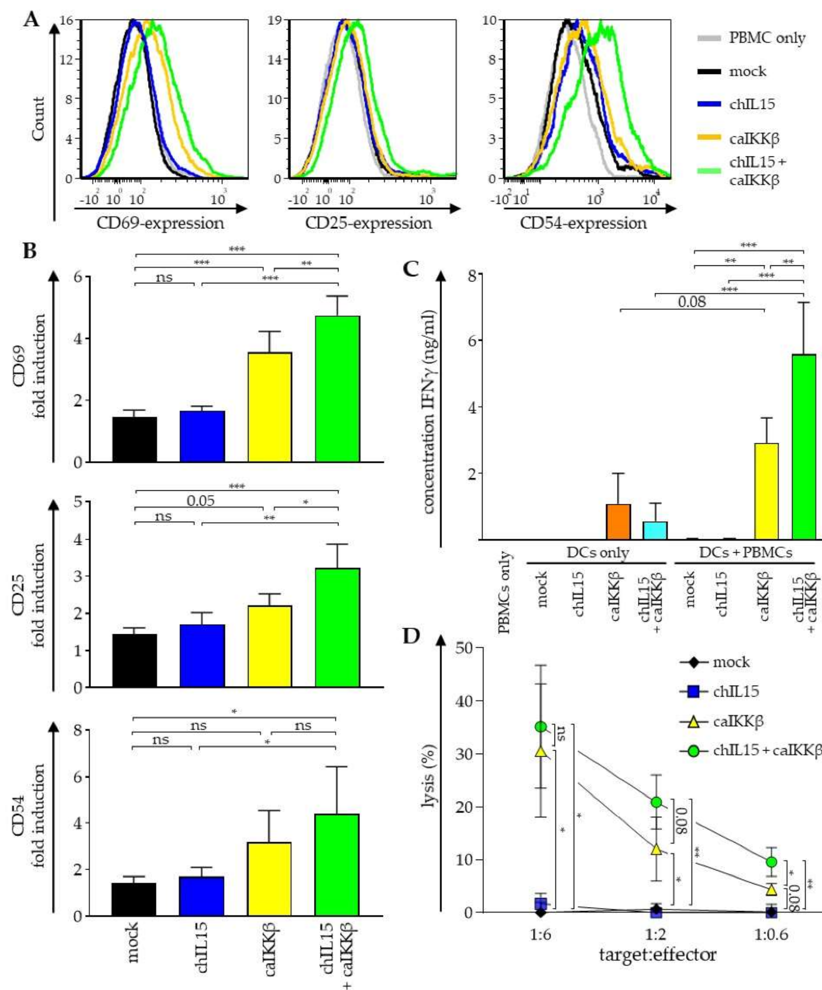

Figure 2. Transfection of DCs with caIKKβ combined with chIL15 leads to an improved NK-cell activation. DCs matured with the standard cytokine cocktail were electroporated with RNA encoding caIKKβ and chIL15 either alone or in combination. As a control, DCs were mock electroporated. Transfected DCs were co-cultured with autologous peripheral blood mononuclear cells (PBMCs) 2–4 h after electroporation at a ratio of 1:10 (final concentrations 2×10^5 DCs/mL and 2×10^6 PBMCs/mL). As controls, transfected DCs and PBMCs were cultured alone. Cells were harvested and supernatant was sampled after 48 h of co-culture. (**A**) The expression of surface markers CD69, CD25, and CD54 was determined via flow cytometry on cells negative for CD3 and positive for CD56 (using the gating strategy shown in Supplemental Figure S3). A representative histogram for each activation marker out of three independent experiments is shown. (**B**) The depicted values show the fold upregulation of each surface marker, calculated relative to the mean fluorescence intensity (MFI) of PBMCs cultured in absence of DCs. The average fold induction of three different donors with SD is shown. *p* values were calculated with a one-way ANOVA and subsequent Tukey test, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, numbers indicate *p* values of $0.05 < p \leq 0.1$. (**C**) The secretion of IFNγ was measured in the supernatant by Cytometric Bead Array. Average cytokine concentrations with SD are shown from three independent donors. *p* values were calculated with a one-way ANOVA and subsequent Tukey test, *** $p \leq 0.001$, ** $p \leq 0.01$, numbers indicate *p* values of $0.05 < p \leq 0.1$. (**D**) The cytolytic capacity of stimulated cells was determined in a ^{51}chromium release assay. The K562 cell line was used as a target at the indicated target ratios. Average values with SD of three independent donors, each analyzed in triplicates, are shown. *p* values were calculated with a one-way ANOVA and subsequent Tukey test, ** $p \leq 0.01$, * $p \leq 0.05$, numbers indicate *p* values of $0.05 < p \leq 0.1$. The respective brackets refer to the mock-transfected co-cultures.

3. Discussion

Activating the NFκB pathway through electroporation with caIKKβ-RNA makes DCs ideal candidates for tumor vaccination, as they unite several beneficial features. Transfection with caIKKβ leads to a higher activation state of DCs providing them with the ability to secrete IL-12. This leads to an advanced stimulation capacity towards both CTLs and NK cells, which in turn gain a superior lytic capacity [10,14]. Currently, the efficacy of these DCs is evaluated in a phase I clinical trial (NCT04335890). Besides IL-12, as a crucial cytokine for the activation of effector cells, also IL-15 plays a pivotal role in the activation of both the adaptive and the innate immune responses [15,24,28,29]. Therefore, it represents an interesting candidate for improving immunotherapeutic approaches [30]. However, NFκB activation does not induce IL-15 secretion by mature monocyte-derived (mo)DCs [10] and merely adding soluble IL-15 has major disadvantages, as IL-15 has a very short half-life [21] and shows high transient toxicity in high plasma concentrations [22]. These negative features can be circumvented when IL-15 is bound to the IL-15Rα. Trans-presentation of IL-15 bound to the IL-15Rα shows high potential for the activation of the adaptive and the innate immune response. Treatment with nanoparticles trans-presenting IL-15 enhanced $CD8^{+}$ T-cell potency [31]. Unfortunately, moDCs do not endogenously express the IL-15Rα but nevertheless showed enhanced activation of NK cells after being equipped with both IL-15 and the IL-15Rα by mRNA electroporation [19]. The fusion of IL-15 to the IL-15Rα might therefore further improve the activation of effector cells through DC vaccination.

We observed that chIL15 alone had only a marginal effect on the activation status of NK cells, probably because IL-12 is critically required for NK activation and standard moDC, matured with the standard cytokine cocktail do not produce this cytokine. When, in contrast, transfected with chIL15 and caIKKβ-RNA, which facilitates IL-12-production, DCs were significantly better in NK cell activation. In a previous paper, we could already show that vast amounts of IFNγ that were secreted in PBMC and DC co-cultures were secreted by NK cells and not other bystander cells [14]. However, the results presented here, only provide an initial proof-of-principle and a deeper characterization of these DCs is necessary to address whether they maintain their T-cell stimulatory capacity, or are by any other means impaired in their immunogenic function. Also, the resulting NK cells should further be characterized for their activity against different tumor cells and their effector function in general.

The use of a single chimeric construct for this purpose is advantageous for several reasons. Under good manufacturing practice (GMP) conditions, which are prerequisites for clinical application of cellular products, the production and transfection of one RNA vs. two separate ones is easier, cheaper and bears less variability. The covalent link prevents any dissociation of the IL-15 from the receptor α chain thus prolonging the activity and avoiding any free IL-15 that might act systemically. A suspected side effect of IL-15 is the induction of autoimmunity [32,33], and Sato et al. postulate that this effect requires *cis* presentation of IL-15 [33], and as chIL15 only presents IL-15 *in trans*, this side effect would be prevented.

As mRNA electroporation is a well-established technique used in clinical trials on cancer immunotherapy [34], taking this approach from the bench to the bedside seems a reasonable task. DCs electroporated with caIKK-RNA are currently tested in a phase I clinical trial on uveal melanoma patients (NCT04335890). If these DCs prove safe and efficient, DCs additionally electroporated with the chIL15 could be clinically tested, provided that a reasonable risk assessment is performed and the regulatory authorities consider the risk:benefit ratio adequate. The intended activation of NK cells, however, would probably have the best impact in the treatment of tumors with impaired HLA expression, which renders the malignant cells invisible for $CD8^{+}$ T-cell responses but increases their sensitivity to NK cells. Uveal melanoma, in contrast, usually shows good HLA class I expression [35]. Hence, tumor entities, which typically use HLA loss as a means of immune escape, would represent better targets. Cervical cancer, colorectal cancer, gastric cancer,

and esophageal squamous cell carcinoma regularly display loss of HLA class I and this loss is often associated with progression or escape [36]. But for most other tumors, HLA-loss or down-regulation has been described to occur with different incidences. The possibility to generate DCs with an increased capacity to activate NK cells which in turn could block this immune escape route for the tumor provides a possibility to further individualize cancer immunotherapy and to specifically respond to tumor immune evasion mechanisms.

Taken together, our experimental data confirm that forced trans-presentation of IL-15 supports the activation of NK cells by DCs. Combining this with caIKKβ transfection into DCs extends their immune-stimulatory function, as it adds a missing piece to the system, overcoming the inability of such DCs to produce endogenous IL-15. This, on the one hand, confirms the importance of IL-15 trans-presentation in NK cell activation, and on the other hand, may represent a possibility to improve designer DC vaccines further.

4. Materials and Methods

4.1. Acquisition of Primary Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from 100 to 360 mL blood, taken from healthy donors after informed consent and approval by the institutional review board (Ethikkommission of the Friedrich-Alexander University Erlangen-Nürnberg, Ref. no. 4158), by density centrifugation with Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) as described previously [37]. For the generation of moDCs, monocytes, were first separated from the non-adherent fraction (NAF) by plastic adherence, to be differentiated into immature DCs (iDCs) over the course of 6 days in DC medium, consisting of RPMI 1640 (Lonza, Verviers, Belgium) supplemented with 1% non-autologous human plasma (Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-glutamine (Lonza), and 20 mg/l gentamycin (Lonza). Fresh DC medium with GM-CSF (800 IU/mL; Miltenyi Biotec, Bergisch Gladbach, Germany) and IL-4 (250 IU/mL; Miltenyi Biotec) was added on days 1, 3, and 5. On day 6, DCs were matured (mDC) with the standard cytokine cocktail consisting of 200 IU/mL IL-1β (CellGenix, Freiburg, Germany), 1000 IU/mL IL-6 (Miltenyi Biotec), 10 ng/mL TNFα (Beromun, Boehringer Ingelheim Pharma, Ingelheim am Rhein, Germany), and 1 µg/mL PGE_2 (Pfizer, Zurich, Switzerland), as described in detail previously [14]. After 24 h of maturation, DCs were used for electroporation. Cells were incubated at 37 °C with 5% CO_2.

4.2. RNA Constructs and Electroporation of DCs

For in vitro transcription of mRNA, the mMESSAGE mMACHINE™ T7 ULTRA Transcription Kit (Life Technologies, Carlsbad, CA, USA) was used and mRNA was purified with an RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. To activate the classical NFκB pathway in DCs, RNA encoding a constitutively active mutant of IKKβ (caIKKβ) was used [10]. A DNA sequence encoding the original fusion construct consisting of the full-length IL-15 variant 1 (NCBI Reference Sequence: NP_000576.1) linked to the IL-15Rα (AA 31-267, NCBI Reference Sequence: NP_002180.1) with a flexible linker (SGGGSGGGGSGGGGSGGGGSGGGSLQ) was ordered from GeneART® (Thermo Fisher Scientific, Waltham, MA, USA) and cloned into the pGEM4Z64A RNA production vector [37]. The sequence encoding the first 48 AA of the IL-15 was replaced with a sequence encoding the first 21 AA of CD25 (NCBI Reference Sequence: NP_000408.1) using two annealed complementary oligonucleotides (Eurofins Genomics, Ebersberg, Germany). The detailed sequences are provided as supplementary Data S1.

DCs were electroporated in a total volume of 100 µL, with a maximum of 6×10^6 DCs, using 30 µg of each mRNA, with a square-wave pulse and 1250 V/cm for 1 ms, as described in detail [38]. As a control, cells were electroporated under identical conditions but without any RNA (mock).

4.3. moDCs and PBMCs Co-Cultures

After transfection, DCs were rested for 2–4 h, then 2×10^5 DCs/mL and 2×10^6 fresh autologous PBMCs/mL were co-cultured, gaining a ratio of 1:10. A maximum of

2×10^5 DCs and 2×10^6 PBMCs, and a minimum of 4×10^4 DCs and 4×10^5 PBMCs were used. PBMCs cultured alone served as control. Cells were seeded into 24- or 48-well plates, depending on cell numbers and incubated in MLPC medium consisting of RPMI 1640 (Lonza), 10% non-autologous human serum (Sigma-Aldrich), 2 mM L-glutamine (Lonza), 20 mg/L gentamycin (Lonza), 10 mM HEPES (PAA Laboratories, GE Healthcare Life Sciences, Pasching/Linz, Austria), 1 mM sodium pyruvate (Lonza), and 1% non-essential amino acid (100×; Lonza). Cells and supernatants were harvested after 48 h. To generate cells for cytotoxicity assays, co-cultures were extended to 1 week and during this period cells were split and fresh MLPC medium was added as necessary.

4.4. Analysis of Marker Expression on the Cell Surface

Cells were harvested either 2, 4, 6, and 24 h after electroporation or after 48 h of co-culture. The expression of surface markers was determined via flow cytometry using anti-CD215-PE (anti-human IL-15Rα, clone JM7A4) (Biolegend, San Diego, CA, USA) and IgG2b-PE isotype control, anti-CD56-FITC, anti-CD3-APC-Cy7 or anti-CD3-V500, anti-CD69-PE, anti-CD25-BV421 or anti-CD25-PE, and anti-CD54-APC or anti-CD54-PE (all from BD Biosciences, Heidelberg, Germany) as described [39]. A FACS Canto II flow cytometer (BD Biosciences) and FACSDiva software [40] were used to measure immunofluorescence and acquire data, which was evaluated with FCS Express software [41].

4.5. Measurement of Cytokine Secretion

The supernatants of the co-cultures were sampled after 48 h. The concentration of IFNγ was determined using the Human Th1/Th2 Cytometric Bead Array Kit II (BD Biosciences) following the manufacturer's instructions. A FACS Canto II flow cytometer and BD FACSDiva software [40] were used to measure immunofluorescence and acquire data, which was evaluated with FCS Express software [41].

4.6. Cytotoxicity Assay

Co-cultures were harvested after 1 week of co-incubation. Then the cytolytic capacity was determined in a standard 4–6 h ^{51}chromium release assay as described in detail before [42]. In short, the target cell line K562 was labeled with 100 μCi of $Na_2{}^{51}CrO_4/10^6$ cells (PerkinElmer, Waltham, MA, USA), washed and subsequently cultured in 96-well plates (Thermo Fisher Scientific) at 1000 cells/well. Effector cells harvested from the co-cultures were added at E:T ratios of 6:1, 2:1 and 0.6:1 (i.e., 6000, 2000, and 600 cells per well, respectively). The supernatant was taken after 4–6 h and the release of chromium was measured via a Wallac 1450 MicroBeta plus Scintillation Counter (Wallac, Turku, Finland). The following formula was applied to calculate the percentage of lysis: (measured release − background release)/(maximum release − background release) × 100%.

4.7. Statistical Analysis

GraphPad Prism [43] was employed to create graphs and statistical analysis. To determine statistical significance and p values, a paired one-way ANOVA, and subsequent multiple comparison analyses using the Tukey test were performed, assuming Gaussian distribution, based on our experience in similar experiments. To analyze the expression kinetic of chIL15, a two-way ANOVA was performed.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms221910227/s1.

Author Contributions: N.C.B., L.-M.M., C.J.V., C.B., B.S., G.S., N.S., and J.D. wrote the manuscript. N.C.B. performed experiments and prepared figures. L.-M.M. cloned the chIL15-construct and performed experiments. B.S., G.S., N.S., and J.D. supervised the project. N.C.B., C.J.V., N.S., and J.D. designed experiments. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board (Ethikkommission of the Friedrich-Alexander-University Erlangen-Nürnberg, Ref. No. 4158).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All original data are available from the corresponding author on request.

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Conflicts of Interest: The authors declare the following potential conflict of interest: GS, NS, and JD are named as inventors on a patent on caIKK-RNA-electroporated DCs (WO/2012/055551), which is held by the Friedrich-Alexander-University Erlangen-Nürnberg (FAU).

References

1. Schuler, G. Dendritic cells in cancer immunotherapy. *Eur. J. Immunol.* **2010**, *40*, 2123–2130. [CrossRef]
2. Terme, M.; Ullrich, E.; Delahaye, N.F.; Chaput, N.; Zitvogel, L. Natural killer cell-directed therapies: Moving from unexpected results to successful strategies. *Nat. Immunol.* **2008**, *9*, 486–494. [CrossRef]
3. Moretta, A. Natural killer cells and dendritic cells: Rendezvous in abused tissues. *Nat. Rev. Immunol.* **2002**, *2*, 957–964. [CrossRef] [PubMed]
4. Lion, E.; Smits, E.L.; Berneman, Z.N.; Van Tendeloo, V.F. NK cells: Key to success of DC-based cancer vaccines? *Oncologist* **2012**, *17*, 1256–1270. [CrossRef] [PubMed]
5. Romani, N.; Gruner, S.; Brang, D.; Kampgen, E.; Lenz, A.; Trockenbacher, B.; Konwalinka, G.; Fritsch, P.O.; Steinman, R.M.; Schuler, G. Proliferating dendritic cell progenitors in human blood. *J. Exp. Med.* **1994**, *180*, 83–93. [CrossRef] [PubMed]
6. Sallusto, F.; Lanzavecchia, A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J. Exp. Med.* **1994**, *179*, 1109–1118. [CrossRef] [PubMed]
7. Jonuleit, H.; Kuhn, U.; Muller, G.; Steinbrink, K.; Paragnik, L.; Schmitt, E.; Knop, J.; Enk, A.H. Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *Eur. J. Immunol.* **1997**, *27*, 3135–3142. [CrossRef]
8. Mailliard, R.B.; Wankowicz-Kalinska, A.; Cai, Q.; Wesa, A.; Hilkens, C.M.; Kapsenberg, M.L.; Kirkwood, J.M.; Storkus, W.J.; Kalinski, P. alpha-type-1 polarized dendritic cells: A novel immunization tool with optimized CTL-inducing activity. *Cancer Res.* **2004**, *64*, 5934–5937. [CrossRef]
9. Garg, A.D.; Coulie, P.G.; Van den Eynde, B.J.; Agostinis, P. Integrating Next-Generation Dendritic Cell Vaccines into the Current Cancer Immunotherapy Landscape. *Trends Immunol.* **2017**, *38*, 577–593. [CrossRef] [PubMed]
10. Pfeiffer, I.A.; Hoyer, S.; Gerer, K.F.; Voll, R.E.; Knippertz, I.; Guckel, E.; Schuler, G.; Schaft, N.; Dorrie, J. Triggering of NF-kappaB in cytokine-matured human DCs generates superior DCs for T-cell priming in cancer immunotherapy. *Eur. J. Immunol.* **2014**, *44*, 3413–3428. [CrossRef]
11. Gerer, K.F.; Erdmann, M.; Hadrup, S.R.; Lyngaa, R.; Martin, L.M.; Voll, R.E.; Schuler-Thurner, B.; Schuler, G.; Schaft, N.; Hoyer, S.; et al. Preclinical evaluation of NF-kappaB-triggered dendritic cells expressing the viral oncogenic driver of Merkel cell carcinoma for therapeutic vaccination. *Ther. Adv. Med. Oncol.* **2017**, *9*, 451–464. [CrossRef]
12. Colombo, M.P.; Trinchieri, G. Interleukin-12 in anti-tumor immunity and immunotherapy. *Cytokine Growth Factor Rev.* **2002**, *13*, 155–168. [CrossRef]
13. Yu, Y.; Hagihara, M.; Ando, K.; Gansuvd, B.; Matsuzawa, H.; Tsuchiya, T.; Ueda, Y.; Inoue, H.; Hotta, T.; Kato, S. Enhancement of human cord blood CD34+ cell-derived NK cell cytotoxicity by dendritic cells. *J. Immunol.* **2001**, *166*, 1590–1600. [CrossRef] [PubMed]
14. Bosch, N.C.; Voll, R.E.; Voskens, C.J.; Gross, S.; Seliger, B.; Schuler, G.; Schaft, N.; Dörrie, J. NF-kappaB activation triggers NK-cell stimulation by monocyte-derived dendritic cells. *Ther. Adv. Med Oncol.* **2019**, *11*, 1758835919891622. [CrossRef]
15. Kennedy, M.K.; Glaccum, M.; Brown, S.N.; Butz, E.A.; Viney, J.L.; Embers, M.; Matsuki, N.; Charrier, K.; Sedger, L.; Willis, C.R.; et al. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. *J. Exp. Med* **2000**, *191*, 771–780. [CrossRef] [PubMed]
16. Stonier, S.W.; Schluns, K.S. Trans-presentation: A novel mechanism regulating IL-15 delivery and responses. *Immunol. Lett.* **2010**, *127*, 85–92. [CrossRef]

17. Bergamaschi, C.; Jalah, R.; Kulkarni, V.; Rosati, M.; Zhang, G.M.; Alicea, C.; Zolotukhin, A.S.; Felber, B.K.; Pavlakis, G.N. Secretion and biological activity of short signal peptide IL-15 is chaperoned by IL-15 receptor alpha in vivo. *J. Immunol.* **2009**, *183*, 3064–3072. [CrossRef]
18. Duitman, E.H.; Orinska, Z.; Bulanova, E.; Paus, R.; Bulfone-Paus, S. How a cytokine is chaperoned through the secretory pathway by complexing with its own receptor: Lessons from interleukin-15 (IL-15)/IL-15 receptor alpha. *Mol. Cell. Biol.* **2008**, *28*, 4851–4861. [CrossRef] [PubMed]
19. Van den Bergh, J.; Willemen, Y.; Lion, E.; Van, A.H.; De, R.H.; Anguille, S.; Goossens, H.; Berneman, Z.; Van, T.V.; Smits, E. Transpresentation of interleukin-15 by IL-15/IL-15Ralpha mRNA-engineered human dendritic cells boosts antitumoral natural killer cell activity. *Oncotarget* **2015**, *6*, 44123–44133. [CrossRef]
20. Hasan, A.N.; Selvakumar, A.; Shabrova, E.; Liu, X.R.; Afridi, F.; Heller, G.; Riviere, I.; Sadelain, M.; Dupont, B.; O'Reilly, R.J. Soluble and membrane bound IL-15 Ralpha/IL-15 complexes mediate proliferation of high avidity central memory CD8+ T-cells for adoptive immunotherapy of cancer and infections. *Clin. Exp. Immunol.* **2016**, *186*, 249–265. [CrossRef]
21. Stoklasek, T.A.; Schluns, K.S.; Lefrancois, L. Combined IL-15/IL-15Ralpha immunotherapy maximizes IL-15 activity in vivo. *J. Immunol.* **2006**, *177*, 6072–6080. [CrossRef]
22. Berger, C.; Berger, M.; Hackman, R.C.; Gough, M.; Elliott, C.; Jensen, M.C.; Riddell, S.R. Safety and immunologic effects of IL-15 administration in nonhuman primates. *Blood* **2009**, *114*, 2417–2426. [CrossRef] [PubMed]
23. Mortier, E.; Advincula, R.; Kim, L.; Chmura, S.; Barrera, J.; Reizis, B.; Malynn, B.A.; Ma, A. Macrophage- and dendritic-cell-derived interleukin-15 receptor alpha supports homeostasis of distinct CD8+ T cell subsets. *Immunity* **2009**, *31*, 811–822. [CrossRef] [PubMed]
24. Waldmann, T.A.; Tagaya, Y. The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell differentiation and host response to intracellular pathogens. *Annu. Rev. Immunol.* **1999**, *17*, 19–49. [CrossRef]
25. Clausen, J.; Vergeiner, B.; Enk, M.; Petzer, A.L.; Gastl, G.; Gunsilius, E. Functional significance of the activation-associated receptors CD25 and CD69 on human NK-cells and NK-like T-cells. *Immunobiology* **2003**, *207*, 85–93. [CrossRef]
26. Robertson, M.J.; Caligiuri, M.A.; Manley, T.J.; Levine, H.; Ritz, J. Human natural killer cell adhesion molecules. Differential expression after activation and participation in cytolysis. *J. Immunol.* **1990**, *145*, 3194–3201.
27. Cossarizza, A.; Chang, H.D.; Radbruch, A.; Acs, A.; Adam, D.; Adam-Klages, S.; Agace, W.W.; Aghaeepour, N.; Akdis, M.; Allez, M.; et al. Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition). *Eur. J. Immunol.* **2019**, *49*, 1457–1973. [CrossRef]
28. Zhang, X.; Sun, S.; Hwang, I.; Tough, D.F.; Sprent, J. Potent and selective stimulation of memory-phenotype CD8+ T cells in vivo by IL-15. *Immunity* **1998**, *8*, 591–599. [CrossRef]
29. Goldrath, A.W.; Sivakumar, P.V.; Glaccum, M.; Kennedy, M.K.; Bevan, M.J.; Benoist, C.; Mathis, D.; Butz, E.A. Cytokine requirements for acute and Basal homeostatic proliferation of naive and memory CD8+ T cells. *J. Exp. Med.* **2002**, *195*, 1515–1522. [CrossRef] [PubMed]
30. Cheever, M.A. Twelve immunotherapy drugs that could cure cancers. *Immunol. Rev.* **2008**, *222*, 357–368. [CrossRef] [PubMed]
31. Hong, E.; Usiskin, I.M.; Bergamaschi, C.; Hanlon, D.J.; Edelson, R.L.; Justesen, S.; Pavlakis, G.N.; Flavell, R.A.; Fahmy, T.M. Configuration-dependent Presentation of Multivalent IL-15:IL-15Ralpha Enhances the Antigen-specific T Cell Response and Anti-tumor Immunity. *J. Biol. Chem.* **2016**, *291*, 8931–8950. [CrossRef] [PubMed]
32. Fehniger, T.A.; Caligiuri, M.A. Interleukin 15: Biology and relevance to human disease. *Blood* **2001**, *97*, 14–32. [CrossRef]
33. Sato, N.; Sabzevari, H.; Fu, S.; Ju, W.; Petrus, M.N.; Bamford, R.N.; Waldmann, T.A.; Tagaya, Y. Development of an IL-15-autocrine CD8 T-cell leukemia in IL-15-transgenic mice requires the cis expression of IL-15Ralpha. *Blood* **2011**, *117*, 4032–4040. [CrossRef] [PubMed]
34. Dörrie, J.; Schaft, N.; Schuler, G.; Schuler-Thurner, B. Therapeutic Cancer Vaccination with Ex Vivo RNA-Transfected Dendritic Cells-An Update. *Pharmaceutics* **2020**, *12*, 92. [CrossRef] [PubMed]
35. Souri, Z.; Wierenga, A.P.A.; Mulder, A.; Jochemsen, A.G.; Jager, M.J. HLA Expression in Uveal Melanoma: An Indicator of Malignancy and a Modifiable Immunological Target. *Cancers (Basel)* **2019**, *11*, 1132. [CrossRef]
36. Hazini, A.; Fisher, K.; Seymour, L. Deregulation of HLA-I in cancer and its central importance for immunotherapy. *J. Immunother. Cancer* **2021**, *9*, e002899. [CrossRef]
37. Schaft, N.; Dorrie, J.; Thumann, P.; Beck, V.E.; Muller, I.; Schultz, E.S.; Kampgen, E.; Dieckmann, D.; Schuler, G. Generation of an optimized polyvalent monocyte-derived dendritic cell vaccine by transfecting defined RNAs after rather than before maturation. *J. Immunol.* **2005**, *174*, 3087–3097. [CrossRef]
38. Gerer, K.F.; Hoyer, S.; Dorrie, J.; Schaft, N. Electroporation of mRNA as Universal Technology Platform to Transfect a Variety of Primary Cells with Antigens and Functional Proteins. *Methods Mol. Biol.* **2017**, *1499*, 165–178. [CrossRef]
39. Schaft, N.; Dorrie, J.; Muller, I.; Beck, V.; Baumann, S.; Schunder, T.; Kampgen, E.; Schuler, G. A new way to generate cytolytic tumor-specific T cells: Electroporation of RNA coding for a T cell receptor into T lymphocytes. *Cancer Immunol. Immunother.* **2006**, *55*, 1132–1141. [CrossRef]
40. *BD FACSDiva*; version 8.0.1; Becton, Dickinson and Company: San Jose, CA, USA, 2013.
41. *FCS Express 5 Flow Cytometry*; version 5.01.0082; De Novo Software: Glendale, CA, USA, 2014.

42. Hofflin, S.; Prommersberger, S.; Uslu, U.; Schuler, G.; Schmidt, C.W.; Lennerz, V.; Dorrie, J.; Schaft, N. Generation of CD8(+) T cells expressing two additional T-cell receptors (TETARs) for personalised melanoma therapy. *Cancer Biol. Ther.* **2015**, *16*, 1323–1331. [CrossRef]
43. Muzyka, O.; Tarkanii, O.; Varchenko, K.; Balantsev, A.; Shved, O. *Prism*; version 8.3.0 (538); GraphPad Software, LLC: San Diego, CA, USA, 2019.

Declarations / Erklärungen

(1) Ich erkläre, dass ich mich an keiner anderen Hochschule einem Promotionsverfahren unterzogen bzw. eine Promotion begonnen habe.

(2) Ich erkläre, die Angaben wahrheitsgemäß gemacht und die wissenschaftliche Arbeit an keiner anderen wissenschaftlichen Einrichtung zur Erlangung eines akademischen Grades eingereicht zu haben.

(3) Ich erkläre an Eides statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst habe. Alle Regeln der guten wissenschaftlichen Praxis wurden eingehalten; es wurden keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht.

Datum, Unterschrift

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