Ex vivo-activated MHC-unrestricted immune effectors for cancer adoptive immunotherapy

Introduction

Cancer adoptive immunotherapy is considered among the most promising new strategies for the treatment of metastatic tumors. In the last decades conventional treatments, including chemotherapy, surgery and radiotherapy, obtained relevant results but also reached a plateau and many tumors in their metastatic stage remain incurable and in great need for new strategies. Adoptive immunotherapy is considered among the most promising of such strategies and a great research effort is ongoing to define the optimal approach and operate the transferability from preclinical to clinical settings. The concept of adoptive immunotherapy includes obviously a great variety of approaches, aiming at transferring immune effectors capable of direct or indirect antitumor activity into tumor bearing patients. Schematically it is possible to distinguish strategies based either on Major Histocompatibility Complex (MHC)-restricted or unrestricted mechanisms, each involving one or multiple immune effectors. MHC-restricted strategies are primarily based on the infusion of tumor-specific T lymphocytes able to recognize, through their T cell receptor (TCR), specific tumor-associated antigens (TAA) presented by the MHC system. MHC-unrestricted approaches do not target specific tumor-antigens and are mainly mediated by effectors of the innate immune system like natural killer (NK) cells or NKT cells, first barrier against pathogens and tumorigenesis process, or by ex-vivo activated lymphocytes acquiring MHC-unrestricted killing capabilities like cytokine-induced killer (CIK) cells. The effective clinical translation of adoptive immunotherapy strategies, either of MHC-restricted or unrestricted type, is currently facing crucial and still partially open issues. The first of such issues is given by the need of clinically relevant quantities of immune effectors capable of tumor-killing activity, with consequent research efforts dedicated to set up and optimize ex-vivo expansion strategies. Besides quantity issues, functional aspects of adoptively transferred immune effectors have to be considered, like their persistence into the host, their tumor/patient-specificity, and safety. Furthermore, ex-vivo expansion and activation protocols need to be compliant with stringent good manufacturing practice (GMP) requirements, implying additional costs and involvement of dedicated facilities and personnel. Even if apparently less tumor-specific, MHC-unrestricted approaches are considered appealing in cancer immunotherapy and presenting some important advantages over the more "evolved" tumor-antigen specific immune responses. MHC-unrestricted effectors are usually more abundant than TAA-specific precursors and easier to expand; furthermore their activity is not restricted to patients with a given HLA-haplotype and is potentially deliverable to all patients and not limited to a single tumor histotype.

Cytokines regulate survival, proliferation, networking and functions of immune effectors and may be consequently considered key interventional points to attempt modulating the activity and expansion of cells for adoptive immunotherapies.

Aim of this work is to review the main cancer immunotherapy strategies that employ MHC-unrestricted immune effectors, approaching the topic from the angle of protocols for their ex-vivo expansion in clinical perspective, as well as potential approaches to favorably modulate their functions. The discussion will be reserved to natural Killer (NK) cells, Cytokine-induced (T-NK) killer cells and invariant NKT cells, not including at this time γδ-T lymphocytes and other cellular subset possibly involved in MHC-unrestricted approaches.

Natural Killer cells

General phenotype and functional features

NK cells represent a small subset, about 10%, of circulating lymphocytes, main effectors of the innate immune system involved in the first response against pathogens and tumor immunosurveillance(1). Their origin is from CD34+ hematopoietic cells in bone marrow and their maturation seems to mainly take place in secondary lymphoid organs and the same bone marrow(2). NK cells are phenotypically characterized by the absence of the CD3 molecule on their membrane and the expression of CD56. NK cells express CD16, FC-γ receptor mediating the interaction with the FC fragment of antibodies and allowing their important antibody-dependant cell-mediated cytotoxicity (ADCC) function. The majority, about 90%, presents a dim expression of the CD56 molecule with brighter CD16 and is responsible for the cytotoxicity activity and ADCC. A smaller group of NK cells is characterized by brighter expression of CD56 and lower CD16, and are considered to be predominantly endowed with a more regulatory function (3-5).

NK cells recognize and kill their target in a MHC-unrestricted manner, without need for previous sensitization. The functional activity of NK cells is mediated by a complex of inhibitory and activating receptors like NKG2D, mainly binding MHC-class I related molecules, death-receptor ligands like TRAIL and Fas-ligand(3, 6, 7). A key and well known mechanism employed by NK cells to patrol and if necessary kill transformed cells is that of "missing self". Such activity is mediated by inhibitory killer immunoglobulin-like receptors (KIR), expressed on NK cells(3, 6, 7). Missing the engagement of KIR with the cognate self-HLA-molecules results in NK cells activation and exertion of their cytolitic activity. This situation of KIR-mismatch has been proved fundamental in mediating the antileukemic effect of donor NK cells after T-cell depleted hematopoietic cell transplant (HCT)(8, 9). The same mechanism may impact on graft versus host diseases (GVHD), depending on which tissue the HLA-mismatched molecules are expressed; conversely it may be involved on graft rejection if operated by host NK with KIR-mismatch in the graft versus donor direction(10, 11). In perspective, the increased knowledge of potential KIR mismatches may have important implications in selecting the best donor for NK adoptive immunotherapy. The infusion of donor haploidentical NK cells may be envisioned also outside HTC context. It was recently introduced also in clinical settings of cancer patients previously pre-conditioned with a non-myeloablative lympho-reductive treatment (12-14). Another mechanism exploited by NK cells to kill their targets is the ADCC mentioned above. It requires the recognition by NK cells of the FC fragments of antibodies specifically bound to the target (3, 15, 16). These mechanisms imply intriguing perspectives in cancer immunotherapy, envisioning the potential synergism of NK-based immunotherapy with anticancer monoclonal antibody (mAb). Examples of these potentialities have been positively reported in the setting of breast cancer, with Trastuzumab mAb binding HER2, or colorectal cancer (CRC) with EGFR-binding Cetuximab(17-19).
The activity of NK cells in surveilling tumor formations and the potential of attacking established tumors make them appealing tools for immunotherapy strategies.
Therapeutic possibilities would include strategies to generate and modulate ADCC tumor attack with tumor-specific mAb, approaches aimed at restoring and potentiate the innate NK activity and the adoptive transfer of NK cells, fresh or ex-vivo activated-expanded.
As previously said, our work will only review this last approach, focusing on cytokine-based strategies to expand NK cells or improve their functions.

Initial Immunotherapy approaches with NK cells

Initial therapeutic approaches were based on fresh or short-term stimulated NK cells. In the eighties the group of Rosenberg treated for the first time patients with metastatic solid tumors with high doses of IL2 in the attempt of inducing activation of endogenous NK. Similarly, subsequent trials were proposed with patients receiving short-term IL-2 activated (LAK) killer cells (20-22). Both approaches provided important proof of principles that activated NK cells were capable of clinical antitumor activity but, in spite of some important clinical results, these approaches did not have much following mostly because of the high toxicity associated with the required systemic infusion of IL2. Further attempts with lower doses of IL2 were definitely less toxic but failed to provide clinical benefit (23).
More recently the idea of adoptive therapy with NK cells has been revitalized by the breakthrough finding of KIR mismatch's role in haploidentical HCT and subsequent encouraging applications of NK infusions following mild lymphodepleting treatments in cancer patients(8, 14, 24, 25). In general, one of the main limitations remains however the low numbers of either fresh or short-term IL-2 stimulated NK. Longer ex-vivo culture conditions have recently been tested or are under investigation with the aim of increasing the number of NK cells to clinically relevant rates and possibly improving their tumor killing capacity (26, 27). Production of large quantities of effectors is crucial even considering the necessity of cryopreservation for multiple and flexible treatments. In general it is possible to schematically divide between adoptive therapies employing autologous or allogeneic ex-vivo cultured NK cells.

The ex-vivo expansion of NK cells is a key procedure required to improve the effectiveness of adoptive immunotherapy strategies, allowing the production of clinically relevant amounts of effectors, their cryopreservation and possibly multiple infusions. The ex-vivo expansion also aims to increase or just reestablish the cytotoxic potential of NK cells, possibly impaired in cancer patients or not sufficiently restored by short term cytokine activation protocols(27). The long term ex-vivo expansion for NK cell for clinical use requires protocols to be compliant with

stringent GMP procedures, to be carried within dedicated facilities by qualified personnel. This issue is of course necessary to guarantee the quality of the final cell-therapy product, but inevitably implies procedural difficulties and cost increasing.

Several expansion protocols have so far been tested, with variable design and alternate results. Cytokines are crucial elements of every expansion protocol, as well as important are the possible presence of feeders, type of medium and the time of culture. Many cytokines have been studied but IL-2 is the principal chemokine influencing NK function and proliferation and is the basis for current expansion protocols (27-29).
We will review the main approaches, highlighting those GMP complaints and that entered clinical settings.

GMP-compliant expansion protocols.
The ex-vivo expansion of NK cells for clinical use is currently exploring several protocols with alternate results and there is not yet a final agreement for a standard schema. The starting cell material may be total peripheral blood mononuclear cells (PBMC), T cell depleted products or purified NK(27). Time of culture may be variable, from few days to a couple of weeks; in general it has to be considered that short cultures may be insufficient to get adequate proliferation and most importantly not enough to restore the NK functionality impaired in tumor patients. The choice of tissue culture medium is a key issue; this can be serum free or include bovine or human serum from certified origin. NK cells can be expanded in tissue culture flasks, bags, or GMP validated bioreactors. Several protocols include stimulatory or feeder cells that need of course to be irradiated to absolutely prevent the risk of contaminating the final cell-product with viable stimulatory cells.
As already said above, the NK source may be autologous or allogeneic.

Autologous NK cells
In the autologous setting, trials have been attempted for the treatment of both hematologic and solid malignancies including renal cell carcinoma (30), malignant glioma (31), breast cancer and lymphomas (32, 33). Overall it is difficult to directly compare the efficiency among the protocols as product source, culture media, IL2 concentrations and other parameters were variable. The time of ex-vivo culture ranged between 1 and 3 weeks, IL-2 was always present, doses were variable between 50 and 6000 IU/ml and NK cells were either cultured in microplates or flasks(27).We can try to draw some general consideration analyzing what apparently was the most effective method. The best expansion rate was obtained by Alici et al. who expanded NK cells from patients with Multiple Myeloma, starting from total non-adherent PBMC, up to 1600 fold (34). The culture medium was CellGro (SCGM) supplemented with 5% human serum, IL2 500U/ml and monoclonal antibody (Ab) anti-CD3 (10 ng/ml). This protocol appears simple and avoids laborious upfront steps like cell adhesion or NK selection. Higher IL2 concentrations used by other authors did not seem to increase the final expansion rate (35).
It has to be considered that the presence of anti-CD3 Ab may lower the "NK purity" of the final cell product, as it was reported by Alici et al ranging around 65% (34). In general the presence of T cells may favor the overall NK expansion, with a final product more similar to the old LAK cells. This may be particularly effective in the autologous setting but could raise some safety concerns in the hypothesis of infusing allogeneic NK cells, introducing the evaluation of potential need for T cell depletion. Even in the allogeneic scenario however, a report from Barkholt et al. described the safe infusion of allogeneic LAK cells after HCT from the same donor (36). Of important note, NK cells expanded by Alici et al. were proved effective in killing autologous myeloma targets. The point of functional and phenotypic evaluation of expanded NK cells is crucial, and not always included in all the protocols. The ex-vivo restoring of NK functional and phenotypic features impaired by the tumor environment is of course determinant for their clinical efficacy. As said, time of ex-vivo culture is another important methodological element, and the best results seemed to require at least 2-3 weeks of ex-vivo activation. Short-term or even overnight activation may not be sufficient to obtain the NK functional and phenotypic recovery mentioned above, with less chances of being translated in clinical benefit (36, 37).

Allogeneic NK cells

The potential infusion of allogeneic NK cells from a donor is an intriguing perspective in light of the KIR-based mechanism of tumor killing. As previously said, the NK cytotoxic potential may be triggered and addressed against targets missing specific HLA-ligands recognized as "self" by KIR receptors(8, 9). A KIR-mismatch situation has been described to occur more likely in certain donor-recipient combinations, and its determination may be predictive of a more intense antitumor effect. The most important demonstration of such effect was provided by clinical results following HLA-haploidentical HCT. The group of Martelli in Perugia demonstrated an increased anti-leukemic effect, with delayed relapses and better engraftment in HCT recipients presenting KIR-mismatches in the donor-recipient direction (8, 24, 25). It is currently not completely defined the impact of KIR-mismatches outside HCT settings and against solid tumors (38). This issue is currently under investigation, preclinical data seem in favor of a similar effect but results are more difficult to be confirmed into clinical settings. Initial trials were more centered on fresh or short-term activated NK cells, pointing at obtaining in-vivo expansion after infusion into patients. While encouraging results were initially obtained against hematologic malignancies, the issue appeared more complex in the setting of solid tumors and longer ex-vivo activation strategies are under investigation along with other innovative strategies(13).

In the first report, Miller et al reported the successful and safe adoptive infusion of allogeneic HLA-haploidentical NK cells in patients with solid tumors (metastatic melanoma, RCC) and hematologic malignancies (14). NK cells were obtained by CD3 depletion of leucapheresis products and activated overnight in Teflon bags with IL2 (1000 U/ml). To sustain in-vivo NK expansion IL2 was subcutaneously administered daily for the first 14 days at dose of $1.75x10^6$ IU (the last 14 patients with AML received ($1x10^7$ U, 3 times per week). This study was conducted outside a HCT setting, however it introduced the concept of lymphodepletion of recipient patient to "provide space" for the incoming NK cells, translating the initial experiences of Rosenberg with the adoptive infusion of tumor-specific lymphocytes (39, 40). Three chemotherapy approaches were explored but interestingly only Cyclofosfamide/Fludarabine (Hi-Cy/Flu) applied to AML patients resulted in the in-vivo expansion of infused NK cells. The important observation is that such regimen produced transitory lymphopenia associated with IL15 surge(14), a key cytokine in NK cell activation and homeostasis, that correlated with NK expansion (41). As clinical results, 5 out of 19 AML patients reached complete remission and NK expansion was confirmed in these patients confirming the necessity of persistent expansion for clinical efficacy.

The overall positive results with AML patients were however not confirmed in similar trials against solid tumors. Geller et al explored a similar preparative regimen (Hi-Cy/Flu) on patients with refractory breast and ovarian cancer, prior the infusion of haploidentical NK cells (12). Patients received low dose of subcutaneous IL2 following NK infusion but the in-vivo expansion reported on AML patients was not observed. Actually, the only patients who experienced in-vivo NK expansion was the one who had to interrupt IL2 infusion after 4 administrations and received high doses steroid treatment following the NK infusion. This could be seen as a contradictory effect, as steroids were known to inhibit both T and NK cell activity. More recent data seem to suggest however that steroids may synergize with IL15 to stimulate and promote NK activity (42, 43). This synergism may be what took place in that unique patient who had NK expansion and where, as previously said, the Hi-Cy/Flu regimen could have contributed determining the IL15 peak. Ren et al. reported limited but encouraging results in 11 patients with refractory renal cell carcinoma (RCC) treated with single infusion of haploidentical, IL2 activated, peripheral blood stem cells (PBSC) (44). PBSC were obtained by G-CSF mobilized apheresis and upon 4 hours exposure to high dose IL2 a significant increase in NK cell content was observed, interestingly such IL2 treatment did not result in any increase of T regulatory cells (Tregs). Investigators reported 1/11 partial response, 1/11 mild response and 6/11 disease stabilization.

Other protocols in the setting of solid tumors have explored longer culture time to ex-vivo expand allogeneic NK cells.

A interesting approach, not involving any preparative lymphodepletion, was proposed by Iliopoulou et al. in the setting of NSCLC (45). NK cells were expanded for about 3 weeks, starting from CD56 selected cells, with IL15 and Hydrocortisone. NK cells retained a high degree of purity at the end of culture, with a median expansion of 23 fold. Furthermore expanded NK cells were confirmed to express the panel of activating receptors including NKp30, NKp44, NKp46, NKG2D and to be able of exerting intense cytotoxic activity.

It was demonstrated that long-term ex-vivo expanded NK cells are able to traffic for the whole body, localize to metastatic sites and survive without signs of immune-mediated destruction by host cells (46).

New perspectives.

New promising clinical graded approaches are based on the use of specific stimulators able to provide additional co-stimulatory signals. These strategies are currently under investigation to improve the ex-vivo expansion of NK cells for clinical use, either in the autologous or allogeneic setting.

Berg et al. obtained high ex-vivo expansion rates by long-term culture of CD56 selected cells with IL2 and GMP manufactures irradiated Epstein-Barr virus-transformed B-cell line (EBV-TM-LCL)(47). The protocol was carried equally well in GMP validated bags or flasks and NK cells were expanded from 800 to more than 3000 fold. Viability of expanded NK cells was confirmed not to be compromised as well as phenotype and cytotoxic potential that was significantly increased compared to resting cells. The important issue of viability after cryopreservation of long-term expanded NK cells was also addressed in this study. Authors confirmed a diminished cytotoxic potential after thawing associated with downmodulation of NKG2D and TRAIL receptors. A short re-exposure to IL2 (500 U/ml) for 16 hours after thawing was reported to restore both the phenotype and cytotoxic activity (47).

Another interesting approach using engineered feeder cell was proposed by the group of Campana(48). NK cells were costimulated with K562 cell lines, plus IL2 10U/ml, engineered to provide important costimulatory signals for NK activation and proliferation. The first of such signals was obtained gene-modifying K562 cells to express the 4-1BB ligand, able to activate the costimulatory CD137 molecule on NK cells. The second costimulatory signal was obtained transducing K562 cells with a construct encoding the IL15 gene with CD8α transmembrane domain. IL15 is known to be important for NK maturation and survival and its effect is optimized when IL15 is bound to presenting-cell membrane rather than in soluble form (49, 50). The association of these 2 signals on irradiated K562 effectively improved NK cell expansion compared with results obtained with IL2, IL12, IL15 and IL21(48). The expansion rates could be significantly improved extending the ex-vivo culture up to 2 or 3 weeks and adding IL2 (100 U/ml) (48, 51). NK cells expanded with this protocol exerted increased preclinical cytotoxic activity against hematologic malignancies and initial data were reported also against solid tumors (52). This system was validated in GMP conditions and represents a promising option to be translated in clinical trials. The use of IL15 is also under investigation as in-vivo stimulator by direct infusion into patients. It is expected to provide intense activatory effect on NK cells but avoiding the limiting toxicities or induction of Tregs expansion, both drawbacks associated with IL2 administration (53, 54).

Other interesting perspectives to potentially improve adoptive NK therapy may include selective depletion of Tregs, approaches to up-regulate NK target molecules and KIR blocking.

Tregs have been demonstrated capable of inhibiting NK activity and preclinical data support a positive effect by their direct depletion (55) or inhibition through immunosuppressive drugs like Cyclosporine, capable of inhibiting T lymphocytes but much less active on NK cells (56). Some new drugs, like bortezomib, have been shown able to sensitize cancer cells to NK-mediated killing, by inducing upregulation of TRAIL and FAS death receptors on tumor cells, opening possibilities for appealing synergisms (57, 58). Moreover, experimental antibodies blocking the KIR receptor have shown interesting preclinical results and hold promising therapeutic implications (59). In general a more profound knowledge and genotyping of KIR gene family, polymorphisms and role of their mismatch in the setting of adoptive immunotherapy of solid tumors will result in improving and favoring the clinical transferability of NK adoptive therapies.

Cytokine-Induce Killer (CIK) cells.

Cytokine-Induced Killer cells represent a peculiar subset of ex-vivo expanded T lymphocytes (60-62). At first look they may remind of LAK cells or long term expanded NK cells previously described. They are instead profoundly different as NK cells are only minimally represented within CIK cells that are instead activated T lymphocytes, sharing some NK phenotypic markers and endowed with a peculiar MHC-unrestricted tumor-killing ability. CIK cells are considered a promising option in cancer immunotherapy, they have been demonstrated active against several types of solid and hematologic malignancies in preclinical models and recent clinical trials (63). CIK cells present simple but important biologic features that address important limitations for the effective clinical application of adoptive immunotherapy strategies. They can be ex-vivo expanded with easy and relatively inexpensive culture conditions starting from PBMC, do not require in-vivo IL2 administration following their infusion, the antitumor activity is not restricted to a precise tumor histotype and display a reduced alloreactivity across major HLA barriers. We will review the main biologic features of CIK cells, focusing on conditions for their ex-vivo expansion, clinical applications and new perspectives.

Ex-vivo expansion and phenotype.

The first important biologic feature that pictures CIK cells as appealing effectors for immunotherapy strategies is their potential to be ex-vivo expanded to clinically relevant rates with simple, cost-effectiveness and GMP-compliant procedures. Precursors of CIK cells are naïve T lymphocytes, mostly with a CD4-CD8- double negative phenotype. As initial source, PBMC are classically used starting from peripheral blood withdrawal or leucapheresis. Alternatively, efficient production of CIK cells have been reported starting from cord blood or G-CSF mobilized products. The standard culture conditions to ex-vivo expand CIK cells require the timed addition of IFN-γ on day0 (1000 U/ml), Ab anti-CD3 (50 ng/ml) on day +1 and IL2 (300 U/ml) added from day + 1 and replaced every 3-4 days when medium is refreshed, up to 3-4 weeks of total time of culture(60-62). The early addition of IFN-γ acts activating the monocytes present in the initial culture which provide contact (LFA-3/CD58) and soluble signals, through IL12, to activate T cells and prompt their development toward a Th1 profile (64-66). The presence of anti-CD3 Ab initiates the activation and proliferation of T cells, further sustained by the presence of IL2. The presence of Ab-anti-CD3 and the intermediate dose of IL2 differentiate CIK cells by the production of LAK cells, where a much shorter time of culture, higher IL2 doses and absence of Ab-Anti-CD3 ended up in a heterogeneous population mainly represented by activated NK cells (67, 68). The phenotype of mature expanded CIK cells is primarily given by 2 subpopulations, either CD3+CD56+ or CD3+CD56-, while the presence of "contaminating" NK cells is usually negligible (< 5%).CD3+CD56+ double positive fraction presents more terminally differentiated phenotype and are considered the main responsible of the tumor killing activity. The CD3+CD56- cell subset is more similar to activated conventional T cells, endowed with higher proliferative potential and functional TCR (61, 69, 70). The expansion rates of CIK cells are usually in the range of hundreds fold however a certain degree of variability exists among individuals, regardless their being cancer patients or healthy donors (60, 70-72). Several research groups had proposed possible variations to the original protocol in the attempt to further ameliorate the expansion rates especially for those patients considered poor expanders. Possible variations are investigating the addition of other cytokines like IL1, IL7, IL2 or IL 15 with the scope of increasing their proliferation and cytotoxic potential (73-75). Our group recently proposed to exploit the residual functionality of TCR expressed on CD3+CD56- CIK cells to provide an allogeneic stimulation and improving the final expansion rate of CIK cells. We demonstrated that allo-stimulated CIK cells displayed significant higher proliferation rates compared to standard protocols, both in patients and healthy donors. Data were confirmed on patients who would otherwise be considered poor CIK-expanders. Allo-stimulated CIK cells retained the full cytotoxic potential and did not present significant phenotypic differences with the standard counterpart. An interesting observation was that allo-stimulated CIK cells were endowed with a decreased alloreactive potential against HLA-mismatched third parties compared to standard CIK cells. In clinical perspectives this could have important implications for those strategies considering the infusion of donor cells across HLA barriers following HCT, with a potential reduction of graft versus host disease (GVHD) risk (76).

In general, even if further improvements are certainly possible, it has to be considered that the current standard protocol for ex-vivo expansion of CIK cells is quite efficient and most importantly has been validated in GMP conditions and already tested in clinical trials (77, 78).

When evaluating new strategies that may supposedly improve the expansion efficiency of CIK cells, besides the quantity parameters, transferability in GMP conditions, costs and requirement of new phase I trials are issues that need to be carefully considered.

Tumor killing activity and clinical applications.

CIK cells have been described able to kill several types of solid tumors and hematologic malignancies in MHC-unrestricted manner (60-62, 79). The main mechanism exploited to recognize tumor cells occurs through the interaction of the NKG2D receptor on CIK cells with its ligand on tumor targets (80-82). The principal ligands recognized by NKG2D are stress-inducible molecules, MIC A/B and proteins of the ULBP family, expressed by the majority of tumor histotype and almost not present on normal tissues (82-85). The activity of NKG2D is associated with the upregulation of the adaptor molecule DAP10 on CIK's membrane induced by the exposure to IL2 along the ex-vivo culture, underlying the importance of this cytokines for the generation of functionally effective CIK cells. NKG2D does not cover however the entire cytotoxic activity of CIK cells and other molecules are likely to be involved even if at lesser extent (80). DNAM -1, NKp-30 and LFA-1 have been recently described as capable of mediating at least in part the tumor killing activity of CIK cells (62, 86). The final killing mechanism of CIK cells is operated by perforin and granzyme (72). As previously mentioned, the cellular subset mainly responsible for the tumor killing is that with a CD3+CD56+ double positive phenotype, even if a certain degree of activity is also retained by the CD56 negative subset and usually CIK cells as used as bulk population (61).

The antitumor activity of CIK cells has been demonstrated in several preclinical models against various solid and hematologic malignancies, reporting a significantly increased efficacy compared to LAK cells (61, 87, 88).

Clinical translation of adoptive immunotherapy with CIK cells have started in the early nineties and clinical trials increased in the very recent years.

In the very first phase I report the group of Schmidt-Wolf described the safety of CIK cells infusion in 10 patients with metastatic solid tumors and refractory Non-Hodgkin lymphomas (NHL), also reporting initial clinical responses. In this first approach, CIK cells were slightly different from the standard as they were also genetically engineered to autonomously produce IL-2 (89). Other following clinical studies subsequently confirmed the safety and activity of adoptively infused CIK cells against NHL and other solid malignancies, especially hepatocellular carcinoma (HCC) and renal cancer carcinoma (RCC). Olioso et al. reported 3 complete responses in a trial involving 12 patients including HCC, RCC and NHL (90). It is important to underline that a large numbers of interesting clinical trials have been reported in the very last years in Asia mostly by Chinese research groups. Important results were described in the setting of advanced NSCLC and gastric cancer, where large studies reported significant impact of CIK cells on overall and progression free survival when associated to conventional chemotherapies (91). Interesting data were also presented with CIK cells infused as adjuvant treatment following surgery for HCC (92-94). A very recent randomized study in metastatic RCC described a significant beneficial effect of CIK cells compared to a combination of IFN-alpha and IL-2 in the setting of metastatic RCC, with improvement on overall survival reported (95). Overall these clinical studies certainly confirm the feasibility and safety of adoptive immunotherapy with CIK cells and are strongly suggestive of a potential clinical activity. It has to be acknowledged that some of these trials present a certain degree of variability in treatment schema or data analysis, making it still premature to draw definitive conclusion about the real impact of CIK cells in such challenging settings. In the attempt to set up standardized and reliable criteria to design new trials with CIK cells, an international registry has been recently activated (96).

New perspectives

Perspective research strategies are currently under investigation in the attempt of improving the antitumor activity of CIK cells. Among the most promising approaches is worth to mention the possibility to redirect the antitumor activity using bispecific antibodies. Such antibodies would link both a specific antigen on tumor cells and the CD3 molecule on CIK cells, determining their localization on tumor targets, promising preclinical data have been reported with this approach against solid and hematologic malignancies (97-99). Another appealing possibility is that of engineering CIK cells with vectors encoding for tumor-specific TCR, with the scope of endowing CIK cells with a simultaneous MHC-restricted and unrestricted activity (100, 101). Even if not directly in the topic of this review, a peculiar application of

immunotherapy with CIK cells that is worth to mention is that as alternative to conventional DLI after allogeneic HCT. The reduced alloreactivity of CIK cells was demonstrated to translate into a reduced risk of GVHD, along with a retained activity against the underlying hematologic disease (72, 102). Very informative and well controlled clinical trials have been conducted in this setting (78, 103).

iNKT cells .

NKT cells are a small subset of human lymphocytes expressing both αβTCR and markers of NK lineage.
The TCR expressed by the majority of circulating NKT cells is invariant, composed by the Vα24-Jα18 α-chain with Vβ11 β chains (37, 38). This is the major NKT subset, referred as type I, and can be either positive for CD4 molecule expression or in alternative CD4-CD8- (double negative) (104). Here we will focus only on type I NKT as mainly involved in antitumor activity and exploited for cancer immunotherapy, while we will not discuss type II NKT endowed with a more heterogeneous TCR repertoire and exerting regulatory and potential immune-inhibitory functions.
NKT cells with α24 β11invariant TCR (iNKT) are activated by glycolipid antigens, restricted by the monomorphic CD1-d molecule (105). MHC-like CD1d molecules are constitutively expressed by antigen-presenting cells (APCs) such as dendritic cells (DCs), B cells, and macrophages capable of internalizing and processing lipid antigens prior to presentation on their surfaces (106). CD1d molecules are also highly expressed in thymic stroma where they are required for development of iNKT cells (107)The physiologic role of iNKT cells seems to be providing a quick response to infections and inflammation but they are also likely to exert tumor immune-surveillance functions.
The most well-characterized glycolipid ligand recognized by iNKT cells is α-galactosylceramide (α-GalCer-KRN7000) discovered initially in marine sponges. Upon activation iNKT cells may exert MHC-unrestricted type antitumor activity. Direct cytotoxicity has been reported against several tumor targets, with mechanisms involving perforin/granzyme, FAS-ligand and TRAIL (43-48). Furthermore iNKT may exert an adjuvant immunotherapy function, bridging innate and adaptive immunity by stimulating other effectors (NK; T cells, B cells, DC) through activation of Th1 cytokines cascade (49, 50).
When activated, NKT cells respond with vigorous cytokine production, IFNγ, IL-4, IL-10, IL-13, IL-17, IL-21 and tumour necrosis factor (TNF) within 1–2 hours of TCR ligation (51-54). The cytokine response is rapid and relates in part to the presence of preformed mRNA for cytokines such as IFN-γ and IL-4. iNKT activated by α-GalCer-pulsed antigen-presenting cells (APCs) have been proved able to exert preclinical antitumor activity in-vitro and in vivo within murine models of various type of cancer (108-110).
Overall, clinical observations and experimental data suggest numeric decrease and often functional impairment of iNKT cells in cancer setting of both solid and hematologic origin. Restoring adequate numbers of functionally active iNKT is a crucial issue for therapeutic strategies based on this lymphocyte subpopulation.

The expansion and activation of iNKT for anticancer therapeutic purposes has been attempted by 3 main methods, first direct infusion of αGalCer into cancer patients, second the infusion of αGalCer–pulsed APC and third the direct infusion of ex-vivo activated iNKT cells. The first attempt to activate endogenous iNKT was explored in a phase I trial by Giaccone et al (111) who treated 24 patients with metastatic solid tumors with intravenous injection of various doses of αGalCer. This trial only observed cytokine responses (TNF-alpha, GM-CSF, IL12) that were interestingly dependent on the pre-existing levels in circulating iNKT cells. Other groups explored clinical treatments based on the infusion of ex-vivo generated autologous antigen presenting cells (APC), pulsed with αGalCer. Preclinical data in murine models supported this strategy, confirming the ability to induce in-vivo proliferation and tumor localization of iNKT, with reports of inhibition of tumor growth (ref.). NSCLC and cancer of head and neck (HNC) has been the 2 clinical settings mainly explored.
In the first phase I report and in subsequent phase I-II trial, respectively by Ishikawa et al. and by Motohashi et al., the safety and tolerability of this approach was confirmed in patients with refractory NSCLC (59-61). The administration of αGalCer-pulsed APC determined the expected in-vivo increase (2 fold or greater) of circulating iNKT cells in 1/11 patients and 6/17

patients. APCs in these trials were ex-vivo cultured for 2 weeks with IL2 (100 JU/ml), GM-CSF (800 U/ml) and pulsed with 100ng/ml of αGalCer on the day before administration. IL2 and GM-CSF stimulated APCs were demonstrated to be more effective than DC induced with IL4 and GM-CSF to activate iNKT (62). The ability of in-vivo stimulated iNKT to increase and localize at tumor site was confirmed by Nagato et al. (112) with preoperative administration of αGalCer-pulsed APCs in patients with operable NSCLC. Even if not clear objective clinical responses were observed, 3 stable diseases were reported (59) and the increase in IFN-γ producing cells, including both NK and NKT cells, was described and correlated with survival (61). Higher rates of in-vivo expansion and persistence (>100 fold) of iNKT were reported by Chang et al, following the intravenous administration of αGalCer-pulsed APCs in patients with Multiple Myeloma, anal and RCC. An interesting observation of this study was the activation and induction of antigen-specific T cells, associated with iNKT expansion, suggestive of their advocate adjuvant effect (64). Furthermore bio-humoral responses were reported in patients with MM and SD in patient with RCC.

A peculiar protocol to in-vivo induce iNKT activation and proliferation was reported in the setting of head and neck cancer, where patients were treated with nasal submucosal injection of αGalCer-pulsed DC. The submucosal injection required sensibly lower doses of DC, compared to intravenous administration, to induce iNKT proliferation that was described in 4 out of 9 patients, with increase in their functional activity (65). A different approach explored the possibility to infuse NSCLC patients with ex-vivo expanded iNKT cells (113). Preclinical data (66) had demonstrated the efficient ex-vivo expansion of iNKT cells by repeated stimulation of PBMC with αGalCer confirming their effectiveness in killing tumor target cells (44). Motohashi et al (113) co-cultured iNKT cells with IL2 and αGalCer for 2 or 3 weeks before their reinfusion in chemorefractory NSCLC patients. Ex-vivo activated iNKT displayed a Th1 phenotype (114, 115) and produced expansion of iNKT upon reinfusion in 2 out of 3 patients, data suggestive for adjuvant effect on NK cell activation were reported (113).

An interesting combinatory approach explored the intrarterial infusion of in-vitro expanded iNKT combined with the submucosal injection of αGalCer-pulsed APC in a small cohort of patients with head and neck cancer. The procedure resulted in iNKT expansion and increased IFN-γ secretion in the peripheral blood of 7 out of 8 patients (116). Three partial responses and 4 SD were encouraging clinical results warranting further investigations of this approach.

In summary, iNKT cells may represent a useful tool in the composite scenario of cancer immunotherapy. Their expansion, either ex-vivo or in-vivo, is required and current protocols need further refinement. Even if clinical responses in the initial clinical trials were few, they represent important proofs of principle and suggest that, considering the iNKT mechanism of action, interesting perspectives may be seen as adjuvant of other immunotherapy approaches.

Conclusive remarks and perspectives

Adoptive immunotherapy strategies with MCH-unrestricted effectors like NK and CIK cells hold promises as innovative and safe treatments in challenging settings. They provide contribute of effectiveness and simplicity and may address some of the main limitations that have so far impaired the clinical transferability of other immunotherapies. MHC-unrestricted effectors, mainly referring to NK and CIK cells, can provide clinically relevant amounts of effective cells by relatively simple and not expensive culture conditions, exploiting their response to specific cytokines. On the contrary, other strategies aiming at expanding T lymphocytes specific for a given tumor antigen, usually struggle with the extreme paucity of circulating precursors and difficulties to get adequate expansion rates. Furthermore, the MHC-unrestricted mechanism of tumor recognition does not limit the applicability of these strategies to only patients with a given HLA-haplotype and could be applicable to multiple tumor histotype, in other words expanding the number of patients that could potentially benefit from a single strategy. It has to be considered that one of the main immune-escape mechanisms of tumor cells is given by the downregulation of MHC molecules, mechanism obviously not affecting but even stimulating MHC-unrestricted approaches. The main features of NK, CIK and iNKT cells are summarized in table 1.

A general drawback of MHC-unrestricted strategies may be seen in the incapacity to generate immunologic memory capable of durable and protective responses. From such angle active immunizations like cancer vaccines may appear more advantageous, however it is counterbalanced by the high ex-vivo expansibility of MHC-unrestricted effectors allowing for

multiple and repeatable infusions. As mentioned in this review, an appealing perspective is emerging with the synergism of MHC-unrestricted immunotherapy with other MHC-restricted approaches (e.g. vaccines, TAA-specific lymphocytes) or even with conventional chemotherapies or molecular targeted approaches. Such perspective is of course facilitated by the extremely favorable toxicity profile of adoptive immunotherapy with NK, CIK or NKT cells. Target molecules recognized by both NK and CIK cells, (e.g. MIC A/B and ULBPs) are stress inducible proteins and research efforts are investigating their potential modulation by conventional or new drugs (e.g. Oxaliplatin, Bortezomib, lenalidomide,), with consequent impact on their susceptibility to immune-mediated killing. Similar possibilities have been described inducing upregulation of TRAIL and FAS ligands on tumor cells. Synergism is probably the key for future applications, even looking at new agents blocking crucial inhibitory immune-checkpoints like CTL-A4 and PD-1 axis, often exploited as immune-escape mechanisms within the tumor microenvironment.

Other types of issues that adoptive immunotherapy strategies will have to face for their wider clinical transferability and to improve their effectiveness are of logistic and regulatory nature. Cell products have to be prepared within certified facilities compliant with stringent GMP requirements. To this end the simplicity and cost-effectiveness previously described for the expansion and generation of MHC-unrestricted effectors like NK and CIK cells are added values to consider. Furthermore, a wishful future scenario to appreciate and benefit from immunotherapy strategies should consider shifting the time of treatment at earlier stages of disease, avoiding high tumor burdens and heavily pretreated patients.

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Table 1. Main features of NK, CIK and iNKT cells.

	Main phenotype	Potential activation in-vivo / Main cytokines	Potential ex vivo activation - expansion / Main cytokines	Precursors for ex vivo activation-expansion	Main molecules involved in direct tumor killing	Cell source/clinical application
NK Cells	CD3-CD56+, CD16+, TCR-	Yes / IL2	Yes (++) / IL2, IL15, Feeder	Circulating NK cells	KIR; NKG2D; TRAIL; FAS-L, DNAM-1	Autologous or Allogeneic /solid and hematologic tumors
CIK Cells	2 subsets: 1) CD3+CD56+, 2) CD3+CD56- αβTCR (variable)	No	Yes (+++) / IL2, Ab anti-CD3, IFNγ	Naive T cells	NKG2D; DNAM-1; NKp30	- Autologous / solid and hematologic tumors; - Allogeneic / allogeneic HCT
iNKT Cells (Type I)	CD3+CD56+, TCR invariant Vα24Vβ11	Yes / αGal, αGal pulsed DC	Yes (+) / αGal, αGal pulsed DC, IL2	Circulating iNKT	CD1d-αGalCer / Vα24Vβ11 TCR	Autologous (solid tumors)

Table 3. Clinical Trials involving CIK and iNKT cells

CIK cells			iNKT cells		
Reference	**Type of Treatment**	**Clinical Setting**	**Reference**	**Type of Treatment**	**Clinical Setting**
Schmidt-Wolf et al.,1999[92]	Autologous IL2-engineered CIK cells	CRC, RCC, NHL	Giaccone et al., 2002[128]	Intravenous αGalCer	Solid Tumors
Olioso et al., 2009[93]	Autologous CIK cells	RCC, HCC	Motohashi et al., 2006[132]	Intravenous infusion of ex-vivo expanded iNKT cells	NSCLC
Wu et al., 2008[94]	Autologous CIK cells + CHT	NSCLC	Ishikawa et al., 2005[131]	Intravenous infusion of αGalCer-pulsed APCs	NSCLC
Hui et al., 2009[95]	Autologous CIK cells	HCC (adjuvant treatment)	Uchida et al., 2008[136]	Nasal submucosal injection of αGalCer-pulsed APCs	HNSCC
Weng et al., 2008[96]	Autologous CIK cells	HCC (adjuvant treatment)	Motohashi et al., 2009[133]	Ex-vivo generated αGalCer activated NKT cells	NSCLC
Jiang et al., 2006[154]	Autologous CIK cells + CHT	Gastric cancer	Nagato, K et al., 2012[134]	Ex-vivo generated APC pulsed with αGalCer	NSCLC
Shi et al., 2004[97]	Allogeneic (CB derived) CIK cells + CHT	HCC	Kunii, N et al., 2009 [139]	Intrarterial iNKT+ Nasal submucosal injection of αGalCer-pulsed APCs	HNSCC
Niu et al., 2011[155]	Autologous CIK cells + CHT	NSCLC, gastrointestinal tumors	Chang et al., 2005[135]	Intravenous infusion of αGalCer-unpulsed and αGalCer-pulsed APCs	Mel, RCC, HCC
Jiang et al., 2010[156]	Autologous CIK cells + CHT	Gastric cancer			
Liu et al., 2012[98]	Autologous CIk cells	RCC			

Abbreviations: CIK, Cytokine-induced killer; iNKT, invariant Natural Killer T cells; CRC, colorectal cancer; RCC, renal cell carcinoma; NHL, Non-Hodgkin lymphoma; HCC, Hepatocellular carcinoma; NSCLC, Non-small cell lung cancer; HNSCC, Head and Neck Squamous Cell Carcinoma; Mel, Melanoma; CHT, chemotherapy; CB, cord blood; αGalCer, α-Galactosylceramide; APC, Antigen-Presenting Cell