NK cell infiltration is associated with improved overall survival in solid cancers: A systematic review and meta-analysis

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A R T I C L E I N F O

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A B S T R A C T

The immune landscape of a tumor is highly connected to patient prognosis and response to treatment, but little is known about how natural killer (NK) cells predict overall survival (OS) among patients with solid tumors. We present the first meta-analysis on NK cell infiltration into solid tumors as a prognostic indicator for OS, considering cancer types independently, and together. Samples were collected from 1973 to 2016 with results published between 1989 and 2020. From 53 studies, we found that NK cell infiltration correlates with decreased risk of death (HR=0.34, 95% CI: 0.26–0.46; p<0.0001). Among studies that investigated the prognostic potential of NK cells in different regions of the tumor, intraepithelial infiltration was better predictive of OS than NK infiltration in the tumor-adjacent stroma. Generally, NK cell infiltration is lower in advanced-stage and lower-grade tumors; nevertheless, it remains prognostically beneficial. This meta-analysis highlights an important prognostic role of NK cells in solid tumors, but exposes that few studies have considered the contributions of NK cells. Toward NK cell-based immunotherapies, it will be important to understand the conditions under which NK cells can be effective agents of tumor control.

Introduction

Infiltration of solid tumors by immune cells with anti-tumor activity is both a strong prognostic factor and a therapeutic goal. Specific immune populations can have anti- or pro-tumor roles; the balance of their activity conditions the tumor “microenvironment” (TME) and can predict responses to treatment and overall survival (OS). Characteristics of the tumor itself, including underlying mutations, progression (stage and grade), vascularization, metabolism and the soluble factors it produces also contribute to the TME with impacts on immune cell infiltration and activation. Interactions in the TME are complex, and identifying key features for prognostic and therapeutic targeting is key to developing effective immunotherapies for solid tumors. Here, we present a meta-analysis that demonstrates NK cell infiltration is correlated with decreased risk of death across solid tumor origins, grades and stages.

Tumors can be considered as two structural compartments: the epithelial region, which encompasses the malignant cells within an epithelial lining, and the stromal region, which represents its supportive tissue. The TME and immune reactivity varies within these compartments, which can result in different functional associations for the same leukocyte population. For example, a study conducted in early-stage tongue cancer found lymphocytes infiltrating the intraepithelial compartment frequently expressed the immune checkpoint receptors, PD-1 and NKG2A, but their counterparts in the stroma did not. This underscores the importance of quantifying immune cells within the context of tumor compartments, which requires in situ analysis of intact tissue.

Select immune cell populations, mainly T cells, have been the focus in immuno-oncology. A number of systematic reviews and meta-analyses have been conducted to explore the prognostic value of T cell infiltration in a variety of solid tumors. T cell infiltration generally predicts better survival, and further phenotyping of T cell subsets can reveal more informative associations. For example, infiltration of regulatory T cells (FoxP3+) can be associated with both improved and poorer survival, while the infiltration of cytotoxic T cells (CD8+) is strongly positively correlated with improved OS. Other assessments have revealed prognostic benefits of B cells and M1 macrophages but relatively few studies, in specific cancer subtypes, have analyzed the impact of other lymphocyte populations, including NK cells. As innate controllers of cancer and emergent targets for immunotherapy, understanding the prognostic value of NK cells in solid tumors is overdue.

© One sentence summary: NK cell infiltration into solid tumors is a positive prognostic factor for overall survival.

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NK cells are innate lymphocytes that originate in the bone marrow from the common lymphoid progenitor and comprise 5–15% of the total peripheral lymphocyte population. They can adapt in response to challenge, but do not require sensitization or specific antigens to mount an effective immune response. NK cells recognize danger signals, or stress induced ligands, upregulated in response to DNA damage, trauma or proinflammatory cytokines, and may underlie tumor development. Human NK cells are most often defined and experimentally marked as CD56+CD3− cells, and broadly characterized based on CD56 expression as either circulating, cytokine-producing NK cells (CD56dimCD16−), or tissue-infiltrating, cytotoxic NK cells (CD56brightCD16+). In reality, NK cells are a diverse collection of functionally dynamic lymphocytes, with up to 30,000 unique NK cell phenotypes comprising the repertoire of each individual.

NK cells express and co-express an array of germline-encoded receptors to engage with putative target cells. The outcomes of these interactions can be activating, regulatory or inhibitory; “self” human leukocyte antigen (HLA) is a major signal for inhibition. Since HLA negatively regulates NK cell activation, loss of “self” HLA lowers the threshold for NK cell activation. This function is important in recognition of tumors. Additionally and important anti-tumor roles played by NK cells involve conditioning of the TME for inflammation, and antibody-dependent cell cytotoxicity (ADCC) to facilitate tumor control by monoclonal antibody therapies such as Trastuzumab, Dinutuzumab, or Cetuximab.

The ability of NK cells to control cancer is underscored by their importance in hematopoietic cell transplantation, where NK immunogenetic configurations predict leukemia control or relapse. These observations prompted clinical trials based on adoptive transfer of unmodified or ex vivo-expanded NK cells, including NK cells genetically modified to express a chimeric antigen receptor (CAR). Current clinical trials investigating adoptive NK cell transfer to treat solid tumors include melanoma (NCT0328861, NCT03470922), kidney cancer (NCT03238661), head and neck cancers (NCT02643550), glioblastoma (NCT02658981), gynecologic malignancies (NCT0245301), and other metastatic (NCT03415100) and non-metastatic solid tumors (NCT01875601, NCT01212341, NCT03940820, NCT02671435, NCT01968109). Compared with hematologic malignancies, solid tumors may present an additional challenge, where infiltration will be key to tumor control.

We retrieved, compiled and meta-analyzed studies that associated NK cell infiltration with survival outcomes. We found that increased NK cell infiltration is associated with a decreased risk of dying in patients with solid tumors. Our findings highlight associations between OS and NK cell sub-tumor localization, grade and stage, endorse assessments of NK cells for prognostication of solid tumors and inform more precise NK-based immunotherapy.

Methodology

Data sources and search strategy

We devised a comprehensive search strategy based on the following three key terms: (1) prognostic value, (2) natural killer cell and (3) tumor. Terms used to represent prognostic value included mortality, survival or outcome. Natural killer cells were searched using the terms natural killer cells, NK cells or innate lymphoid cells. To limit our search to those applicable to cancer we searched tumor (tumor), tumor (tumor) infiltration or neoplasm (for full search terms, see Supplemental Data 1). We applied these search terms to EMBASE and PubMed on February 11th, 2020. Through our search we captured a total of 13,591 peer-reviewed studies; 7639 from EMBASE and 5952 from PubMed (Fig. 1). The title, author and study details, from each database were exported and pooled in Microsoft Excel 365.

Eligibility criteria

Once a final list of articles was obtained and duplicates removed, 10,716 studies remained. These studies then underwent abstract review to assess article suitability on the following exclusion criteria: not written in English, not full primary research articles or not within the scope of this review (not on topic) were removed (i.e. studies in animals, on nucleic acids exclusively or that otherwise did not meet our requirement for NK cell evaluation in solid human tumors). Following this, the remaining articles were assessed to ensure they met all of the inclusion criteria: 1) employed immunohistochemistry (IHC) for NK cells using an appropriate marker/markers on intact solid tumor tissue; 2) studied treatment-naive adult tumors; and 3) reported an endpoint survival analyses with correlated NK cell infiltration data.

Data extraction and quality assessment

From each of the final articles (n = 53), we collected the following information: name of first author, DOI, year of publication, number of patients, female: male ratio (biologic sex), tumor stage, tumor grade, NK cell marker used for IHC, other markers analyzed, method of NK cell quantification and stratification (definition of “high” versus “low” NK infiltration), mean number of NK cells, hazard ratio (HR) for death (95% CI and p-value) and survival-based outcome (OS), disease-free survival (DFS), progression-free survival (PFS) and associated p-values (Table 1). The overall quality of the article was evaluated but due to the low number of studies, we did not exclude studies based on quality assessment (Table 1 and Supplementary Table 1). The studies were given a quality score of 12 based on the inclusion of: subtype identification, sex ratios, age (average or range), stage identification, period of cohort collection, ethics reported, >1 IHC antibody, quantification strategy, stratification strategy, pathologist validation, HR for NK cell infiltration, and p-value for NK cell infiltration (Supplemental Table 1).

Analysis

Studies that reported HR values representing the risk of death in patients with NK cell infiltration were compiled for meta-analysis. We conducted specific sub-studies to evaluate differences that may be attributable to differences in markers or methods used. Meta-analysis was conducted in R Studio (version 3.6.3; for code see Supplemental Data 2) using the R package ‘meta’ and “metaphor” following the random-effects model, specifically the Hartung-Knapp-Sidik-Jonkman method. This method was chosen to account for the maximum study heterogeneity. Interstudy heterogeneity was quantified using the I2 statistic, with an I2 value>50% as our a priori threshold for substantial heterogeneity. All graphical representation of statistical results including Forrest plots and p-value visualization were created using GraphPad Prism 8.

RESULTS

NK cell infiltration predicts improved OS in patients with solid tumors

We identified 53 studies representing a total of 9624 patients and encompassing a variety of tissue origins and cancer subtypes. The details of these studies including cancer subtype, sample size, IHC marker, infiltrating NK cell stratification, HR values and impact on survival are summarized in Table 1.

Cancer types explored in the studies included head and neck, breast, colorectal, gastric, liver, ovarian, endometrial, vulvar, kidney, sarcoma, melanoma, peritoneal adenocarcinoma, gallbladder and glioblastoma. Most studies identified NK cells using antibodies against CD57 (56.6%) followed by CD56 (41.5%) and NKP46 (9.4%); four of the 53 studies used both CD57 and CD56. All 53 studies (54 studies separating the esophageal and gastric patients in Svensson
Table 1
Study characteristics. (Studies conducted on multiple cancer tissue sites were separated into respective cancer categories).

| Study                | Year  | Period of Sample Collection | Cancer Type          | Cancer Subtype                        | n    | Sex Ratio (F:M) | Age (Mean (Range)) | NK Cell Marker (clone) | Stratification                  | NK Cell Number | HR of Death (95% CI, p-value), multivariate analysis | Impact on survival
|----------------------|-------|-----------------------------|----------------------|---------------------------------------|------|----------------|---------------------|-------------------------|---------------------------------|----------------|------------------------------------------------|-----------------|
| Cho et al.\(^{16}\) | 2003  | 1989 – 1999                  | Head & Neck Cancer   | Esophageal squamous cell carcinoma    | 122  | 17:105         | 62.3 (NR)           | CD57 (Leu 7)         | Quantified in the stroma into 4 groups: • most abundant • abundant • moderate • scanty | Median = 0.9NK cells/ HPF | NR                                                      | No change, OS | (p = 0.47)                                |
| Fang et al.\(^{35}\) | 2017  | 2007 – 2009                  | Head & Neck Cancer   | Oral squamous cell carcinoma          | 78   | 21:57          | 60 (24–82)          | CD57 (ab82749)       | Mean NK cell number: • (15.75 NK cells) • ) 15.75 NK cells | Median = 15.75 cells/ HPF | NR                                                      | Improved, OS   | (p < 0.001)                                |
| Hsia et al.\(^{36}\) | 2005  | 1994 – 1996                  | Head & Neck Cancer   | Esophageal squamous cell carcinoma    | 38   | 0:38           | NR                  | CD57 (NR)             | Median NK cell number: • (25 NK cells/ 25 fields) • ) 25 NK cells/ 25 fields | Median = 25 cells/ 25 fields | 0.61 (0.21 – 1.82, p = 0.378) | Improved, OS | (p = 0.007)                                |
| Lazaris et al.\(^{37}\) | 2007  | NR                          | Head & Neck Cancer   | Laryngeal carcinoma                  | 31   | 1:30           | 61.3 (42–75)         | CD56 (T119), CD16 (VIFcRII) | Quantified in the parenchyma: • low, <5% of lymphocytes • intermediate, 5–20% • high, >20% | Median NK cell number | NR                                                      | No change, DFS | (p = 0.66)                                |
| Lu et al.\(^{38}\) | 2017  | 2002 – 2003                  | Head & Neck Cancer   | Nasopharyngeal carcinoma              | 197  | 51:146         | 45.22 (NR)           | CD56 (NR)             | Median NK cell number | NR                                                      | Improved, OS | (p = 0.001)                                |
| Lv et al.\(^{19}\) | 2011  | 2002 – 2003                  | Head & Neck Cancer   | Esophageal squamous cell carcinoma    | 181  | 40:141         | 56 (33–79)           | CD57 (NR)             | Median NK cell number | NR                                                      | Improved, OS | (p = 0.002)                                |
| Schoenfeld et al.\(^{40}\) | 2017  | 2004 – 2013                  | Head & Neck Cancer   | Oropharyngeal squamous cell carcinoma | 81   | 17:64          | 64 (49–87)           | CD56 (NR)             | NK cell presence; • present • absent | NR                                                      | No change, OS | (NR)                                     |
| Svensson et al.\(^{41}\) | 2017  | 2006 – 2010                  | Head & Neck Cancer   | Esophageal squamous cell carcinoma    | 97   | NR             | NR                  | NKP46 (NR)            | Median NK cell number | Median (based on age) = 1.89 (<avg age) or 1.93 (> avg age) NK cells/ field | Median (based on gender) = 1.99 (female) or 1.89 (male) | 0.49 (0.28 – 0.86, p = 0.012) | Improved, OS | (p = 0.008)                                |

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| Study                  | Year  | Period of Sample Collection | Cancer Type                              | Cancer Subtype                              | n     | Sex Ratio (F:M) | Age (Mean (Range)) | NK cell Marker (clone) | Stratification                        | NK Cell Number | HR of Death (95% CI, p-value), multivariate analysis | Impact on survival [High NK cell population] |
|-----------------------|-------|-----------------------------|------------------------------------------|---------------------------------------------|-------|----------------|-------------------|-----------------------|------------------------------------------|----------------|------------------------------------------------------------------------------------------|-------------------------------------------------|
| Taghavi et al.       | 2016  | NR                          | Head & Neck Cancer                       | Oral squamous cell carcinoma                | 57    | 30:27          | 62.89 (34–91)     | CD57 (2H7), CD16 (NK-1) | Median NK cell number:                   | NR             | 0.058 (0.013 – 0.26, p < 0.001)                                                       | Improved, OS (p = 0.001)                          |
| Tsuchikawa et al.    | 2011  | 1989 – 1999                 | Head & Neck Cancer                       | Esophageal squamous cell carcinoma          | 98    | 14:84          | 62.9 (51.9–71.9)  | CD57 (Leu 7)          | Quantified in the stroma:                 | NR             | No change, OS (p = 0.31)                                                                |                                                                                                           |
| Wagner et al.        | 2016  | 2000 – 2009                 | Head & Neck Cancer                       | Oropharyngeal squamous cell carcinoma       | 140   | 34:105         | 59 (38–84)        | CD56 (1B6)            | Quantified as:                           | NR             | 0.32 (0.10 – 0.96, p = 0.042)                                                          | Improved, OS (p = 0.038)                          |
| Xu et al.            | 2016  | 2006 – 2011                 | Head & Neck Cancer                       | Esophageal squamous cell carcinoma          | 138   | 36:102         | NR                | CD57 (NR)             | Quantified as:                           | NR             | 0.60 (0.39 – 0.91, p = 0.016)                                                          | Improved, OS (p = 0.019)                          |
| Zancopé et al.       | 2010  | NR                          | Head & Neck Cancer                       | Oral and lip squamous carcinoma, Oral: 40 Lip: 30 | 70    | 64:36          | NR                | CD57 (NK1)            | Median NK cell number:                   | NR             | No change, OS Intra-epithelial (p = 0.70)                                                   | Stromal (p = 0.69)                                |
| Honkanen et al.      | 2017  | 2009 – 2014                 | Breast Cancer                            | HER2+                                      | 48    | 48:0           | NR                | CD56 (MRQA-42)         | Quantified as:                           | NR             | No change, OS (NR)                                                                       |                                                                                                           |
| Muntasell et al.     | 2018  | 2008 – 2016                 | Breast Cancer                            | HER2+                                      | 113   | 113:0          | 57 (36–88)        | CD56 (123C3)           | Quantified as:                           | NR             | Improved, DFS (DC: p = 0.04, VC: p = 0.10)                                               | (continued on next page)                         |
| Study                  | Year  | Period of Sample Collection | Cancer Type          | Cancer Subtype       | n     | Sex Ratio (F:M) | Age (Mean (Range)) | NK cell Marker (clone) | Stratification                                      | NK Cell Number | HR of Death (95% CI, p-value), multivariate analysis | Impact on survival High NK cell population |
|-----------------------|-------|-----------------------------|----------------------|----------------------|-------|----------------|---------------------|----------------------|--------------------------------------------------|----------------|------------------------------------------------|-----------------------------------------------|
| Park et al.           | 2012  | 1997 – 2002                 | Breast Cancer        | Invasive ductal carcinoma | 204   | 204:0          | ≤46= 113 patients >46= 85 patients 49.13 (25–86) | CD57 (TB01) | Quantified as: • low (absent) • High (otherwise) | Str: 1.10 cells/NR | NR                                           | No change, OS (p = 0.367) DFS (p = 0.358) Poorer, OS (p = 0.05) |
| Rathore et al.        | 2014  | NR                          | Breast Cancer        | Invasive ductal carcinoma | 175   | 175:0          | Mean NR (28–75) | NKp46 (ab199128) | Quantified in Str and Epi each as: • low <25 cells/25 fields • high >25 cells/25 fields | NR                                           | 1.92 (1.08 – 3.57, p = 0.05) | |
| Tian et al.           | 2016  | 2006 – 2008                 | Breast Cancer        | Invasive ductal carcinoma | 278   | 278:0          | Mean NR (28 – 75) | CD56 (1BC) | Quantified in Str based on density: • 0 (absent) • 3 (dense) | NR                                           | 0.54 (0.39 – 0.74, p = 0.001) | Improved, OS (p = 0.018) Improved DFS, (p < 0.001) |
| Triki et al.          | 2019  | NR                          | Breast Cancer        | NR                    | 158   | 158:0          | 118 patients > 40 40 patients < 40 | CD56 (1BC) | Quantified as: • low (negative or weak infiltration) • high (moderate or strong infiltration) | NR                                           | 0.17 (0.039 – 0.73, p = 0.017) | Improved, OS ER+; p = 0.007 PR+; p = 0.018 HER+ p = 0.287 |
| Vgenopoulou et al.    | 2003  | NR                          | Breast Cancer        | Invasive ductal carcinoma | 64    | 64:0           | NR                  | CD57 (TB01) | Quantified based on staining intensity as: • Weak-moderate • Strong | NR                                           | NR                                           | No change, D5S (NR) |
| Wang et al.           | 2014  | 2006 – 2007                 | Breast Cancer        | ALDH1 high            | 212   | 379:0          | 212 high, 379 low | CD56 (NR) | Quantified as: • CD56 low <5 CD56+ cells • CD56 high >5 CD56+ cells | NR                                           | 1.10 (0.50 – 2.44, p = 0.81) | No change, OS (NR) |
| Alderdice et al.      | 2017  | DC: 2004 – 2013 VC: 2001 – 2005 | Colorectal Cancer | Locally advanced rectal cancer | 150   | NR            | NR                  | CD56 (NCL-L-C56-1B6) | Quantified as: • 4 CD56+ cells • 4 CD56+ cells | NR                                           | 0.11 (0.014 – 0.81, p = 0.031) 0.28 (0.11 – 0.73, p = 0.005) | Improved, OS (NR) |

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| Study | Year | Period of Sample Collection | Cancer Type | Cancer Subtype | n | Sex Ratio (P:M) | Age (Mean (Range)) | NK cell Marker (clone) | Stratification | NK Cell Number | HR of Death (95% CI, p-value), multivariate analysis | Impact on survival High NK cell population |
|-------|------|-----------------------------|-------------|----------------|---|----------------|------------------|----------------------|---------------|--------------|-------------------------------------------------|-------------------------------------------|
| Coca et al.56 | 1997 | 1977–1990 | Colorectal Cancer | | 157 | 76:110 | 63.4 (29–84) | CD57 (IOT-10, Immunotech, SA) | NK cell infiltration classified as little (≤50 NK cells), moderate (50–150 NK cells), and extensive (>150 NK cells) | NR | 0.4 | Improved, OS and DFS (p < 0.01) |
| Lim et al.57 | 2014 | 1998 – 2007 | Colorectal Cancer | Locally advanced rectal carcinoma | 52 | 18:34 | 63 (NR) | CD56 (NR) CD57 (NR) | Quantified based on staining intensity: • None-poor • Moderate-marked | NR | 0.4 | Improved, OS (p = 0.035) |
| Liska et al.58 | 2012 | 2004 – 2007 | Colorectal Cancer | | 150 | 53:97 | 66.33 (NR) | CD57 (NK1) | Quantified as: (4 NK cells) | NR | 0.4 | Improved, DFS (p = 0.05) |
| Menon et al.59 | 2004 | | Colorectal Cancer | | 93 | 37:56 | 69 (26 – 85) | CD56 (123C3) CD57 (HNK1) | Quantified at high power: • >4 cells/mm² Epi: 5 cells/mm² CD57+ Epi: 2 cells/mm² | NR | 0.43 (0.17 – 1.01, p = 0.03) | Improved, DFS (p = 0.05) |
| Sconocchia et al.60 | 2011 | | Colorectal Cancer | Mucinous (1301) Non-mucinous (174) | 1420 | 741:673 (3 NR) | 71 (30 – 96) | CD16 (NR) CD56 (NR) CD57 (NR) | Quantified as: • <4 cells • >4 cells | Mean = 0.14 ± 0.002 | No change, OS (NR) |
| Sconocchia et al.61 | 2014 | | Colorectal Cancer | | 1410 | NR | NR | CD56 (NR) CD57 (NR) | Quantified as: (4 CD56+ cells) | NR | 0.43 (0.3 – 0.7, p = 0.002) | Improved, OS (p = 0.039) |
| Amoueian et al.62 | 2011 | 2004 – 2008 | Gastric Cancer | | 50 | 12:38 | 68 (NR) | CD56 (123C3) | Quantified at both low (100x) and high (400x) power as: • low • high | Mean = 8 cells/400x | NR | Improved, OS (NS) |
| Ishigami et al.63 | 2000 | 1988 – 1996 | Gastric Cancer | | 169 | 48:121 | 63.8 (30 – 87) | CD57 (NR) | Quantified as: low <25 cells/25 fields • high >25 cells/25 fields | Mean = 0.9 cells/400x | NR | Improved, OS (p < 0.05) |
| Pernot et al.64 | 2020 | | Gastric Cancer | | 40 | NR | 64 (34 – 83) | CD57 (NK1) | Quantified as: low <17% • high >17% | Mean = 2.8 cells/mm² Epi: 3.7 cells/200x Str: 12.3 cells/200x | 0.40 (0.15 – 1.06, p = 0.04) | Improved, OS (p = 0.02) |
| Rusakiewicz et al.65 | 2013 | | Gastric Cancer | Gastrointestinal stromal tumors | 91 | 39:52 | 57 (NR) | Nkp46 (195,314) | Median NK cell number | 0.2 (NR) | Improved, OS (p = 0.0001) | (continued on next page)
| Study              | Year       | Period of Sample Collection | Cancer Type | Cancer Subtype | n     | Sex Ratio (F:M) | Age (Mean (Range)) | NK cell Marker (clone) | Stratification | NK Cell Number | HR of Death (CI, p-value) | Impact on survival |
|--------------------|------------|-----------------------------|-------------|----------------|-------|-----------------|---------------------|------------------------|----------------|----------------|---------------------------|-------------------|
| Svensson et al.    | 2017       | 2006 - 2010                 | Gastric Cancer | Gastric adenocarcinoma | 75    | NR              | 63.5 (39 – 76)     | NKp46 (NR)            | Median NK cell number | Median (based on age) = 1.89 (< avg age) or 1.93 (> avg age) NK cells/field | 0.84 (0.41 – 1.70, p = 0.619) | No change, OS (p = 0.38) |
| Platonova et al.   | 2011       | NR                          | Lung Cancer  | Non-small cell lung carcinoma, squamous cell | 86    | 35:51           |                      | NK46 (NR)              | Quantified as:   | Mean Intratumoral NK cell = 15 cell/mm² |                      | No change, OS (NR)       |
|                    |            |                             |             |                |       |                 |                      |                        |                           | Mean stromal NK cells = 21 cells/mm² |                      |                         |
|                    |            |                             |             |                |       |                 |                      |                        |                           | Mean NK cells = 32 cells/field |                      |                         |
|                   |            |                             |             |                |       |                 |                      |                        |                           | 0.41 (p = 0.12) | Improved, OS (p = 0.0002) |
| Takanami et al.    | 2001       | 1989 - 1994                 | Lung Cancer  | Pulmonary adenocarcinoma | 150   | 67:83           | 61 (30 – 81)        | CD57 (IOT-10)         | Mean NK cell number | Mean NK cell number | 0.99 (0.40 – 0.99, p = 0.046) | Improved, OS and DFS (NR) |
| Villegas et al.    | 2002       | 1986 - 1997                 | Lung Cancer  | Squamous cell   | 50    | 1:49            | 67.2 (50 – 81)      | CD57 (NR)              | Quantified as:    | Mean NK cell number | Improved, OS (p = 0.032) |
|                    |            |                             |             |                |       |                 |                      |                        |                           | Mean NK cell number | Mean NK cell number | 0.66 (0.25 – 1.78, p = 0.41) | Improved, OS (p = 0.032) |
| Yamada et al.      | 2010       | 2007 - 2008                 | Lung Cancer  | Malignant Pleural Mesothelioma: Epithelioid; 26 Biphasic; 14 Sarcomatoid: 4 | 44    | 4:40            | 59 (35 – 85)        | CD56 (1B6)             | Median NK cell number | Median NK cell number | 0.12 (0.043 – 0.31, p < 0.001) | Improved, OS (p = 0.001) |
| Chew et al.        | 2012       | 1991 - 2009                 | Liver Cancer | Hepatocellular Carcinoma | 40    | NR              | 59 (20 – 84)        | CD56 (NR)              | Median NK cell number | Median NK cell number | 0.63 (0.40 – 0.99, p = 0.046) | Improved, OS (p = 0.001) |
| Wu et al.          | 2013       | 2000 - 2004                 | Liver Cancer | Hepatocellular Carcinoma | 256   | NR              | NR                  | CD57 (NK1)             | Median NK cell number | Median NK cell number | 0.38 (0.17 – 0.85, p = 0.019) | Improved, OS (p = 0.005) |
| Zhao et al.        | 2014       | 2003 - 2004                 | Liver Cancer | Hepatocellular Carcinoma | 163   | 32:131          | NR                  | CD57 (NR)              | Median number of NK cells | Median number of NK cells | 0.38 (0.17 – 0.85, p = 0.019) | Improved, OS (p = 0.005) |
| Zhu et al.         | 2009       | 2002 - 2004 2006 – 2007     | Liver Cancer | Hepatocellular Carcinoma | 81    | 6:13, 9:53      | 55 (34 – 75)        | CD56 (NR)              | Quantified as:   | Epi (high) = 11.8 cells/field |                      |                         |
|                    |            |                             |             |                |       |                 |                      |                        |                           | Str (high) = 2.3 cells/field |                      |                         |
|                    |            |                             |             |                |       |                 |                      |                        |                           | Epi (low) = 18 cells/field |                      |                         |
|                    |            |                             |             |                |       |                 |                      |                        |                           | Str (low) = 8.5 cells/field |                      |                         |

(continued on next page)
| Study                  | Year        | Period of Sample Collection | Cancer Type   | Cancer Subtype                          | n   | Sex Ratio (F:M) | Age (Mean (Range)) | NK cell Marker (clone) | Stratification | NK Cell Number | HR of Death (95% CI, p-value), multivariate analysis | Impact on survival | High NK cell population |
|-----------------------|-------------|-----------------------------|---------------|-----------------------------------------|-----|----------------|---------------------|-----------------------|----------------|---------------|-------------------------------------------------|---------------------|------------------------|
| Henriksen et al.³⁶    | 2019        | 2005                        | Ovarian Cancer| High grade serous carcinoma             | 283 | 283:0          | 63 (NR)             | CD57 (NK1)            | Quantified by ROC curve: low <9 cell  
|                       |             |                             |               |                                         |     |                |                     |                      | high >9 cell    | Median = 5 cells/mm²  | 0.67 (0.46 – 0.98, p = 0.041) | Improved, OS (p = 0.031) |                     |
| Li et al.³⁵           | 2009        | 1993 – 2003                 | Ovarian Cancer| Serous, Clear, Transitional, Endometrioid | 82  | 82:0           | 55.3 (26 – 80)     | CD57 (NR)            | Quantified as: CD56+ tumor & stroma   
|                       |             |                             |               |                                         |     |                |                     |                      | CD56+ stroma    | Epi: > 1 cell/200x field in 61% of samples  
|                       |             |                             |               |                                         |     |                |                     |                      | Str: > 2 cells/200x field in 40% of samples | Epi: 0.55 (0.188 – 1.607, p = 0.27) | Epi: Improved, OS (p < 0.05) |                     |
| Ino et al.³⁶          | 2008        | 1992 – 2001                 | Endometrial Cancer |                               | 65  | 65:0           | 57.7 (NR)           | CD57 (NR)            | Quantified as: low <5 cells  
|                       |             |                             |               |                                         |     |                |                     |                      | high >5 cells    | Median = 2 cells/200x  | NR (p = 0.23) | No change, OS (p = 0.17) |                     |                     |
| Versluis et al.³⁷     | 2017        | 1984 – 2004                 | Endometrial Cancer |                               | 355 | 355:0          | NR                  | NKp46 (195,314)      | Patients divided into:  
|                       |             |                             |               |                                         |     |                |                     |                      | Unfavourable outcome (recurrence or death within 5 years of diagnosis) | NR                  | Improved only if with HLA-3 upregulation (NR) |                     |
| Zinovkin et al.³⁸     | 2016        | 2008 – 2009                 | Endometrial Cancer |                               | 82  | 82:0           | NR (45 – 80)        | CD57 (NR)            | Patients divided into:  
|                       |             |                             |               |                                         |     |                |                     |                      | Unfavourable group = 24.3% Favourable outcome | Median CD57 in: Unfavourable group = 45.6% | Improved, OS (p = 0.001) |                     |

(continued on next page)
| Study | Year | Period of Sample Collection | Cancer Type | Cancer Subtype | n | Sex Ratio (F:M) | Age (Mean (Range)) | NK cell Marker (clone) | Stratification | NK Cell Number | Impact on survival | HR of Death (95% CI, p-value), multivariate analysis |
|-------|------|----------------------------|-------------|----------------|----|----------------|-------------------|---------------------|---------------|----------------|------------------|------------------------------------------------|
| Sznurkowski et al. | 2014 | NR | Vulvar Cancer | Squamous cell carcinoma | 76 | 76:0 | 69.5 (36 – 85) | CD56 (123C3) | Median NK cell number | Median = 2 cells/ field | NR | Improved, OS (p = 0.0004) |
| Jasinski-Bergner et al. | 2015 | 1998 – 2011 | Kidney Cancer | Clear cell: 345 Papillary: 49 Chromophobe: 29 Other: 17 | 445 | 166:279 | 63.6 (23 – 92) | CD56 (MRQ-42) | Quantified as: | Median = 28 cells/mm² | NR | No change, OS (p = 0.91) |
| Jensen et al. | 2009 | 1992 – 2001 | Kidney Cancer | | 121 | 61:74 | 61 (19 – 82) | CD57 (NK1) | Median NK cell number | Mean = 0.55 cells/400x field | NR | No change, OS (p = 0.22) |
| Sorbye et al. | 2012 | 1973 – 2006 | Sarcoma | Soft tissue | 249 | 139:110 | NR (20 – 60) | CD57 (NR) | Quantified AS: | • Gr 3 (20+ cells) • Gr 2 (6 – 19 cells) • Gr 1 (1 – 5 cells) • Gr 0 (no cells) | NR | No change, OS (p = 0.62) |
| Erdag et al. | 2012 | 1982 – 2007 | Melanoma | | 147 | 63:84 | 58 (19 – 89) | CD56 | Median NK cell number | Epi = 5.1 cells/mm² Str = 2.5 cells/mm² | NR | No change, OS (p = 0.43) |
| Lundgren et al. | 2016 | 2001 – 2013 | Periampullary adenocarcinoma | Intestinal: 65 Pancreatobiliary: 110 | 175 | 82:90 | 67 (38 – 84) | CD56 (MRQ-42) | Quantified as: | • low <2.75 cells/core • high >2.75 cells/core | NR | Intestinal: 0.23 (0.07 – 0.78, p < 0.05) Pancreatobiliary: 0.59 (0.34 – 0.82, p < 0.05) | Improved, OS (p = 0.002) |
| Nakakubo et al. | 2003 | 1989 – 1999 | Gallbladder cancer | Primary gallbladder adenocarcinoma | 45 | 28:17 | 66.7 (NR) | CD57 (Leu-7) | Quantified as: | • (10 NK cells/ HPF + 10 NK cells/ HPF) Divided into NK cell present or absent groups | Mean = 1.2 cells/200x | Mean = 0.56 (0.20 – 1.58, p = 0.27) | No change, OS (p = 0.27) |
| Vaquero et al. | 1989 | NR | Glioblastoma | | 25 | NR | 55.6 (19 – 69) | CD57 (IOT-10) | Quantified as: | Mean = 6 cells/core | NR | No change, OS (NR) |
et al.\textsuperscript{41} reported the impact of NK cell infiltration on survival measures (Fig. 2A). The majority of studies \((n = 32, 59.3\%)\) reported significantly improved OS, while 21 (38.9\%) reported no significant impact on survival. Of the studies that did not achieve statistical significance by dichotomizing between high and low NK cell infiltration, four reported \(p\) values <0.10, demonstrating trends towards improved OS in the NK cell high groups (Fig. 2B). Notably, only one study (1.9\%) reported significantly poorer OS with higher NK cell infiltration\textsuperscript{50}.

Thirty studies reported HR values, 95% confidence intervals and \(p\)-values and therefore could be included in our meta-analysis. Importantly, these studies employed similar, but not identical, methods of NK cell quantification and stratification (dichotomizing or grouping patient populations based on NK cell infiltration). Meta-analyzed, these studies demonstrated a lower risk of dying in populations with increased NK cell infiltrations (HR=0.34, 95% CI: 0.26-0.46, \(p<0.0001\)) (Table 2, Fig. 3A). Notably, this specific meta-analysis yielded a high level of heterogeneity (\(I^2 = 86.9\\%\), Table 2, Fig. 3B). This statistic indicates that while the majority of studies, across tumor types, found NK cells to be associated with improved OS, additional sub-meta-analyses is warranted by tissue compartment, marker, or within cancer types to strengthen and validate conclusions.

**NK cell infiltration into intraepithelial compartments is most strongly associated with improved OS**

To determine whether the association between NK cells and patient survival extends to different regions within tumors (stromal or epithelial regions), we next examined studies that reported NK cell infiltration at specific tumor regions. Locations included intraepithelial (intra-tumoral, cancer cell nest or center of tumor\textsuperscript{45,47,50,59,75,82,84} \((n=8)\), peritumoral (at the margin of the tumor)\textsuperscript{47,59,82} \((n=3)\) or stromal\textsuperscript{44,45,50,59,75,84} \((n=6)\). A general trend was observed that intraepithelial NK cells had a greater impact on survival compared to NK cells in the surrounding regions\textsuperscript{45,47,50,75,84}. For example, patients with advanced stage esophageal cancer had the highest OS concurrent to high NK cell infiltration into the intraepithelial region and low infiltration in the stroma; conversely, the worst OS was found in patients with low NK cell infiltration into the intraepithelial region, and high infiltration in the stroma\textsuperscript{45}. Overall, NK cell infiltration into the cancer nest significantly improved OS (\(p=0.019\)), whereas infiltration into the stroma did not (\(p=0.65\))\textsuperscript{45}. In another study, patients with ovarian cancer who had only intraepithelial NK cell infiltration had significantly longer OS than those who had stromal infiltration (\(p<0.05\))\textsuperscript{74}; patients with NK cells restricted to the stroma had the lowest OS, even compared with patients who had no NK cell infiltration to their tumors at all\textsuperscript{70}. Noteworthy, only five (6\%) of patients were classified in the stroma infiltration only group\textsuperscript{70}. One notable exception was a study in invasive ductal carcinoma where OS was significantly worse when more NK cells infiltrated into the intraepithelial region (\(p=0.0029\)), but no difference was noted when they infiltrated the stromal region (\(p=0.21\))\textsuperscript{76}. In general, NK cells in closer physical proximity to tumor cells may lead to better survival, though further study across an array of tumor types is required.

**NK cell infiltration is greatest in low stage and high-grade tumors**

Tumor staging incorporates tumor size, nodal involvement and metastases to describe tumor progression, inform treatment and predict survival. Later stage tumors are typically larger, metastatic and generally carry poorer prognosis\textsuperscript{67}. Fourteen studies assessed how tumor stage is associated with NK cell infiltration in breast, colorectal, endometrial, gallbladder, head and neck, lung, ovarian, and vulvar cancer. There was a general trend toward decreased NK cell infiltration with increasing tumor stages\textsuperscript{51,59,67,75,77,84,85,88} \((n=8)\), of which colorectal (\(p=0.005\))\textsuperscript{59} and lung cancer (\(p=0.001\))\textsuperscript{67} reached statistical significance (Fig. 4B). Two studies observed more NK cells in advanced stages of high grade serous ovarian (\(p=0.47\))\textsuperscript{68} and invasive ductal carcinomas (\(p=0.01\) (intraepithelial), and \(p=0.12\) (stromal))\textsuperscript{55} (Fig. 4A). Comparing within stages, NK cell infiltration was most strongly associated with improved OS among patients with high stage tumors in colorectal cancer (stage I: \(p=0.24\); stage II: \(p=0.11\); stage III \(p=0.0008\))\textsuperscript{89}.

Cancer grade, the microscopic description of the cells and tissues, is also used to classify tumors: the higher the grade, the more abnormal, or undifferentiated, the cells appear\textsuperscript{27}. Five studies found a significant correlation of NK cell infiltration to grading\textsuperscript{50,53,58}. Four found that...
at higher grades, there were more NK cells in advanced rectal cancer ($p = 0.0080^{55}$ breast cancer ($p = 0.0003^{56}$), and invasive ductal carcinoma ($p = 0.0003^{56}$, $0.0001^{50,52}$) (Fig. 4C). Conversely, one study in invasive ductal carcinoma observed a significant association between lower NK cell infiltration and higher grades ($p = 0.040^{51}$) (Fig. 4D). Although the majority of studies did not report sub-analyses of tumor stage and grade, these pioneering investigations reveal that they should be considered when exploring the prognostic value of immune infiltration.

**NK cell identifying marker impacts the interpretation of the importance of NK infiltration**

There is no universal marker to define NK cells, and subsets of NK cells express CD56, CD57 and NKP46 differently$^{19}$. We meta-analyzed HR values from studies based on their primary NK cell marker (Fig. 5A). Studies staining for CD56$^{38,44,48,50,52,54,55,59,60,69,70,73,84}$ (n=16, HR=0.27, 95% CI: 0.18–0.41; $p = 0.0001$) and CD57$^{36,42,45,58,59,64,67,68,71,74,75,85,89}$ (n=12, HR=0.38, 95% CI: 0.23–0.63, $p = 0.0014$) demonstrated a similarly reduced risk of death with NK cell infiltration. Although studies staining for NKP46 also revealed a significant trend, the prognostic value was weaker$^{41,51,65,77}$ (n=4, HR=0.64, 95% CI: 0.41–1.00; $p = 0.020$) (Fig. 5B). Next, we similarly pooled the studies examining the relationship between NK cell infiltration and survival outcome (improved, no impact or poorer survival). Of the 53 studies, we found CD56 and CD57 yielded consistent conclusions and that the majority of studies (61%) that used these markers indicated improved OS. In contrast, a larger percentage of studies (57%) that evaluated infiltration by NKP46 found no impact on survival (Fig. 5C). Although the importance of this trend remains to be validated and functionally described, the relatively poor predictive capacity of NKP46 could reflect the inclusion of both immunosuppressive ILC subsets and conventional NK cells within the marked populations, the natural variation in expression of NKP46 that occurs between people, or downregulation of NKP46 prompted by the tumor$^{60-94}$.

**Head & neck cancers (13 studies, n=1328 patients)**

Head and neck cancers represent a heterogenous group of cancers that arise from tissues of the mouth, nose, throat, larynx, sinuses or salivary glands. A benefit of NK cell infiltration in these tumors, including longer DFS, was first reported in 1986 and confirmed in a recent meta-analysis$^{95,96}$. We identified 13 studies that assessed the value of NK cell infiltration in head and neck cancers including esophageal$^{34,36,39,41,43,45}$ (n=6), oropharyngeal$^{40,44}$ (n=2), nasopharyngeal$^{38}$ (n=1), oral$^{45,46}$ (n=3), laryngeal$^{57}$ (n=1), and lip$^{46}$ (n=1) carcinomas (Table 1, Fig. 2B). Of the 13 studies, 8 found that NK cell infiltration was associated with significantly improved OS; the remainder found no significant impact. Six of these studies reported HRs and together represent a total of 667 patients. Meta-analyzed, these studies revealed an overall decreased risk of dying in patients with NK cell infiltration or higher numbers of infiltrating NK cells (HR=0.31; 95% CI: 0.11–0.85, p<0.030, Table 2, Fig. 3A). Based on the extremely high heterogeneity between the studies ($I^2 = 94.7$%), further studies and stratification by specific head and neck cancer subtype are warranted. In our meta-analysis, this could be accomplished for esophageal cancer only.

**Esophageal (6 studies, n=674 patients)**

Four studies reported a significant association of NK cell infiltration with improved OS in patients with esophageal cancer ($p = 0.002^{39}$, $0.007^{39}$, $0.008^{41}$, $0.019^{45}$, Table 1, Fig. 2B). Meta-analysis conducted on the three studies that reported HRs demonstrated decreased risk of dying with high NK cell infiltration (HR=0.55; 95% CI: 0.41–0.74,
Table 2
HR values of meta-analysis overall, by cancer site and individual studies.

| Study                        | n  | HR   | Lower CI | Upper CI | p-value | F  | Heterogeneity |
|------------------------------|----|------|----------|----------|---------|----|---------------|
| **ALL SOLID TUMORS**         |    |      |          |          |         |    |               |
| Head and Neck Cancer         | 5337| 0.34 | 0.26     | 0.46     | <0.0001 | 86.9%|               |
| Haia et al. 2005             | 667 | 0.31 | 0.11     | 0.85     | 0.030   | 94.7%|               |
| Lu et al. 2017               | 197 | 0.46 | 0.27     | 0.77     | 0.004   |     |               |
| Svensson et al. 2017         | 97  | 0.49 | 0.28     | 0.86     | 0.012   |     |               |
| Taghavi et al. 2016          | 57  | 0.06 | 0.26     | 0.01     | 0.26    |     |               |
| Wagner et al. 2016           | 140 | 0.32 | 0.10     | 0.96     | 0.042   |     |               |
| Xu et al. 2016               | 138 | 0.60 | 0.39     | 0.91     | 0.016   |     |               |
| **Breast Cancer**            |    |      |          |          |         |    |               |
| Muntasell et al. 2018        | 71  | 0.07 | 0.01     | 0.60     | 0.01    |     |               |
| Muntasell et al. 2018        | 41  | 0.30 | 0.08     | 1.30     | 0.10    |     |               |
| Rathore et al. 2014          | 175 | 1.92 | 1.08     | 3.57     | 0.05    |     |               |
| Tian et al. 2016             | 278 | 0.54 | 0.39     | 0.74     | 0.001   |     |               |
| Triki et al. 2019            | 158 | 0.17 | 0.04     | 0.73     | 0.017   |     |               |
| Wang et al. 2014 (ALDH1 High)| 379 | 1.10 | 0.50     | 2.44     | 0.811   |     |               |
| Wang et al. 2014 (ALDH1 Low)| 379 | 0.12 | 0.01     | 0.81     | 0.031   |     |               |
| **Colorectal Cancer**        |    |      |          |          |         |    |               |
| Aldeidza et al. 2018         | 150 | 0.28 | 0.11     | 0.73     | 0.005   |     |               |
| Menon et al. 2014            | 93  | 0.43 | 0.17     | 1.01     | 0.030   |     |               |
| Sconocchia et al. 2011       | 1420| 0.43 | 0.30     | 0.70     | 0.0020  |     |               |
| **Gastric Cancer**           |    |      |          |          |         |    |               |
| Pernot et al. 2020           | 40  | 0.40 | 0.15     | 1.06     | 0.040   |     |               |
| Svensson et al. 2017         | 75  | 0.84 | 0.41     | 1.60     | 0.019   |     |               |
| **Lung Cancer**              |    |      |          |          |         |    |               |
| Villegas et al. 2002         | 50  | 0.43 | 0.20     | 0.95     | 0.036   |     |               |
| Yamada et al. 2010           | 44  | 0.66 | 0.25     | 1.78     | 0.41    |     |               |
| **Liver Cancer**             |    |      |          |          |         |    |               |
| Chew et al. 2012             | 40  | 0.12 | 0.04     | 0.31     | 0.0010  |     |               |
| Wu et al. 2013               | 256 | 0.63 | 0.40     | 0.99     | 0.046   |     |               |
| Zhu et al. 2009              | 81  | 0.38 | 0.17     | 0.85     | 0.019   |     |               |
| **Ovarian Cancer**           |    |      |          |          |         |    |               |
| Henrikson et al. 2019        | 283 | 0.67 | 0.46     | 0.98     | 0.031   |     |               |
| Li et al. 2009 (intra-epithelial) | 41 | 0.38 | 0.15     | 0.99     | 0.048   |     |               |
| Li et al. 2009 (stromal)     | 41  | 1.62 | 0.15     | 5.32     | 0.27    |     |               |
| **Endometrial Cancer**       |    |      |          |          |         |    |               |
| Versluis et al. 2017         | 355 | 0.85 | 0.56     | 1.29     | 0.445   |     |               |
| **Periampullary Cancer**     |    |      |          |          |         |    |               |
| Lundgren et al. 2016 (Intestinal) | 87.5 | 0.23 | 0.07     | 0.78     | 0.05    |     |               |
| Lundgren et al. 2016 (PA)    | 87.5| 0.59 | 0.34     | 1.02     | Not reported |     |               |
| **Gallbladder Cancer**       |    |      |          |          |         |    |               |
| Nakakubo et al. 2003         | 45  | 0.56 | 0.20     | 1.58     | 0.2655  |     |               |

*p = 0.013). These studies demonstrated substantially diminished heterogeneity ($I^2 = 0\%$). Hence, NK cells are positive prognostic markers for improved survival in esophageal cancer.

Breast cancer (8 studies, n = 1631 patients)

Breast cancer is highly heterogenous, with targeted treatment informed by molecular subtypes characterized by the expression or constitutive activation of receptors: human epidermal growth factor receptor 2 (HER-2$^+$), estrogen receptor (ER$^+$), progesterone receptor (PR$^+$) or triple negative breast cancer (TNBC), which does not express any of these receptors. The eight breast cancer studies we evaluated included: HER2$^{152}$ (n = 211), ER$^{152}$ (n = 100), PR$^{152}$ (n = 87), and TNBC$^{151}$ (n = 278). Four studies found that higher NK cell infiltration was associated with significantly improved OS (Table 1, Fig. 2B)$^{57,51,52,54}$; one reported significantly improved DFS$^{48}$, and two reported no significant impact on survival$^{69,53}$. The eighth study did not report patient subtype and was the only one to find NK cells associated with significantly worse survival$^{69}$. Notably, this study stained for NK cells using a single marker, CD56, with no exclusionary markers and therefore NK cell frequency may be overrepresented$^{69}$. Meta-analysis of these studies demonstrated a decreased risk of death (HR=0.27, 95% CI: 0.09–0.68, p = 0.027) however with a high level of heterogeneity ($I^2 = 85\%$) (Table 2, Fig. 3A). Breast cancer is a multi-faceted disease with significant heterogeneity and as such, it is not surprising that a clear trend was not observed, therefore, further analysis with stratification by subtype may provide a clearer association.

Colorectal cancer (7 studies, n = 3432 patients)

Colorectal cancer is highly heterogenous, with a large number of hereditary predispositions and environmental risk factors that both contribute to a high mutational burden$^{97}$. We evaluated seven studies that investigated the impact of NK cell infiltration into colorectal tumors on OS. Two studies identified the subtype as either large bowel adenocarcinoma$^{56}$ (n = 157) or mucinous (n = 119)$^{60}$ and nonmucinous (n = 1301)$^{56}$; the other five did not disclose a subtype$^{55,57,59,61}$ (n = 1855). Two of the studies identified patients as having locally advanced rectal cancer (n = 202)$^{55,57}$. The studies included the NK cell markers CD56 and CD57. Of the seven studies, five observed a significantly improved OS with high NK cell infiltration (Table 1, Fig. 2B)$^{55,56,58,61}$. Sconocchia et al. (2011)$^{56}$ observed a trend towards better OS in those with greater NK cell infiltration, and Lim et al. (2014)$^{57}$ did not identify any associations between OS and NK cell infiltration. Meta-analysis of the three studies that reported HR revealed a significant decrease in risk of death with high NK cell infiltration with little heterogeneity between studies (HR=0.38; 95% CI: 0.22–0.68, p = 0.019, $I^2 = 0\%$, Table 2, Fig. 3A)$^{55,53,68}$. Noteworthy, Menon et al. (2004)$^{59}$ observed that women and older patients had the highest NK cell infiltration into the stroma. The prognostic implications of this were not explored, though these observations indicate that the role of sex and
Fig. 3. NK cell infiltration is associated with a decreased risk of dying in patients with solid tumors. (A) A random effects model meta-analysis was conducted on the 30 studies revealing a decreased risk of death in patients with greater NK cell infiltration (HR=0.34, 95% CI: 0.26–0.46; p<0.0001). Forest plots demonstrate pooled meta-analysis results by solid tumor type and pooled meta-analysis results from all solid tumor types. (B) All studies which reported HR values were visualized by Forest plot, grouped by tumor type (dots indicate the size of patient population studied).

age should be assessed in the further study of NK cell infiltration into tumor regions. Patient survival with colorectal cancer has strong evidence to be influenced by NK cell infiltration, and therefore further analysis should be completed to understand this relationship.

Gastric cancer (5 studies, n = 425 patients)

Gastric cancer is an aggressive adenocarcinoma largely affecting the lining of the stomach and exemplified by genetic heterogeneity. Several subtypes were represented across the five studies examined, including intestinal-type gastric adenocarcinoma (n = 50), and gastrointestinal stromal (n = 91); the remaining samples were of unspecified subtype (n = 284). Three of the five studies identified NK cells by staining for CD56 or CD57 and found that NK cell infiltration significantly improved OS (n = 284). Meta-analysis of the gastric cancer studies reporting a HR (n = 2; patients n = 115) did not reach significance for an overall decreased risk of dying when NK cells were present or found at high frequencies within the tumor (HR=0.52, 95% CI:0.01–47.35, p = 0.32, Table 2, Fig. 3A). Two of the five studies included in this meta-analysis used NKP46 as their NK cell-defining marker; with increasing NK infiltration, one demonstrated a trend toward improved OS, but not OS. NK cell infiltration into gastric cancer can act as a positive prognostic marker for OS; these associations may be made clearer with further studies and careful choice of NK marker.

Lung cancer (4 studies, n = 330 patients)

Lung cancer, the leading cause of cancer death worldwide, is divided into two major categories: non-small cell (>80% of cases) and small cell lung carcinomas. We identified four lung cancer studies (n = 330 patients) that evaluated the association between NK cell infiltration and OS. The lung cancer subtypes examined included malignant pleural mesothelioma (n = 44), squamous cell carcinoma (n = 67), and adenocarcinoma (n = 219). Of the three studies that identified tumor stage, 126 (51.6%) patients were stage 1, 47 (19.3%) were stage 2, 68 (27.9%) were stage 3, and 3 (1.2%) were stage 4. Two of the studies found that high CD57+ NK cell infiltration into the tumor was associated with significantly improved OS. Two studies did not find associations between NK cell infiltration and prognosis: one stained using CD56 and their cohort included more than 50% of patients at advanced stages; the other used NKP46 to mark NK cells and found them mostly localizing to the invasive margin of the tumor. Our meta-analysis included two lung cancer studies and found a non-significant trend towards a decreased risk of death with NK cell infiltration (HR=0.47, 95% CI: 0.05–4.11, p = 0.14, Table 2, Fig. 3A). There may be associations between NK cell infiltration and lung cancer, but additional studies are required to understand how NK cells can be used as a prognostic factor, and whether this differs with subsets of lung cancer.

Liver cancer (4 studies, n = 540 patients)

Liver cancer is most often preceded by cirrhosis and chronic liver disease. This precancerous chronic disease is associated with decreased numbers of circulating NK cells compared with age- and sex-matched individuals without cancer. Four studies of five patient cohorts examined the value of infiltrating NK cells in hepatocellular carcinoma (n = 540) using either CD56 or CD57 to identify NK cells. All four studies found NK cell infiltration was significantly associated with improved OS. Meta-analysis of the liver cancer studies (n = 3; n = 377 patients) revealed a trend towards decreased risk of death when NK cells were present within the tumor (HR=0.29, 95% CI:0.03–2.67,
Table 2, Fig. 3A). While these studies all agree that NK cells are prognostically beneficial in liver cancer, a high level of heterogeneity ($I^2 = 91.8\%$) supports further investigation to clarify the variability observed.

**Gynecological cancers**

All gynecologic cancers similarly involve the female reproductive tract, but they arise from various tissues. The impact of NK cell infiltration on survival has been studied in ovarian\cite{Génin2012, Van2015}, endometrial\cite{Kaliniczka2011, Perniciaro2013}, and vulvar cancer\cite{Kanagawa2013}

**Ovarian cancer (2 studies, n = 365 patients)**

The majority of ovarian cancers arise from the epithelial lining of the fallopian tubes\cite{Thompson2009}. They are subclassified into serous, endometrioid, mucinous, clear cell, or undifferentiated; however, tumors can also arise from germ and stromal cells\cite{Thompson2009}. Two studies explored the impact of NK cell infiltration in ovarian cancer using CD57 as their principal NK cell marker and observed a decreased risk of dying in patients with greater NK cell infiltration\cite{Taylor2010, Van2015}, or more infiltration of NK cells into the tumor epithelium (Table 1, Fig. 2B)\cite{Chen2017}. Meta-analysis of these two studies demonstrate a trend towards a decrease in risk of death for those with higher NK cell infiltration (HR = 0.57, 95% CI: 0.26–1.24, $p = 0.089$, Table 2). Taken together, these studies suggest NK cell infiltration, particularly into the intra-epithelial region, appears to be associated with better prognosis in ovarian cancer.

**Endometrial cancer (3 studies, n = 502 patients)**

Unopposed estrogen stimulation can lead to the rapid proliferation of the endometrial lining, resulting in endometrial cancer\cite{Yoshida2010}. Three studies evaluated NK cell infiltration in endometrial cancer; PFS trended towards improvement in patients with NK cell counts greater than five but did not reach significance ($p = 0.17$, Table 1, Fig. 2B)\cite{Ozkara2014}. Overall survival was significantly higher in the population with a greater proportional infiltration of CD57+ NK cells ($p = 0.001$)\cite{Schwartz2014}. In the third study, DFS was significantly improved with NKp46+ NK cell presence but only in the context of HLA-E overexpression ($p = 0.035$)\cite{Schwartz2014}. A HR was only reported in this last study and was not significant (Table 2, Fig. 3B)\cite{Ozkara2014}. The mixed results between studies highlight that further investigation is warranted to confirm the trend towards improved prognosis with greater NK cell infiltration.

**Vulvar cancer (1 study, n = 76 patients)**

Unlike many other solid tumors, presence of T cells does not affect the prognosis of vulvar cancer\cite{Schwartz2014}. Szurkowski et al. (2014) used CD56 as a NK cell marker along with Granzyme B to mark cytotoxicity (Table 1, Fig. 2B)\cite{Schwartz2014}. No significant improvement in OS was observed between high and low NK-infiltrated cases, both generally and in metastatic cases\cite{Schwartz2014}. Although it appears that NK cell infiltration may not be associated with better prognosis in vulvar cancer, further studies will be required to confirm this finding.

**Kidney cancer (2 studies, n = 566 patients)**

Kidney cancer occurs in the renal tubular epithelium and includes a heterogenous group with various cancer subtypes, including clear cell, papillary, and chromophobe renal cell carcinoma\cite{Chen2017}. The value of infiltrating NK cells in renal cell carcinoma (including subtypes) was examined in two studies (n = 566) with staining for CD56\cite{Ozkara2014} or CD57\cite{Schwartz2014} (Table 1, Fig. 2B). In contrast to many of the other cancers examined in
Fig. 5. The associations concluded for the impact of NK cell infiltration and may be influenced by IHC marker. (A) Forest plot visualizing the reported HR’s of studies organized by marker used; NKp46 (orange), CD56 (dark blue), CD56 & CD57 (yellow) or CD57 (light blue). A random-effects model meta-analyses was conducted on studies using each marker. (B) This Forrest plot demonstrates the difference in the pooled risk of dying was larger in studies staining by CD56 (n = 16, HR:0.27, 0.18–0.41, p = 0.0001) and CD57 (n = 12, HR:0.38, 0.23–0.63, p = 0.0014) than NKp46 (n = 4, HR:0.58, 0.40–0.85, p = 0.020). (C) Bar graph demonstrating the difference in proportion of studies finding associations between NK cell infiltration and survival when separated by marker used.

this systematic review, NK cells were not significantly associated with improved survival in either of these studies. Neither study reported a HR and therefore these could not be included in the meta-analysis. Although further study is warranted, the available evidence suggests that NK cells are not associated with improved prognosis in renal cell carcinoma, a finding consistent with reports that T cell infiltration is also not beneficial in this cancer type.

**Sarcoma (1 study, n = 249 patients)**

Soft tissue sarcoma, a rare group of cancers, develop in the mesenchyme, a portion of the embryo that establishes the connective and skeletal tissues. One study examined the prognostic effect of infiltrating NK cells in soft tissue sarcoma. Sorbye et al. (2012) evaluated 249 patients by staining for NK cells using CD57. High numbers of CD57+ NK cells in the peritumoral capsule non-significantly trended towards better OS of soft tissue sarcoma patients (p = 0.797) (Table 1, Fig. 2B). The median survival for high NK cell infiltration in this tumor compartment (29 patients, 36%) was 138 months, compared to 47 months for low expression (50 patients, 63%). There was no trend observed between CD57+ NK cell infiltration into the tumor and OS. Overall, the potential association between better prognosis and NK cell infiltration warrants further studies.

**Melanoma (1 study, n = 147 patients)**

Melanoma is the most aggressive form of skin cancer and the global incidence is currently 160,000 new cases per year and steadily increasing. Melanoma is notable for its resistance to classical therapies and susceptibility to immunotherapy. Only one study examined infiltration of NK cells into melanoma, using the CD56 marker (Table 1). No significant association between NK cell infiltration and OS was reported, but further studies are needed in order to understand the role of NK infiltration in melanoma.

**Periampullary adenocarcinoma (1 study, n = 175 patients)**

Periampullary cancer arises from tissue surrounding the ampulla of Vater, the area where the common bile duct and pancreatic duct come together and open into the duodenum. This cancer type is a heterogeneous group of malignancies which arise from tissue from the distal bile duct, pancreatic duct and pancreas itself. Lundgren et al. (2016) was the only study we identified that explored immune infiltration in periampullary adenocarcinomas and stratified into intestinal and pancreatobiliary subtypes (Table 1). Those with high NK cell infiltration had significantly longer OS (p = 0.002) compared to those without. Risk of dying in the NK cell infiltrated population was lower in the intestinal (HR=0.23, 95% CI: 0.07–0.78, p<0.05) but not pancreatobiliary (HR=0.59, 95% CI: 0.34–1.02, ns) subtype. This study suggests NK cells are associated with improved survival in periampullary adenocarcinoma but may be more protective in intestinal type periampullary carcinoma; additional studies will help to validate this association.
Galbladder (1 study, n = 45 patients)

We identified one study that evaluated the prognostic impact of NK cell infiltration in galbladder cancer, specifically galbladder adenocarcinoma. Nakakubo et al. (2003) evaluated 45 tissue samples using CD57 to identify NK cells. While significance was not reached, a trend toward improved survival in the high NK cell infiltrated group emerged (Table 1, Table 2). With growing interest in immunotherapeutic options for galbladder patients, additional studies evaluating the prognostic value of infiltrating NK cells are warranted.

Glioblastoma (1 study, n = 25 patients)

Glioblastoma is the most common primary central nervous system neoplasm and is most often located in the brain. Vaquero et al. (1989) evaluated 25 patients with glioblastoma, and found no change in survival with respect to the frequency of infiltrating NK cells. More research into the prognostic value of NK infiltration in glioblastoma is required to confirm these findings.

Discussion

We report the first systematic review describing the prognostic value of NK cell infiltration into solid tumors. Thirty two of the 54 (59.3%) reported positive associations between OS and NK cell infiltration; twenty (38.9%) reported no impact and just one (1.9%) found a negative association between NK cell infiltration and OS. NK infiltration was more common with earlier stage and higher-grade tumours. When considering localization, the reviewed studies revealed that NK cells infiltrating intraepithelial regions impacted survival more than NK cells infiltrating the adjacent stroma. These findings define NK cell infiltration in solid tumors as a positive prognostic factor and prompt further research to understand and maximize NK cell function in solid tumors.

The total number of NK cells infiltrating solid tumors – including those considered “highly” infiltrated – was relatively low compared with other immune populations, and several studies asserted that the number of NK cells was too low to pursue prognostication. Notwithstanding, the presence of a single NK cell within a high powered microscopic field was associated with significantly improved OS and DFS in colorectal cancer, HER2 breast cancer, and hepatocellular carcinoma. Although the exact role(s) of infiltrating NK cells remains to be determined, that NK cells are competent “serial killers,” capable of polarizing the TME, recruiting and activating additional effector cells suggests that their low frequency should not be interpreted as a lack of power or importance.

Historically, IHC was limited both by the number of chromogens available and the relatively few species in which antibodies can be raised, but modern multiplex IHC technology now allows for simultaneous identification of up to nine markers on a single slide, regardless of the species in which the antibodies are raised, and machine learning to identify cellular infiltration, sub-tumor localization and sociology. With this new technology, we look forward to better understanding the features that impact NK cells’ relationship with solid tumor control, including the function of NK cells within tumors.

In addition to infiltration, the solid tumor microenvironment restricts NK cell reactivity. In tumors infiltrated with NK cells that are suppressed, interventions to support NK cell reactivity and overcome immunosuppression, such as checkpoint blockade or local cytokine production may prove efficacious. NK cells isolated from patients with solid tumors including head and neck, gallbladder, and ovarian cancers exhibit increased expression of inhibitory receptors and decreased expression of activating receptors. In the TME, high levels of TGF-β directly suppress NK cell proliferation and cytotoxicity, leading to impaired anti-tumor activity in vivo. Hypoxia and adenosine signaling through the high-affinity A2A receptor can interfere with NK cell development and cytotoxicity. Immunosuppression, particularly that driven by myeloid-derived suppressor cells, increases with tumor stage; this has been observed in bladder, pancreatic, hepatocellular, gastric, non-small cell lung, and head and neck squamous cell cancers. Our meta-analysis reveals that these higher stage tumours are more often infiltrated by NK cells; in them, highlighting that NK cells may have an important role in these hard-to-treat tumors.

Within sub-tumor regions, NK cell phenotypes and infiltration differ. The best survival outcomes were observed specifically with high NK infiltration to the intraepithelial region. In hepatocellular carcinoma, NK cells in the peritumoral (stromal) region expressed high levels of CD69 (a marker of activation), while intraepithelial NK cells from the same patients did not. Although intraepithelial NK cells expressed CD107a, a marker associated with degranulation, they also exhibited low perforin, TRAIL, and granzyme B, suggesting that NK cells within this region may be exhausted. In tongue cancer, intraepithelial NK were more likely to express the NKG2A inhibitory receptor than their stromal counterparts. Together, these results suggest that like T cells, NK cells can be activated for antitumor activity, but become suppressed as they enter further into the TME and closer to the tumor cells.

The phenotype of NK cells can be highly informative of their reactive capacity or suppression. Germline-encoded receptors for activation and inhibition are differentially expressed and co-expressed, and modified through NK cell “education” for missing self reactivity. NK cytotoxicity occurs through several mechanisms including perforin and granzyme release and the death receptor pathways (i.e., Fas, TRAIL). Other markers often expressed or upregulated by activated NK cells include CD69, NKG2D, NKP46, or DNAM-1 (all associated with activation), immune checkpoints (i.e. TIGIT, PD1/PD-L1, NKG2A and TIM-3) and inhibitory receptors for HLA (i.e. killer immunoglobulin-like receptors, NKG2A, LIR-1). Whether the ligands for these receptors are present within solid tumors, or may be attenuated (inhibitory) or triggered (activating) are active areas of research in the field of NK-based immunotherapy. An overabundance of inhibitory ligands or infiltration of cells with immunosuppressive activity, including the ILC1 subset which has several overlapping features with conventional NK cells, would be expected to be associated with poor prognosis.

With novel multiplexing technologies and improved understanding of the distinction between NK cells and ILC subsets, it is now possible to distinguish conventional NK subsets from other populations that share markers with NK cells, including ILC1. Understanding the roles and distribution of ligands for NK cells in solid tumors will be important toward refining and precisely delivering NK-based immunotherapies.

Of the 53 studies evaluated in this review, only five used more than one NK cell marker to quantitate NK cell infiltration; the rest used CD56, CD57, or NKP46 exclusively. Compared with NKP46, definitions of NK cells using CD56 or CD57 were more consistently associated with improved survival. NKP46 is an activating receptor that is more dynamic in its expression than CD56 and CD57, and whose expression varies between tissues and may be diminished in patients with solid tumors. While informative as an activation marker, the definition of NK cells using NKP46 may be problematic because it is expressed by innate-like lymphocyte populations, including subsets associated with immunosuppression. In fact, the only study in our meta-analysis to report a significantly negative association between NK cell infiltration and OS used NKP46 to define NK cells and reported a substantially higher number of infiltrating cells compared with other studies. Note-worthy, CD56 and CD57 expression also varies on human NK cells, and CD56 can be expressed on cells of neural origin, so studies aiming to identify NK cells in vivo should include exclusion staining and redundancy in NK cell markers.

Cancer therapy – especially immunotherapy – is a rapidly evolving field, and the roles and benefits of NK cells likely intersect with treatment. Our analysis included data from treatment-naïve samples but reported survival of patients who may have undergone treatment. Patient diagnoses and therapy occurred over a period of three decades, during
which treatment, cancer detection and the understanding of NK cell biology have improved. Studies did not report systematically on the impact of patient/tumor genetics, driver mutations, mutational burdens, virus infections and patient characteristics such as environmental or workplace exposures, smoking, obesity and alcohol use histories; these features may all impact the immunologic landscape of a tumor and the resulting necessary immune responses to control its growth.

Our meta-analysis reveals that NK cells are prognostically beneficial across an array of solid tumor types. Consideration of NK cells as therapy or therapeutic collaborators will be important for successful immunotherapy of solid tumors. For example, strategies to preserve or engineer NK cell expression of chemokine receptors could enhance their efficacy for solid tumor therapy 148. Clinical approaches to expand and activate patients’ NK cells ex vivo may result in loss of CXCR2 expression, and genetic modification of NK cells to express CXCR2 increases migration to renal cell carcinoma in vitro 149. Across an array of cancer types, infiltration of NK cells is associated with improved response to immunotherapy 121. In sum, either alone or with an extra “boost” from therapeutic interventions, NK cells are highly promising effectors in solid tumor therapy. Optimizing NK cell anti-cancer efficacy will require supporting their infiltration, activation and resilience against immunosuppression in the TME.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Sarah Neresian: Conceptualization, Formal analysis, Data curation, Writing - review & editing, Investigation, Methodology, Supervision, Visualization. Sarah L. Schwartz: Data curation, Writing - review & editing, Investigation, Stephanie R. Grantham: Data curation, Writing - review & editing, Investigation. Leah K. MacLean: Data curation, Writing - review & editing, Investigation. Stacey N. Lee: Data curation, Writing - review & editing, Investigation. Morgan Pugh-Too: Data curation, Writing - review & editing, Investigation. Jeanette E. Boudreau: Conceptualization, Formal analysis, Writing - review & editing, Investigation, Methodology, Supervision, Funding acquisition.

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Supplementary materials

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