Human NK Cytotoxicity against Porcine Cells Is Triggered by NKp44 and NKG2D

Pietro Forte, Benjamin G. Lilienfeld, Bettina C. Baumann and Jörg D. Seebach

*J Immunol* 2005; 175:5463-5470;
doi: 10.4049/jimmunol.175.8.5463
http://www.jimmunol.org/content/175/8/5463

---

**Why The JI?**

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

---

**References**

This article cites 45 articles, 10 of which you can access for free at:
http://www.jimmunol.org/content/175/8/5463.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Human NK Cytotoxicity against Porcine Cells Is Triggered by NKp44 and NKG2D

Pietro Forte, Benjamin G. Lilienfeld, Bettina C. Baumann, and Jörg D. Seebach

Pig-to-human xenotransplantation has been proposed as a means to alleviate the shortage of human organs for transplantation, but cellular rejection remains a hurdle for successful xenograft survival. NK cells have been implicated in xenograft rejection and are tightly regulated by activating and inhibitory receptors recognizing ligands on potential target cells. The aim of the present study was to analyze the role of activating NK receptors including NKp30, NKp44, NKp46, and NKG2D in human xenogeneic NK cytotoxicity against porcine endothelial cells (pEC). 51Cr release and Ab blocking assays were performed using freshly isolated, IL-2-activated polyclonal NK cell populations as well as a panel of NK clones. Freshly isolated NK cells are NKp44 negative and lysed pEC exclusively in an NKG2D-dependent fashion. In contrast, the lysis of pEC mediated by activated human NK cells depended on both NKp44 and NKG2D, since a complete protection of pEC was achieved only by simultaneous blocking of these activating NK receptors. Using a panel of NK clones, a highly significant correlation between anti-pig NK cytotoxicity and NKp44 expression levels was revealed. Other triggering receptors such as NKp30 and NKp46 were not involved in xenogeneic NK cytotoxicity. Finally, Ab-dependent cell-mediated cytotoxicity of pEC mediated by human NK cells in the presence of xenoreactive Ab was not affected by blocking of activating NK receptors. In conclusion, strategies aimed to inhibit interactions between NKp44 and NKG2D on human NK cells and so far unknown ligands on pEC may prevent direct NK responses against xenografts but not xenogeneic Ab-dependent cell-mediated cytotoxicity. The Journal of Immunology, 2005, 175: 5463–5470.

The clinical use of porcine organs to alleviate the current shortage of human organs in transplantation medicine is impeded by the occurrence of several types of vigorous rejection mechanisms that lead to rapid graft failure (1, 2). Recently, advances in the prevention of hyperacute rejection in preclinical models using genetically engineered pigs suggest the importance of both coagulation disorders and cellular immunity in human anti-pig xenogeneic responses (3, 4). Despite the fact that prolonged survival of these xenografts has been achieved without specifically inhibiting NK cells, they might play an important role in endothelial injury and delayed rejection of porcine xenografts. This hypothesis is supported by the finding that in vitro NK cells activate porcine endothelium upon direct contact and act as a potent source for proinflammatory cytokines such as IFN-γ (5, 6). In contrast, NK cell activation depends, among others, on T cell-derived IL-2 stimulation, an interaction between the innate and adaptive immune system that might be interrupted by conventional immunosuppressive protocols used in preclinical models for xenotransplantation. Human NK cells have further been demonstrated to adhere to and lyse porcine target cells both directly and, in the presence of human serum containing xenoreactive Ab, by Ab-dependent cell-mediated cytotoxicity (ADCC) (7, 8). In addition, pig organs perfused with human blood ex vivo are predominantly infiltrated by NK cells (9, 10), and NK cells are present in histological samples of graft rejection in concordant and discordant rodent and preclinical pig-to-baboon models (11–13). Thus, although the potential role of NK cells is still controversial, strategies to inhibit both direct NK cytotoxicity and ADCC against porcine cells might facilitate successful clinical xenotransplantation.

Under physiological conditions, NK cytotoxicity is accurately regulated to lyse only transformed or infected cells (14). Two major checkpoints control target cell susceptibility to NK cytotoxicity: 1) the expression of ligands for various NK-activating receptors and 2) the presence of MHC class I molecules interacting with inhibitory NK receptors. The killing signals transduced by activation receptors are balanced by several groups of inhibitory receptors that bind HLA class I molecules, including killer Ig-like receptors (KIR), ILT2, and the CD94/NKG2 family (15). Consequently, NK cytotoxicity occurs when NK cells encounter ligands for activating receptors on potential target cells that, in addition, have lost or down-regulated MHC class I expression. Porcine endothelial cells (pEC) are susceptible to human NK-mediated lysis possibly due to the inability of their MHC class I molecules to signal through human NK cell inhibitory receptors (16).

Theoretically, xenogeneic NK cytotoxicity could be avoided either by expressing HLA class I molecules on porcine cells or by blocking the function of activating receptors. Indeed, we and others have previously demonstrated that the expression of human MHC class I molecules including HLA-B27, -Cw3, -E, and -G on porcine cells provided partial protection from lysis mediated by...
polyclonal human NK cells (17–22). However, complete inhibition of NK cytotoxicity has not been achieved by transgenic HLA class I expression because the corresponding NK inhibitory receptors for each HLA molecule were expressed only on NK subpopulations. Moreover, protection from ADCC by HLA class I expression has been demonstrated only for NK clones, but not for polyclonal NK cells (19, 23).

Activating receptors on NK cells include Nkp30, Nkp44, and Nkp46 (24), collectively named natural cytotoxicity receptors (NCR), and NKG2D (25). NCR play a major role in NK-mediated killing of tumor cell lines as revealed by mAb-mediated receptor-masking experiments. Their surface density on NK cells correlates with the magnitude of cytolytic activity against NK-susceptible target cells. Despite considerable efforts, the cellular ligands recognized by NCR are still not defined. However, as revealed by cytolytic assays, NCR ligands are expressed by cells belonging to different histotypes (24). Although Nkp30 and Nkp46 are detected on all NK cells regardless of their activation status, Nkp44 is selectively expressed by activated NK cells (26). In contrast to NCR, several ligands of NKG2D have been identified, including the stress-inducible MHC class I chain-related proteins A (MICA) and B (MICB), or UL16-binding proteins (27). Other triggering surface molecules expressed by NK cells appear to function primarily as coreceptors (24), because their ability to signal depends on simultaneous coengagement of a main triggering receptor. This group includes NK-specific receptors such as Nkp80, 2B4, NTB-A, and receptors that are not unique to NK cells such as CD59, CD2, ICAM, CD69, β1 integrins, and DNAM-1. Apart from the direct cytotoxicity described above, NK cells can also be triggered by ADCC. This mechanism is mediated by FcγRIII receptors (CD16) on NK cells interacting with Igs bound to target cells.

The aim of the present study was to explore the hypothesis that human NK cytotoxicity against porcine cells might be overcome by blocking activating NK receptors. We demonstrate that human NK cytotoxicity against pEC depends exclusively on Nkp44 and NKG2D signals.

Materials and Methods

Cells

Two SV40-immortalized pEC lines, the aortic PEDSV.15, and the bone marrow-derived microvascular 2A2 cell lines were established and characterized in our laboratory (28). A primary aortic pEC (PAEC) was isolated using a protocol previously described (28) and cultivated in S199 (Invitrogen Life Technologies) supplemented with 15% FCS (PAA Laboratories), 1% penicillin/streptomycin (Invitrogen Life Technologies), 20 mM HEPES (pH 7.2; Invitrogen Life Technologies), 50 μg/ml endothelial cell growth supplement (BD Biosciences), and 100 μg/ml heparin (Sigma-Aldrich). The human melanoma cell line MEL-15 (a gift from M. Urosevic, University Hospital, Boston, MA) (19, 29), and the human lymphoblastoid cell line 721.221 (American Type Culture Collection) (30) were cultured in RPMI 1640 (Invitrogen Life Technologies) supplemented with 12.5% FCS. Iso- marrow-derived microvascular 2A2 cell lines were established and characterized in our laboratory (28). A primary aortic pEC (PAEC) was isolated using a protocol previously described (28) and cultivated in S199 (Invitrogen Life Technologies) supplemented with 15% FCS (PAA Laboratories), 1% penicillin/streptomycin (Invitrogen Life Technologies), 20 mM HEPES (pH 7.2; Invitrogen Life Technologies), 50 μg/ml endothelial cell growth supplement (BD Biosciences), and 100 μg/ml heparin (Sigma-Aldrich). The human melanoma cell line MEL-15 (a gift from M. Urosevic, University Hospital, Boston, MA) (19, 29), and the human lymphoblastoid cell line 721.221 (American Type Culture Collection) (30) were cultured in RPMI 1640 (Invitrogen Life Technologies) supplemented with 12.5% FCS. Iso-
A minor fraction of polyclonal NK populations was NKp30 (8%, MFIR of 1.6) or NKp46 (2%, MFIR of 2.5) positive. Flow cytometry analysis of the activating NK receptors was always repeated at the day of the cytotoxicity assay.

Cytotoxicity of freshly isolated and IL-2-activated NK cells against immortalized pEC

Freshly isolated NK cells and polyclonal NK populations generated in the presence of IL-2 were tested for their ability to lyse the immortalized pEC lines PEDSV.15 and 2A2. A substantial difference in NK cytotoxicity against PEDSV.15 mediated by freshly isolated vs IL-2-activated NK cells at an E:T ratio of 20:1 was evident (21 ± 9% vs 48 ± 22% median specific lysis; n = 10 and 37, respectively; Fig. 2A). The lysis of 2A2 cells by activated NK cells was comparable (43 ± 21% median specific lysis; n = 38), whereas lysis of 2A2 cells by freshly isolated NK cells was clearly lower (8 ± 5%; n = 9), as shown in Fig. 2B.

The variability among different donors in the ability of their freshly isolated NK cells to lyse pEC was remarkable. This finding is illustrated in Fig. 2. Overall, freshly isolated NK cells displayed a cytolytic activity ranging between 3 and 54% at an E:T ratio of 40:1. Importantly, freshly isolated NK cells displaying a weak cytotoxicity against porcine targets were still able to efficiently lyse the MHC class I-deficient human target cell line 721.221 (data not shown). Blocking of NKp30, NKp44, and NKp46 independently or in combination did not lead to a reduced cytotoxicity of freshly isolated NK cells against PEDSV.15 (Fig. 3). Regardless of the individual killing efficiency of different donors, blocking of NKG2D was always able to reduce xenogeneic cytotoxicity of freshly isolated NK cells significantly without further reduction of cytotoxicity by additional blocking of NKp30 and NKp46 (Fig. 3B).

In contrast, the cytolytic activity of IL-2-activated human polyclonal NK cells against PEDSV.15 depended on both NKG2D and NKp44, but not on NKp30 or NKp46. A partial inhibition of xenogeneic NK cytotoxicity was observed by mAb-mediated masking of either NKp44 (44% inhibition) or NKG2D (56% inhibition), whereas isotype-matched control mAb as well as blocking of NKp30 or NKp46 had no effect (Fig. 4A). Neither did a combination of anti-NKp44 mAb with either anti-NKp30 or anti-NKp46 mAb enhance the inhibitory effect of anti-NKp44 (data not shown). As a positive control for the activity of the anti-NKp30 and anti-NKp46 mAb to inhibit NK killing, we used the human melanoma cell line MEL-15. NK-mediated lysis of MEL-15 was clearly reduced in blocking assays using NKp30 and NKp46 mAb alone or in combination...
For 1 of 5 (B) and 1 of 15 (C) independent experiments performed with NK cells purified from 10 different donors.

**FIGURE 4.** NKp44 and NKG2D but not NKp30 and NKp46 receptors trigger cytotoxicity of NK cells against PEDSV.15 cells. The cytotoxic activity of IL-2-activated NK cells was tested against PEDSV.15 cells in 2- and 4-h 51Cr release assays in the presence of different combinations of the following mAb: IgG1 isotype control, anti-NKG2D, anti-NKp30, anti-NKp44, anti-NKp46, and anti-NKG2D/-NKp44 in combination. A, Summary of four independent experiments where cytotoxicity is expressed as percentage of relative lysis of PEDSV.15 cells in the presence of the indicated mAb as compared with the respective intra-assay control (lysis with isotype control mAb, index = 100). The mean relative cytotoxicity was calculated at four different E:T ratios (40:1 to 5:1); error bars indicate SEM. B and C, Representative experiments for 1 of 5 (B) and 1 of 15 (C) independent experiments performed with NK cells purified from 10 different donors.

(datum not shown). Nearly complete blocking of activated NK cell cytotoxicity against PEDSV.15 was achieved by simultaneous blocking of the NKp44 and NKG2D receptors (Fig. 4, B and C). No difference with regard to the blocking efficiency was observed when comparing 2- to 4-h 51Cr release assays (Fig. 4, B and C); we only observed a higher specific lysis when co-cultivating target and effector cells for longer time periods. The cytotoxic response of freshly isolated and IL-2-activated NK cells against pEC did not correlate with NKG2D receptor surface densities that were similar between different donors (data not shown).

Altogether, these data indicate that NKG2D plays a pivotal role in xenogeneic NK cytotoxicity mediated by both freshly isolated and IL-2-activated human NK cells, whereas NKp44 triggers lysis mediated by IL-2-activated human NK cells. In contrast, NKp30 and NKp46 do not play a role in human NK cytotoxicity against pEC.

**Xenogeneic cytotoxicity of NK clones against immortalized porcine aortic endothelial cells correlates with NKp44 expression levels**

To further analyze the role of NCR and NKG2D in NK-mediated xenogeneic lysis of PEDSV.15, a panel of NK clones was generated. NK clones expressing different levels of NKG2D and NCR provide a unique tool to study the role of these activating receptors. The potential of NK clones to lyse PEDSV.15 cells was tested in the absence or presence of mAb against NCR and NKG2D. Lysis of PEDSV.15 mediated by an NK clone expressing high levels of NKp30 (MFIR, 9) and NKp46 (MFIR, 6) was markedly reduced (93% inhibition) only by blocking with NKp44- and NKp46-specific mAb (Fig. 5A). The combined use of anti-NKp44, anti-NKG2D, anti-NKp30, and anti-NKp46 mAb did not further enhance the inhibitory effect of anti-NKp44-NKG2D mAb, providing additional evidence that NKp30 and NKp46 receptors do not recognize any ligands on PEDSV.15.

In addition, 10 different NKG2D-positive NK clones, with MFIR for NKp44 expression ranging from 3 to 26, were analyzed for their capacity to lyse PEDSV.15. In the absence of NKG2D-mediated activation, i.e., in the presence of saturating concentrations of anti-NKG2D mAb, NK cytotoxicity was proportional to the expression level of NKp44. Thus, as shown in Fig. 5B, PEDSV.15 cells were efficiently killed by NKp44bright NK clones while they were partially resistant to cytotoxicity mediated by NKp44 dull NK clones. In contrast, clonal NK cytotoxicity did not correlate with NKp30 and NKp46 cell surface densities.

**Lysis of primary aortic and immortalized bone marrow-derived pEC as well as porcine lymphoblastoid cells depends on NKp44 and NKG2D**

The finding that NKp44 and NKG2D are responsible for IL-2-activated xenogeneic NK lysis of PEDSV.15 cells raised the question about the role of these receptors in the lysis of other porcine target cells. Thus, NK cytotoxicity against PAEC (Fig. 6A) and immortalized bone marrow-derived pEC (2A2) (Fig. 6B) was analyzed. Both PAEC and 2A2 were protected from IL-2-activated human NK cytotoxicity in the presence of anti-NKp44 and anti-NKG2D mAb, whereas isotype-matched and anti-HLA class I mAb had no effect. NK cytotoxicity against 2A2 seemed to depend predominantly on NKp44 as compared with NKG2D, whereas PEDSV.15 cells were mainly killed through NKG2D (Fig. 4). In agreement, we observed that 2A2 cells were clearly less susceptible than PEDSV.15 to NK cytotoxicity mediated by freshly isolated NKp44-negative NK cells (Fig. 2B). Taken together, these results show that NKp44 and NKG2D function as unique receptors involved in human NK-mediated cytotoxicity against pEC independent of the respective NK donor. To expand our study to cell types other than endothelial cells, xenogeneic human NK cytotoxicity against porcine lymphoblastoid cells was tested. In parallel to pEC, blocking of NKG2D and NKp44 inhibited the lysis of lymphoblastoid cells (Fig. 6C), whereas NKp30 and NKp46 had no effect (data not shown). In contrast to pEC, blocking with NKG2D and NKp44 mAb were not sufficient to completely prevent the lysis of lymphoblastoid cells, indicating that other activating receptors are involved in the lysis of non-xenogenic hemopoietic target cells.

**Xenogeneic ADCC is not prevented by interfering with NKG2D activation**

ADCC of human NK cells against pEC represents a very efficient mechanism of lysis. Although the involvement of CD16 in ADCC is well documented, the role of other activating receptors has currently not been investigated. Therefore, the contribution of NCR and NKG2D in xenogeneic NK-mediated ADCC against pEC was...
analyzed. NK cytotoxicity mediated by freshly isolated NK cells against PEDSV.15 cells was evaluated in the presence of heat-inactivated human serum containing saturating amounts of xenoreactive natural Ab. The addition of human serum clearly increased the lysis of PEDSV.15 by freshly isolated NK cells, demonstrating a strong NK cell-mediated ADCC. Blocking with NCR- and NKG2D-specific mAb in 51Cr release assays revealed no inhibitory effect on NK cell-mediated ADCC against PEDSV.15 cells (Fig. 7). These findings indicate that activation signals transmitted by CD16 are sufficient to induce efficient lysis of pEC and do not require additional activation signals through NKG2D, NKp30, or NKp46.

Discussion

The generation of galactose-α1,3-galactose knockout pigs has overcome the barrier of anti-galactose (Gal) mediated rejection in pig-to-nonhuman primate xenotransplantation. Therefore, strategies to reduce other rejection mechanisms need to be explored. These may include natural or elicited non-Gal-specific xenoreactive Ab and cellular immunity mediated by direct NK cell responses, all of which lead to porcine endothelial activation, injury, and eventually to graft dysfunction. Human NK cytotoxicity against pEC is readily observed in vitro, whether or not it contributes to xenograft rejection in vivo remains unknown. Nevertheless, recognition of xenogeneic pEC by human NK cells in vitro leads to activation and injury of pEC by several mechanisms. Taking the limitations of in vitro studies into careful consideration, NK cytotoxicity studies may represent relevant NK cell responses in vivo. Similarly, the level and specificity of allogeneic cytotoxic T cell responses in vitro reproduce in vivo graft rejection which also does not necessarily involve cytotoxic functions. In this study, we further analyzed the molecular mechanisms leading to NK cell activation and cytotoxicity in response to porcine endothelium, the first immunological target following vascularized pig-to-human xenotransplantation. Human NK cytotoxicity against porcine target cells requires cross-species compatibility between human NK-activating receptors and their respective porcine ligands. At the same time it reflects the absence of sufficient interactions between NK inhibitory receptors and MHC class I molecules across the species barrier. Whereas human NK cells are inhibited only weakly, if at all, by swine MHC class I molecules (32), human activating NK receptor interactions with porcine ligands have not been investigated so far.

The data presented here provide clear evidence that NKG2D and NKp44 function as triggering receptors involved in direct human NK cytotoxicity against pEC, whereas NKp30 and NKp46 do not play a role. NKG2D was the only activating receptor involved in pEC lysis mediated by freshly isolated NK cells. Intriguingly, the significant variability among donors in the response of freshly isolated NK cells against pEC did not correlate with NKG2D receptor surface densities that were similar between different donors. Therefore, the reason for this variability might depend on the different efficiency of the respective cytotoxic effector pathways such as the perforin-granzyme release that is important in xenogeneic
Indeed, it has been shown that NK cell culture conditions, in particular IL-2 and IL-15 supplements, up-regulate the expression levels of molecules involved in the perforin-granzyme cytolytic pathway (34). Moreover, the cytolytic potential of individual freshly isolated NK populations might depend on the immunological condition of the donor since NK cells collected at different time points from the same donor differed in their ability to kill pEC without apparent differences in NKG2D expression (data not shown). The importance of NKp44 for NK cytotoxicity mediated by activated human NK cells against pEC was clearly supported by 1) Ab blocking studies, 2) the correlation of lysis with NKp44 expression levels on NK clones, and 3) the association of poor xenogeneic NK cytotoxicity against 2A2 cells with the absence of NKp44 on freshly isolated NK cells. Since it was not addressed in the present study, we can only speculate on the role of the numerous additional coreceptors involved in human NK cytotoxicity. Their triggering function appears to depend on the simultaneous engagement of the main activating receptors (24).

The involvement of NKp44 and NKG2D in NK-mediated lysis of pEC indicates the presence and recognition of porcine homologues of human NKp44 and NKG2D ligands on pEC or of “unique” ligands in pigs without apparent human homologues. Compatibility of NK-activating receptors and their respective ligands has also been described in other species combinations. In fact, the putative ligand for the human NKp46 receptor is responsible for xenogeneic NK-mediated lysis of murine cells (35). The notion that NCR and NKG2D receptors are, at least partially, conserved between humans and other species is clearly different from the divergent evolution that has been documented for the MHC class I-specific inhibitory receptors (36–38). Allelic specificity of human KIR for HLA class I molecules suggests a coevolution of inhibitory KIR with the respective MHC genes. This might explain why human KIR have a low affinity for MHC class I molecules of unrelated species (39). In contrast, it is conceivable that NCR and NKG2D as well as their ligands have not been subjected to the pressure that caused the evolution of MHC genes and their receptors. The porcine NCR and NKG2D ligands are unknown; however, comparison of porcine and human genomic sequences suggests the presence of one ULBP gene and one MIC-A/MIC-B-like gene (MIC2) in the pig genome (40, 41). NKG2D ligands are generally poorly expressed by normal cells but are up-regulated in transformed, infected, and stressed cells in both mice and humans (25). These findings are compatible with the supposed expression of porcine NKG2D ligands on transformed pEC lines like PEDSV.15 and 2A2 but are in contrast with the involvement of NKG2D in NK-mediated lysis of primary pEC. However, stress generated by isolation and cell culture conditions could be responsible for NKG2D ligand expression on primary pEC and subsequent susceptibility to xenogeneic NK lysis. Similarly, it is easy to predict that triggers of inflammation, ischemia reperfusion injury, and rejection mechanisms following xenotransplantation might also allow the expression of porcine ligands of human NKG2D.
This hypothesis is supported by the finding that human kidney allografts undergoing both acute and chronic rejection episodes have been shown to express polymorphic MIC molecules which may even induce allospecific Ab (42, 43). Future studies are needed to identify the porece ligands for NKG2D and NKp44, thereby it will be of interest whether only two or more ligands are involved in this interspecies NK recognition. Besides cytokine secretion, endothelial cell activation, and direct NK cytototoxicity, human NK cells are also able to kill porcine cells via ADCC. As reported previously, the lack of galactose-α1,3-galactose on porcine cells strongly reduced NK-mediated ADCC, whereas binding and direct cytototoxicity of human NK cells were not inhibited (44, 45). The fact that ADCC was not influenced by blocking NCR and NKG2D indicated that the two mechanisms of human NK cytopotoxicity against porcine target cells are independent.

Emerging evidence over the past few years indicated that the expression of HLA class I molecules in porcine tissues did not provide complete protection from direct xenogeneic human anti-pig NK cytotoxicity through inhibitory receptors. In this article, we demonstrate that the abrogation of human NKp44 and NKG2D interactions with their porcine ligands was able to completely prevent direct human NK cytototoxicity against pEC. Thus, the identification and elimination of porece ligands of NKp44 and NKG2D might have important implications by representing a complementary approach to protect porcine xenografts from human NK cell responses, including direct xenogeneic NK cytototoxicity.

Acknowledgments

We thank A. Moretta (University of Genova, Genova, Italy) for kindly providing Ab, G. Waneck (Massachusetts General Hospital, Boston, MA) for kindly providing the 13271.10 cell line, and M. Urosevic (University Hospital Zurich, Switzerland) for kindly providing the MEL-15 cell line. M. D. Crew (University of Arkansas for Medical Sciences, Little Rock, AR) and M. K. J. Schneider (University Hospital Zurich) are acknowledged for careful reading of this manuscript and helpful comments.

Disclosures

The authors have no financial conflict of interest.

References


