Contrast-enhanced ultrasound with sub-micron sized contrast agents detects insulitis and β-cell mass decline in mouse models of type 1 diabetes

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ABSTRACT

Type 1 diabetes (T1D) is characterized by the infiltration of autoreactive T-cells into the islet of Langerhans, and depletion of insulin-secreting β-cells. This immune cell infiltration (insulitis) first occurs during an asymptomatic phase of T1D that can take place many years prior to clinical diagnosis. Methods to diagnose insulitis and changes in β-cell mass during this asymptomatic phase are limited, thus precluding early therapeutic intervention. While therapeutic treatments can delay T1D progression, treatment efficacy is limited and widely varying, and a method to track this efficacy is also lacking. During T1D progression, the islet microvasculature increases permeability as a result of insulitis, in both mouse models of T1D and humans with T1D. This increased permeability can allow nanoparticles, such as contrast agents for diagnostic imaging, to access the islet microenvironment. Contrast enhanced ultrasound (CEUS) uses shell-stabilized gas bubbles to provide high acoustic backscatter in vasculature and tissue and is clinically approved. A novel, sub-micron sized ‘nanobubble’ (NB) ultrasound contrast agent has been developed and shown to extravasate and accumulate in tumors, where microvascular permeability is high. To test whether CEUS can be used to measure increased islet microvasculature permeability and indicate the asymptomatic phase of T1D, we applied CEUS measurements with NBs in pre-clinical T1D models. NOD mice and mice receiving an adoptive-transfer of diabetogenic splenocytes showed accumulation of NBs specifically within the pancreatic islets, and only in the presence of insulitis. This accumulation was measured by both ultrasound contrast and histological analysis, and accumulation only occurred for sub-micron sized bubbles. Importantly, accumulation was detected as early as 4w in NOD mice. Thus, CEUS with sub-micron sized NB contrast agent may provide a predicative marker for disease progression early in asymptomatic T1D, as well as monitoring of disease prevention or reversal.
INTRODUCTION

Type 1 diabetes (T1D) is caused by infiltration of auto-reactive T-cells into the pancreatic islets and the destruction of the insulin-producing $\beta$-cells\(^1\). Prior to the clinical presentation of diabetes there exists an asymptomatic phase where insulitis and immunological irregularities are present, but there is sufficient $\beta$-cell mass and insulin secretion to regulate blood glucose levels. After substantial $\beta$-cell loss (e.g. >80\%)\(^2\) patients present with hyperglycemia and are diagnosed with T1D\(^3\). The asymptomatic phase presents an opportunity for therapeutic intervention to blunt insulitis and preserve $\beta$-cell mass\(^4,5\). However, the inability to effectively diagnose insulitis and $\beta$-cell mass decline will limit attempts to treat patients prior to T1D onset. The presence of circulating auto-antibodies can predict eventual T1D onset, but these antibodies are not pathogenic and represent an aggregate risk of developing the disease\(^6\). Therefore, a method to identify and track the underlying disease progression during the asymptomatic phase is paramount for diagnosing and treating patients at risk for developing T1D.

To date, therapeutic interventions aimed at preventing islet autoimmunity and insulitis and preserving $\beta$-cell mass have had limited success. For example, the immunotherapy anti-CD3 ($\alpha$CD3) has been shown to preserve insulin secretion (c-peptide levels) for up to two years. However, $\alpha$CD3 treatment is highly heterogenous, where some patients retained robust insulin secretion more than two years (‘responders’), but others lacked any preservation of insulin secretion (‘non-responders’)\(^7\). A means to assess the reversal of insulitis and preservation of $\beta$-cell mass would also enable the efficacy of preventative treatments to be assessed. However, there are limited means to monitor the response to preventative treatments.

There are challenges associated with imaging the state of the islet *in-vivo*. The islets represent a low volume (1-2\%) of the total pancreas mass\(^7\). As such any label for the islet can suffer from poor signal-to-background; for example the background can be ~50\% greater than islet signal for GLP1R radio-labels\(^8\). The pancreatic islets do receive a disproportionately high proportion of the pancreas blood flow compared to the exocrine tissue (10-20\%)\(^9,10\) and islet blood flow can vary during the progression of insulitis and T1D\(^11-13\). Within the islet the microvasculature becomes more permeable during insulitis and inflammation, as demonstrated by the uptake of nanoparticles into the islet microenvironment of non-obese diabetic (NOD) mice\(^14\) or STZ-treated mice\(^15\), which model T1D. Magnetic resonance imaging (MRI) has been used to visualize magnetic nanoparticles (MNPs) uptake and retention within the inflamed islets during the development of T1D in rodent models\(^14-17\) and in human T1D\(^18,19\). As such, measurements of increased islet vascular permeability and changes in these measurements over time could be used more broadly to diagnose and monitor islet infiltration and decline during the progression of T1D.

Ultrasound is a cost-effective, easily deployable, and safe medical imaging modality. Contrast-enhanced ultrasound (CEUS) utilizes lipid- or protein stabilized gas
core microbubbles (MBs) that provide a strong acoustic backscatter of ultrasonic waves. MBs are FDA approved for cardiac and liver imaging in adult and pediatric populations\textsuperscript{20}, and have been used ‘off label’ for other indications \textsuperscript{21,22}. While MBs are restricted to the blood stream, smaller sub-micron sized nanobubbles (NBs) are capable of extravasating through the hyper-permeable microvasculature of injured tissue. This includes accumulation of \(\sim\)100-300 nm diameter NBs within tumors as a result of increased tumor vascular permeability\textsuperscript{23-25}. Such NBs are echogenic at clinically relevant ultrasound frequencies (6-18 MHz), allowing regions of high permeability microvasculature to be imaged non-invasively and in real time.

In this study, we tested whether CEUS measurements of sub-micron sized NBs can detect changes in islet microvascular permeability as a result of increasing insulitis during the asymptomatic phase of T1D. We found that NBs accumulated within the pancreas and islets in multiple mouse models of T1D compared to non-diabetic controls, with accumulation detectable at very early stages of disease. This accumulation was measured by both ultrasound contrast signal and by histology, and correlated with the level of insulitis.
RESULTS

*Disease-dependent increase in nanobubble (NB) contrast signal within the pancreas.*

Previous work has demonstrated that submicron sized 'nanobubbles' (NBs) accumulate within tumors that exhibit increased microvascular permeability. As such, we hypothesized that NBs would accumulate within the pancreatic islets undergoing insulitis where there is increased vascular permeability. Prior work examining vascular permeability using NBs and CEUS has shown an elevated signal immediately following injection corresponding to rapid filling of the vasculature, followed by a persisting signal for ~10 minutes corresponding to tissue accumulation. To examine these kinetics we first infused NBs via tail vein catheter into 10w non-obese diabetic (NOD) mice, at which age islets show heavy insulitis, and examined the kinetics of NB signal within the pancreas. We also performed these measurements utilizing different NB concentrations, center frequency and peak negative pressure (mechanical index, MI).

The pancreas tail can be readily located in B-mode ultrasound using the kidney, spleen and stomach as guide markers, as well by the distinct texture (Fig. 1a, Fig. S1). Measurements in nonlinear contrast mode following NB infusion showed increased sub-harmonic contrast signal within ~1min of infusion in the pancreas at 12.5MHz center frequency (4% power), that returned to baseline around 5min, likely corresponding to vascular filling (Fig. 1b) 11. This immediate elevation in contrast signal was diminished when using a center frequency of 18MHz. However, with a center frequency of 18MHz and higher transmission power (10%), an elevation in contrast signal that was maintained over ~30 minutes was observed in the pancreas (Fig. 1c). Thus, a sustained increase in contrast signal is observed within the pancreas associated with NB infusion.

Given increased islet microvascular permeability during the progression of T1D, we next sought to determine if NB signal within the pancreas was associated with disease. In 10w NOD mice NB contrast in the kidney, where no immune infiltration occurs, did not increase significantly after ~30 mins, and was significantly less than the substantial elevation in contrasts signal within the pancreas (Fig. 1d,e). Following saline vehicle infusion both the kidney and pancreas did not show a signal increase (Fig. 1f), indicating signal specifically associated with the infused NB formulation. In healthy 10w female C57BL/6 mice there was a lack of any significant signal change following NB infusion (Fig. S2). To further examine the disease-dependence of NB signal changes within the pancreas, we compared immunodeficient NOD;Rag1ko (Rag1ko) mice which do not develop spontaneous diabetes. After NB infusion, there was no significant increase in NB contrast signal in the pancreas of Rag1ko mice (Fig. 1g) compared to the robust elevation in NOD mice.

These results show that there is increased NB contrast signal specifically within the pancreas in the NOD mouse models of T1D, where increased islet microvascular permeability occurs. The signal does not increase in the kidney nor in healthy C57BL/6
and immune deficient Rag1ko mice. This is consistent with the changes in islet microvascular permeability induced by insulitis prior to T1D onset.

**NB accumulation specifically within pancreatic islets.**

To characterize the biodistribution of NBs within the pancreas in the presence of insulitis, we infused rhodamine-labeled NBs into 10w NOD and Rag1ko mice. Following infusion of these labeled NBs the contrast signal within the pancreas and kidney was similar to that following infusion of unlabeled NBs: only in the pancreas of the NOD mice was there a robust contrast signal increase (Fig. 2a,b). In NOD and Rag1ko mice we performed histological analysis of NB-rhodamine accumulation within the islets and exocrine tissues of the pancreas, where islets were identified by their characteristic autofluorescence and DAPI staining (Fig. 2c,d). In 10w NOD mice, islet regions of the pancreas showed significantly greater fluorescence coverage compared to exocrine regions (Fig. 2c,e), representing greater NB accumulation. Furthermore, islet regions from 10w NOD mice had significantly greater fluorescence coverage compared to islet regions from 10w Rag1ko mice (Fig. 2c,e). In NOD mice over 80% of the islets had some level of rhodamine coverage, representing NB targeting, and this was much lower in Rag1ko mice (Fig. 2f). The exocrine tissue from both models showed significantly less coverage than in 10w NOD islets. Thus NBs target the islet regions preferentially compared to exocrine regions in NOD mice but not immunodeficient mice that do not develop diabetes.

Prior studies have indicated nanoparticles can extravasate into the islet microenvironment during insulitis as a result of increased microvascular permeability. To confirm NBs were extravasating from the microvasculature, as opposed to adhering to vessel walls, we performed histological analysis following infusion of rhodamine-labeled NBs and fluorescently conjugated tomato lectin to label blood vessels (Fig. 2g). Within the islets, we observed NB-rhodamine coverage was significantly less within the vasculature regions (lectin+) compared to outside the microvasculature regions (lectin-) (Fig. 2h), indicating extravasation of NBs into the islet microenvironment was occurring.

To test whether NB accumulation depended on the level of insulitis and thus disease, we scored the level of insulitis in 10w NOD and Rag1ko immunodeficient mice (Fig. 2i). Utilizing adjacent slices stained with hematoxylin and eosin (H&E) or in which rhodamine coverage is examined, we correlated the insulitis scoring and the fluorescent coverage in the islet region of both mouse models. The mean rhodamine coverage across islets of the pancreas of 10w NOD mice was significantly correlated with the mean insulitis score across the same islets (Fig. 2j). Thus, mice with higher immune cell infiltration within the islets show greater NB targeting to the islets across the pancreas. However, when examined as an islet-by-islet basis, interestingly there was no significant relationship between islet insulitis score and fluorescent coverage (Fig. 2k). Thus, NB targeting of infiltrated islets is based on the pancreas-wide level of insulitis within the NOD mouse.
Taken together, these data suggest that increased islet microvascular permeability occurs alongside islet immune cells infiltration, and thus we can observe this increased insulitis by using extravasating NBs and ultrasound contrast signal measurements, as a measure for diabetes progression.

**NB accumulation within the pancreas is size-dependent.**

The NBs are fractionated from a polydisperse sample that will also contain micron sized bubbles (microbubbles, MBs). Given that the MB scattering cross-section scales strongly with bubble radius (~r^6)^26, even a very small population of MBs could explain the contrast signal increase within the pancreas. To determine whether sub-micron sized bubbles are required to generate the contrast within the pancreas we observe, we first characterized the sizes of the sub-micron NB fraction we infuse. The NB fraction contained approximately four orders of magnitude more 'nano-sized' (<1µm diameter) objects than 'micron-sized' (>1µm diameter) objects (Fig. 3a,b), as measured by microscopy analysis. Similar results were obtained using Resonant Mass Measurement (RMM, Archimedes) (Fig. S3), where the ‘nano-sized’ (196±32nm diameter) objects were also approximately four orders of magnitude more abundant than ‘micron-sized’ (2356±483nm diameter) objects. The micron-sized MB fraction was substantially depleted of ‘nano-sized’ objects, but contained a similar number of ‘micron-sized’ objects as in the NB fraction (Fig. 3c,d). We infused 10w NOD mice with these different fractions on alternating days to test if the MB fraction contributed to the pancreas contrast signal. While the NB fraction shows a sustained increase in contrast signal in the pancreas over 30 minutes, the MB fraction within the pancreas did not significantly differ from background (Fig. 3e). We also infused ~10 million size-isolated microbubbles (SIMBs) of either 3-4µm diameter or 1-2µm diameter to test whether specific sized MBs could generate disease-dependent contrast signal within the pancreas. SIMBs of each size range showed a strong contrast signal within ~1min, consistent with their high echogenicity and vascular filling. However, no signal increase was observed within the pancreas at >10min after infusion (Fig. 3f), although 1-2µm SIMBs cleared slightly slower than 3-4µm SIMBs.

The lack of any pancreas contrast signal elevation from micron-sized bubbles therefore indicates that NB pancreas contrast signal elevation and NB accumulation within the islets is dependent on the NB size.

**NBs also target the pancreas and islets in NOD mice at 4w.**

To determine how early in the disease progression NB signal can indicate changes in the islet microvasculature, we examined 4w NOD and 4w Rag1ko mice. Following NB infusion, a sustained increase in contrast signal was also observed in the pancreas of 4w NOD mice (Fig. 4a). This contrast signal was significantly greater than the contrast signal in the pancreas of 4w Rag1ko mice, which remained similar to background levels (Fig.
4a,b). However the contrast signal in 4w NOD mice was less than the contrast signal we observed in 10w NOD mice (Fig. 1e,g). We also performed histological analysis on these same mice to quantify NB-rhodamine fluorescence coverage (Fig. 4c). The islet regions of 4w NOD mice had significantly increased rhodamine coverage compared to the islet regions of 4w Rag1ko mice (Fig. 4d). However, again the rhodamine fluorescence coverage was less than we observed in the islet regions of 10w NOD mice (Figs. 2e). These data suggest that measuring NBs accumulate within the pancreas upon insulitis early in the progression of T1D.

*NOD-scid mice treated with diabetogenic splenocytes have increased CEUS signal.*

Our histological data (Fig. 2) suggests that NB accumulation depends on the level of insulitis across the pancreas. To further test whether NB accumulation reports insulitis and is not a specific feature of the NOD mouse, we utilized an adoptive transfer (AT) mouse model in which immunodeficient NOD-scid mice receive a transfer of diabetogenic splenocytes from recently diabetic NOD mice. In this AT model, mice develop diabetes between 5-8 weeks after splenocyte transfer (Fig. 5a). Following NB infusion, AT mice and mice lacking an adoptive transfer showed similar contrast signal measurements at baseline prior to transfer (Fig. 5b,c). At 2 weeks post transfer the AT mice had significantly greater contrast signal within the pancreas compared to control mice (Fig. 5d). Therefore, NB contrast signal can detect increased insulitis across the pancreas across multipole models of T1D, and therefore is a robust reporter of insulitis and concurrent inflammation.
DISCUSSION

There are limited means for detecting the asymptomatic phase of type1 diabetes (T1D) development. Accurately detecting the progression of immune infiltration and islet decline would allow diagnosis of diabetes development to enable early preventative treatment, as well being able to monitor the efficacy of this treatment. Here, our goal was to test whether changes in islet microvascular permeability that occurs concurrently with insulitis, local inflammation and islet decline could be detected using standard nonlinear ultrasound available on most clinical scanners and sub-micron sized nanobubble (NB) ultrasound contrast agents. We demonstrated that nanobubbles (NBs) accumulate specifically within the pancreas in multiple animal models of T1D as detected by persistent increased ultrasound sub-harmonic contrast, and that histological measurements further localize the accumulation to the infiltrated pancreatic islets. These results suggest that contrast-enhanced ultrasound using NB contrast agents can be used to detect and longitudinally track pre-symptomatic T1D progression (Fig. 6).

**CEUS with NB contrast agents can detect islet infiltration and asymptomatic T1D.**

Our results show strong evidence that CEUS with sub-micron sized NBs can detect insulitis and islet inflammation associated with the preclinical asymptomatic (pre-symptomatic) stage of T1D. This includes significant targeting to the pancreas, but not other imaged organs, as measured by ultrasound (Fig. 1); as well as specific targeting to the islets but not exocrine tissue as measured by histology (Fig. 2). The lack of pancreas and islet targeting in immune deficient mice that do not get diabetes further indicates this targeting is specific to models of type1 diabetes (Fig. 1,2). We observed similar results in both NOD animals (Fig.1) and the adoptive transfer model (Fig. 5) in which insulitis leads to islet decline and diabetes. This indicates that islet targeting and accumulation is not model-specific, but occurs as a result of insulitis. The correlation between insulitis across the pancreas and nanobubble accumulation within the islets further indicates an ability to detect insulitis, although we cannot be sure that the accumulation is directly as a result of insulitis itself (see below). Nevertheless, non-invasive measurement of sub-micron sized bubbles accumulation within the islets using contrast-enhance ultrasound provides a convenient measure of the progression of insulitis and pre-symptomatic type1 diabetes.

We observed nanobubble accumulation and contrast signal within the pancreas in 10w NOD mice. However, we also observed significant ultrasound contrast within the pancreas and nanobubble accumulation within the islets in 4w NOD mice, albeit lower than measured in 10w NOD mice. At this age insulitis is very mild, consisting mostly of peri-insulitis, yet nanobubble accumulation readily detected. Thus, measurement of nanobubble accumulation is very sensitive to disease development. This also compares favorably to other modalities that have been applied to pre-clinical models. For example, insulin autoantibodies are elevated after 4 weeks in NOD mice. Magnetic nanoparticles show significant accumulation within the islets as early as 6 weeks in NOD mice or 4
weeks in NOD-BDC2.5 mice\textsuperscript{14}. Notably, clear changes were observed with measurements at a single time point: multiple longitudinal measurements may capture the progression of insulitis more clearly. Nevertheless, such highly sensitive accumulation and thus measurement of insulitis may have drawbacks. For example, when detecting disease reversal following therapeutic treatment, if some inflammation or islet damage remains post-treatment, this could still cause some significant nanobubble accumulation. Testing whether effective disease reversal is predictable by nanobubble accumulation measurements will be a key goal for future work.

Insulitis and inflammation contribute to the increased NB signal within the pancreas and islets, likely as a result of increased microvascular permeability within the islet, Interestingly while the level of NB accumulation across the pancreas correlated with the level of insulitis across the pancreas, there was no such correlation by islet: islets with substantial infiltration on average showed similar NB targeting as islets showing peri-insulitis or absent insulitis within the same pancreas. Such islet-by-islet analysis has not been presented within the literature. Given the sensitivity for NB accumulation, we speculate this may reflect early macrophage recruitment and action of inflammatory mediators across the islets\textsuperscript{31}. Thus, NB accumulation reflects the level of infiltration and inflammation across the pancreas. While islet microvascular changes occur in mouse models of T1D and human T1D\textsuperscript{6,16,18}, it has not been demonstrated exactly what causes these microvascular changes and whether they solely reflect the islet microenvironment or are influenced by events elsewhere in the pancreas. For example, sympathetic activity is a strong tonic regulator of islet pericyte function\textsuperscript{32}. Determining the molecular mechanism underlying these microvascular changes that reflect NB accumulation, and likely the accumulation of other imaging contrast agents, is therefore needed.

Our measurements indicate that nanobubbles specifically accumulate within the islets in models of T1D. Nanobubbles can be readily functionalized with antibodies and peptides, and can also incorporate hydrophobic small molecules. This property has been utilized for both molecular imaging\textsuperscript{25} and also for specific therapeutic delivery\textsuperscript{33}. While microbubbles have been used for cargo delivery\textsuperscript{34,35}, their localization is restricted to the vasculature and requires ultrasound-induced ‘ablation’ for cargo release. Given the passive delivery of nanobubbles to the islet, they may also provide an effective vehicle for therapeutic delivery to this region.

\textbf{Increased CEUS signal correlates to islet vascular changes.}

Our results show a clear correlation between NB accumulation and insulitis. The islet-vascular interface has many fenestrae to facilitate the transfer of cellular products, with the microvasculature within the islet normally being permeable to particles of $<70\text{kDa}$, equivalent to $\sim6\text{nm}$ size\textsuperscript{36}. This is much greater than the microvasculature within the exocrine tissue which is permeable to particles of $<20\text{kDa}$, equivalent to 2-3nm size\textsuperscript{36}. However, under inflammatory conditions in T1D the islet microvascular permeability
specifically increases, allowing passage of larger molecules, for example ~25nm magnetic nanoparticles. The NBs we utilized have a size of 100-300nm diameter which is consistent with the object size that is permeable in models of T1D. We also demonstrated that bubbles of >1μm diameter did not extravasate and accumulate, which is also consistent with our prior measurements of microbubbles within the pancreas in models of T1D, as well as being consistent with use of high MW fluorophore dextrans for labelling the vasculature. These results are also consistent with prior imaging of NB accumulation within tumors by ultrasound and histology.

Our results support that extravasation through more permeable islet microvasculature and accumulation within the parenchyma is occurring in models of T1D. However, it is important to note that we observed a very small component of micron-sized bubbles within our preparation (Fig.3). While <0.01% by percentage, this fraction could generate a comparable sub-harmonic contrast signal owing to the strong dependence of scattering cross section on bubble radius (~r^6). However, our results infusing the micron-sized MB fraction and 1-2μm size isolated microbubbles which lack any persistent contrast signal argue against any contribution from microbubbles to the disease dependent islet contrast signal. We also measure bubble accumulation more effectively using higher transmission frequencies which is consistent with sub-micron sizes. Conversely the initial vascular filling is measured less effectively using higher center frequencies, which is consistent with microbubbles being detected, but only during the initial vascular filling. Therefore, our results strongly indicate that the signal indicating insulitis results from sub-micron sized bubbles extravasating through the microvasculature rendered more permeable due to insulitis and local inflammation.

Studies using magnetic nanoparticles and MRI to detect inflammation rely on islet-resident macrophage uptake of the magnetic nanoparticles to retain contrast at the site of insulitis and inflammation. In this study we did not measure the kinetics for nanobubble clearance from the islet microenvironment, being >30min. However, the kinetics of clearance from the vasculature is <10min. This rapid vascular clearance, consistent with many bubble contrast agents for ultrasound, allows the specific islet signal to be separated within the same measurement window. Nevertheless, testing whether accumulation persists over many hours or days will be a goal for future work and is highly relevant to using NBs as a vehicle for therapeutic delivery to the islet, as discussed above.

Potential for Translation of CEUS and nanobubbles for clinical diagnosis.

Our results also suggest imaging NB accumulation within the pancreas may be a clinically deployable approach for diagnosing insulitis and pre-clinical asymptomatic T1D. There have been limited studies of human islet microvascular function compared to those in mouse. However, the use of MRI to measure magnetic nanoparticle accumulation within the pancreas has been reported in human subjects with T1D, and these studies have demonstrated clear differences between healthy patients and those recently
diagnosed with T1D\textsuperscript{18,19}. This indicates that the islet microvascular permeability is likely similarly altered in human, despite differences in the intensity and duration of insulitis. Therefore, nanobubbles will likely also accumulate in the human islet microenvironment in T1D, although the precise microvascular permeability changes are still unknown. There are also other important considerations to discuss before this approach can be clinically deployable.

Microbubbles (e.g. Lumason\textsuperscript{®}, Bracco) are FDA approved for use in liver applications in adult and pediatric patients. Furthermore, they have had additional off label applications, including visualizing pancreatic tumors\textsuperscript{41,42}. The use of ultrasound modalities provides several advantages over other clinical imaging modalities, including wide-spread deployment, cost effectiveness, high spatial and temporal resolution and the lack of ionizing radiation enabling repeated measurements. While nanobubbles are not clinically approved, they are synthesized using many similar components to clinically applied formulations. These approved formulation are polydisperse, and thus include a significant fraction of sub-micron-sized bubbles. Thus, achieving clinical approval is not insurmountable. We also utilized a small animal ultrasound machine in these studies using frequencies greater than those clinically applied for pancreas imaging\textsuperscript{43,44}. However, nanobubble imaging has been performed using clinically-relevant frequencies, including the detection of increased persistence in certain classes of tumors over other classes\textsuperscript{39}. Likewise, higher frequency clinical transducers are also becoming more widely available and utilized especially on the pediatric population. Finally, while insulitis is less aggressive in human compared to mouse, the sensitivity of our measurements to insulitis even in 4w NOD indicates this approach will be applicable during the pre-symptomatic phase and may provide sensitivity surpassing other approaches in detecting the progression of diabetes at an early stage.

In summary, we present a non-invasive, deployable, ultrasound imaging modality for assessing insulitis in pre-symptomatic type1 diabetes. As a result of increased islet vascular permeability during the progression of disease, sub-micron sized nanobubble agents accumulate within the islets. The increase in ultrasound contrast signal originating from the islets allows for diagnosis of disease progression. This may provide a convenient approach to examine human islet microvascular permeability in patients in the presymptomatic phase of T1D.
METHODS

Animals

All animal procedures were performed in accordance with guidelines established by the Institutional Animal Care and Use Committee of the University of Colorado Anschutz Medical campus. Female NOD mice were purchased from Jackson Laboratories (Bar Harbor, ME) at age 4-10 weeks, and imaged at either 4 weeks or 10 weeks of age. Female Rag1ko animals were bred in house and imaged at either 4 weeks or 10 weeks of age. NOD-SCID animals were purchased from Jackson Laboratories at age 10-14 weeks, and imaged at 12-24 weeks of age. Throughout the study, animals were monitored weekly for blood glucose concentration utilizing a blood glucometer (Bayer).

Nanobubble synthesis and characterization

Nanobubbles were synthesized using previously published protocols. Briefly, unlabeled NBs were made dissolving a lipid mixture of DPPC (1,2-dipalmitoyl-sn-glycerol-3-phosphocholine), DPPA (1,2-dipalmitoyl-sn-glycerol-3-phosphate), DPPE (1,2-dipalmitoyl-sn-glycerol-3-phosphoethanolamine) (Avanti Polar Lipids, Pelham, AL), and mPEG-DSPE (1,2-distearoyl-phosphatidylethanolamine-methyl-poly ethylene glycol conjugate-2000) (Laysan Lipids, Arab, AL) in chloroform in a 4:1:1:1 mass ratio. Upon complete dissolution, chloroform was removed via evaporation. The lipid film remaining was hydrated by adding glycerol (50 µl) and 1 ml of 0.6 mg/ml Pluronic L10 solution in PBS (Sigma Aldrich, Milwaukee, WI) at 75 °C for 30 min. To prepare labeled NBs, 50 µl (1 mg/mL solution in chloroform) of a fluorescent lipid (Liss Rhod PE) (1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt)) was added to the unlabeled lipid mixture. To activate the NBs, the hydrated lipid solution was shaken for 45 seconds with a mechanical shaker (Vialmix, Lantheus Holds) and centrifuged at 50 RCF for 5 minutes. Following activation, a 28.5-gauge needle was used to remove the NB fraction, which was then diluted in PBS by a factor of 1:5 and pipetted into a 28.5G insulin syringe for subsequent use. NBs were used immediate after activation. NBs were activated within 30 days of the synthesis of the hydrated lipid solution.

Bubbles were first characterized at Case Western via resonant mass measurement (RMM) (Archimedes, Malvern Pananalytical Inc., Westborough, MA, USA) which measures particle size, size distribution, and concentration. In RMM, measurements are performed using a sensor with a microelectromechanical systems (MEMS) resonator, which contains a microfluidic channel embedded in a resonating cantilever under vacuum to detect, count, and measure the buoyant mass of the particles in the liquid passing through the channel. The RMM nanosensor was used to characterize the nanobubbles, which provides measurement between 100 nm to 2 µm. Sensors were calibrated using NIST traceable 565 nm polystyrene bead standards (ThermoFisher
Nanobubbles were diluted 1:100 with phosphate buffered saline (pH 7.4) and a total of 1000 particles were measured for each trial performed (n=3). The sensor and microfluidic tubing were cleaned with deionized water in between each run.

Size-isolated microbubble (SIMBs) contrast agent was purchased from Advanced Microbubbles Laboratories (Boulder, CO) and infused with a 28.5G insulin syringe. Approximately 10 million microbubbles were infused in a 100 µl solution.

Contrast-Enhanced Ultrasound (CEUS) imaging

General anesthesia was established with isoflurane inhalation for a total of 40-50 minutes for all animal imaged. Prior to imaging, a custom made 27G ½” winged infusion set (Terumo BCT, Lakewood, CO) was attached to a section of polyethylene tubing (0.61 OD x 0.28 ID; PE-10, Warner Instruments) and was inserted in the lateral tail vein and secured with VetBond (3M). Abdominal fur was removed using depilatory cream, and ultrasound coupling gel placed between the skin and transducer. Foot pad electrodes on the ultrasound machine platform monitored the animal’s electrocardiogram, respiration rate, and body temperature. All animals were constantly monitored throughout the imaging session to maintain body temperature and respiration rate.

A VEVO 2100 small animal high-frequency ultrasound machine (Visual Sonics, Fujiﬁlm, Toronto, Canada) was used for all experiments. For CEUS imaging a MS201 linear array transducer was initially used (Fig. 1b,c) at a frequency of 12.5 MHz or 18 MHz; and subsequent experiments utilized a MS250 linear array transducer at a frequency of 18 MHz. B-mode imaging (transmit power 100%) was performed prior to NB or MB infusion to identify anatomy of the pancreas body, based on striated texture and location in relation to the spleen, kidney, and stomach (Fig. 1a, Fig. S1). Following identification of the pancreas and selection of a region of interest, sub-harmonic contrast mode was initiated. For the initial contrast mode experiments, acquisition settings were set at: transmit power 4% or 10%, frequency 12.5 MHz or 18 MHz, standard beamwidth, contrast gain of 30 dB, 2D gain of 18 dB, with an acquisition rate of 26 frames per second. For all subsequent experiments using contrast mode, acquisition settings were set at: transmit power 10%, (MI=0.12), frequency 18 MHz, standard beamwidth, contrast gain of 30 dB, 2D gain of 18 dB, with an acquisition rate of 26 frames per second. Gating to remove movements as a result of animal breathing was carried out manually or in MATLAB (MathWorks, Natick, MA).

Following nanobubble (NB) activation and separation (see above), NBs were injected as a single bolus of 100μl solution of 2x10^{11} NB/ml into the lateral tail vein via the catheter. Background data were acquired for 3 minutes prior to injection. Time courses were stored at 30 second intervals up to a 30 minute duration post injection. Identical procedures were used with MBs. The continual imaging for >30min using 18MHz and
10% transmission power did not significantly impact the contrast signal generated by pancreas-accumulating NBs (Fig. S4).

For analysis of NB contrast, the NB signal was background subtracted by the averaged contrast intensity taken before injection. Each time course was normalized to its pre-injection background. Regions of interest for the pancreas and kidney, were identified by the B-mode image based upon anatomical features and texture, and manually adjusted prior to analysis of the infusion to avoid small regions of very high contrast in the background image. Similar trends were seen if the background subtracted signal was not normalized to background, albeit increased variability (Fig. S5).

Isolation and Adoptive Transfer of Diabetogenic Splenocytes

Splenocytes were isolated from diabetic female NOD mice (hyperglycemic for <1 week), manually dissociated and counted in cold HBSS (without MgCl₂ and CaCl₂). Leukocytes were counted to determine an estimate of cellular density. 12-14 week old NOD-SCID mice received a single intraperitoneal (I.P.) dose of 20 x 10⁶ leukocytes resuspended in HBSS. Control animals were injected with an equivalent volume of HBSS without leukocytes.

Histology and Insulitis Morphology

For assessment of insulitis, all animals were anesthetized by I.P. injection of Ketamine (80 mg/kg) and Xylazine (16 mg/kg) until no longer reactive to toe pinch, pancreata were dissected and mice were euthanized by exsanguination and/or Bilateral thoracotomy. Pancreata were fixed in paraformaldehyde at 4°C rocking overnight and embedded in OCT blocks similar to published protocols. 8μm sections from at least three tissue depths were stained by Hematoxylin and Eosin (H&E) for evaluation of islet monocyte infiltration. Images were acquired on an Eclipse-Ti wide field microscope with a 20X 0.75 NA Plan Apo objective with a color CCD camera.

Images of islets were scored based on the extent of infiltration/insulitis: grade 0, no insulitis; grade 1, peri-insulitis with immune infiltrate bordering; grade 2, immune infiltrate penetrating the islet, covering <50% of the islet area; grade 3, immune infiltrate penetrating the islet, covering >50% of the islet area. A minimum of three different tissue depths and at least 30 non-overlapping islets per animal were analyzed. Weighted averages were calculated for each animal.

For histological assessment of NB extravasation 4w or 10w female NOD mice (JAX) or Rag1ko mice received a single bolus injection of 100μl solution of 2x10¹¹ NB/ml of rhodamine-labeled NBs. Following contrast imaging, mice were anesthetized by I.P. injection of ketamine (80 mg/kg) and xylazine (16 mg/kg). The pancreas was dissected and fixed in 4% PFA on ice for 1 hour and cryoprotected in 30% sucrose overnight or until the tissue sank. Pancreata were embedded in OCT medium, frozen in cryomolds, and cryosectioned at 8μm sections. Sections were imaged on an LSM800 confocal
microscope (Zeiss), with at 561 nm excitation using a x63 1.2NA objective and pinhole settings to provide 1µm z-section thickness throughout the tissue depth. Separate images were taken of exocrine tissue at locations anatomically isolated from the islets. Rhodamine coverage was calculated in MATLAB as the area of rhodamine positive pixels (pixels with fluorescence intensity significantly above the background fluorescence intensity) across the islet and expressed as a fraction of total islet area.

Assessment of NB and MB size

For optical quantification, the NB fraction was activated and the 100µl NB solution was serially diluted by a factor of either 10$^3$, 5x10$^3$ or 10$^4$ with PBS and plated on glass slides. The slides were sealed with a coverslip and CoverGrip (Biotium, Fremont, CA). The plated fractions were imaged using 561nm excitation on an LSM800 confocal microscope with a x63 1.2NA oil immersion objective (Zeiss), using a pixel size of 198nm. Nano-sized and micron-sized objects were identified using the Analyze Particle function in ImageJ software. Nano-sized' objects were classified as having a diameter <1µm, and 'micron-sized' objects a diameter >1µm. Only samples diluted by a factor of 10$^4$ were used to count nano-sized objects owing to the high density preventing accurate counting under lower dilutions. Total counts were estimated based on a colony-forming unit calculation: bubbles/ml = (N * df) / (Vpl * AIm/Apl). Where N is the number of objects counted of the size classification; df is the dilution factor applied; Vpl is the volume of bubble solution that was plated; AIm is the area of the plated bubble solution that was imaged; and Apl is the total area covered by the plated bubble solution.
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AUTHOR CONTRIBUTIONS

DR designed and performed experiments, analyzed data, wrote the manuscript; CH and EA synthesized and characterized the nanobubbles; LAP performed experiments and analyzed data; SP analyzed data; AAE conceived of the idea, designed experiments and edited the manuscript; RKPB conceived of the idea, designed experiments and edited the manuscript.
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FIGURE LEGENDS

Figure 1: Contrast Enhanced Ultrasound measurements of NB targeting the pancreas in T1D. (A) Schematic illustrating the transducer placement on the animal models (left) and the anatomical landmarks used to identify the pancreas during the ultrasound scan (right). The pancreas (green), spleen (purple), and kidney (yellow) are shown in B-Mode and Contrast Mode. (B) Mean time-course of contrast signal in the pancreas of 10 week old female NOD mice following NB infusion, using 12.5MHz or 18MHz center frequency, each at 4% transmission power. (C) Mean time-course of contrast signal in the pancreas of 10 week old female NOD mice following NB infusion, using 18MHz center frequency, at 4% or 10% transmission power. (D) Representative B-mode and sub-harmonic contrast ultrasound images of pancreas (green) and kidney (yellow), before and after NB infusion, at 18 MHz and 10% power. (E) Mean time-course of contrast signal in the pancreas and kidney of 10 week old female NOD mice following NB infusion (left), together with the mean contrast signal averaged between 20-25 minutes (right). (F) Mean time-course of contrast signal, and mean signal between 20-25 minute as in E comparing pancreas and kidney of 10 week old female NOD mice following saline infusion. (G) Mean time-course of contrast signal, and mean signal between 20-25 minute as in E comparing pancreas of 10 week old female NOD mice and 10 week old female NOD;Rag1ko (Rag1ko) mice following NB infusion. Dashed lines in B-G represent 95% CI, error bars in B-G represent s.e.m. Data in B,C representative of n=7 mice. Data in E representative of n=19 mice. Data in F representative of n=3 mice. Data in G representative of n=6 mice. * p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 comparing groups indicated (Paired Student’s t-test).

Figure 2: Histological assessment of NB targeting the islets in T1D. (A) Mean contrast signal between 20-25 minutes following Rhodamine-labelled NB infusion, in the pancreas and kidney of 10 week old female NOD mice. (B) as in A for 10 week female Rag1ko mice. (C) Representative confocal images of an islet within a pancreas section of 10 week female NOD mouse following rhodamine-labeled NB infusion (orange). Islet is circled with a dotted line, as determined from autofluorescence (green) and DAPI-labeling morphology (blue). (D) as in C for 10 week old Rag1ko female mice. (E) Mean rhodamine coverage in islet (Endocrine) and acinar (Exocrine) tissue in 10 week old NOD female mice and 10 week old Rag1ko female mice. (F) Mean proportion of islets with rhodamine labelling in 10 week old female NOD and Rag1ko mice. (G) Representative maximum-projection confocal image of an islet within a pancreas section of a 10w female NOD mouse infused with rhodamine-NBs (orange) and DyLight 488 tomato lectin (green). Islet circled with a dotted line, as determined from brightfield and DAPI-labeling morphology. (H) Mean rhodamine coverage within vascular areas (lectin positive) and non-vascular areas (lectin negative) in 10w NOD female mice. (I) Representative images of hematoxylin and eosin (H&E) stained pancreas sections of 10w NOD mice (left) and
Rag1ko mice (right). Islet circled with a dotted line, as determined from brightfield morphology. (J) Scatterplot of NB-rhodamine fluorescent coverage of islets within the pancreas against the mean insulitis score within the pancreas, for NOD mice. (K) Mean NB-rhodamine fluorescent coverage of islets within the pancreas that show insulitis scores of 1, 2, 3, or 4 (see methods). Error bars in A,B,E,F,H,K represent s.e.m. Trend line in J indicates linear regression with 95% confidence intervals. Data in A represents n=5 mice, B represents n=6 mice. Data in E, F represents 284 islets and 34 exocrine regions from n=6 NOD mice and n=6 Rag1ko mice. Data in H represents 66 islets from n=3 mice. Data in J represents n=6 NOD mice and n=6 Rag1ko mice. Data in K represents 62 islets from n=5 NOD mice. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 comparing groups indicated (paired Student's t-test for data in A, B, H; ANOVA for data in E, F, I, K). A mixed-effects model was used to assess the statistical significance and generate the regression in J.

Figure 3: Size-dependence to NB targeting the pancreas in T1D. (A) Representative images of nano-sized (label 1) and micron-sized (label 2) objects within the nanobubble fraction. (B) Calculation of the number of nano-sized and micron-sized objects within the nanobubble fraction. (C) as in A for the microbubble fraction. (D) as in B for the microbubble fraction. (E) Mean time-course of contrast signal in the pancreas of 10 week old female NOD mice (left), together with the mean contrast signal between 20-25 minutes (right) following NB infusion (NB Fraction) or MB infusion (MB Fraction). (F) Mean time-course of contrast signal in the pancreas of 10 week old female NOD mice (left), together with the mean contrast signal between 20-25 minutes (right) following infusion of size-isolated MBs of diameter 1-2 µm (SIMB 1-2) or 3-4 µm (SIMB 3-4). Dashed lines in E,F represent 95% CI, error bars in B,D,E,F represent s.e.m. Data in a-d represent n=3 samples, with each sample assessed under 3 separate dilutions. Data in E represents n=4 NOD mice. Data in F represents n=3 NOD mice. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 comparing groups indicated (paired Student’s t-test). Scale bars in a,c represent 10 µm (large image) and 6 µm (small closeup images).

Figure 4: NBs target the pancreas and islet early in disease development. (A) Mean time-course of sub-harmonic contrast signal in the pancreas of 4 week old female NOD mice and 4 week old female Rag1ko mice. (B) Mean contrast signal between 20-25 minutes in the pancreas of 4 week old female NOD and Rag1ko mice. (C) Representative images of an islet within the pancreas of a 4 week female NOD and a 4 week female Rag1ko mouse following rhodamine-NB infusion. (D) Mean rhodamine coverage in islet (Endocrine) and acinar (Exocrine) tissue in 4 week old NOD female mice and 4 week old Rag1ko female mice. *p<0.05, comparing groups indicated (paired Student’s t-test). Data in A-D represents n=3 NOD mice and n=3 Rag1ko mice.
Figure 5: NBs targeting the pancreas is dependent on immune cell infiltration. (A) Time course of ad-lib blood glucose in the adoptive transfer (AT) model treated with splenocytes from diabetic female NOD donors (AT, green) or treated with vehicle alone (control, black). Black arrows indicate time points of contrast enhanced ultrasound scans, green arrow indicates delivery of splenocytes/vehicle. (B) Mean time-course of sub-harmonic contrast signal in the pancreas of female NOD-scid mice prior to splenocyte/vehicle delivery. (C) Mean contrast signal between 20-25 minutes in the pancreas of NOD-scid female mice prior to splenocyte/vehicle delivery. (D) as in C for the pancreas of NOD-scid female mice 2w post splenocyte transfer. Data in A represents n=8 NOD-scid mice. Data in C, D represents n=6 A.T. mice and n=2 Control mice. *p<0.05, **p<0.01, comparing groups indicated (paired t-test).

Figure 6: Summary of experiments demonstrating nanobubbles extravasate in diseased islets which increases the contrast signal within the pancreas. After NBs are delivered, the healthy islet microvasculature are not permeable to NB extravasation so the NBs are retained in the vasculature and cleared. Infiltrated islets in T1D have high vascular permeability and therefore NBs accumulate and are retained in the tissue following infusion, providing a source of sub-harmonic contrast. This sub-harmonic contrast signal therefore provides a diagnostic measure of insulitis and pre-clinical asymptomatic T1D.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6

Transducer

Pancreas

Microvasculature

Islet of Langerhans

- bubbles

- $\beta$ cells

- $\alpha$ cells

Bubbles

UNHEALTHY ISLET

Nanobubble Extravasation
Supplemental Figures

Contrast-enhanced ultrasound with sub-micron sized contrast agents detects insulitis and β-cell mass decline in mouse models of type 1 diabetes

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Supplemental Figure S1: Identification of the pancreas in relation to other organs in the abdomen. (A) Following ultrasound transducer placement, the abdomen and stomach was located in B-mode. Using micromanipulators, the transducer was moved along the transverse plane until the pancreas, kidney, and spleen were identifiable. The striated appearance of the pancreas was used to confirm transducer placement in B-mode and then Contrast mode was engaged. (B) Screen shot showing representative images in B-mode and contrast mode in a NOD mouse prior to NB infusion using 18MHz frequency and 10% transmission power. (C) as in B in NOD mouse 20min after NB infusion indicating pancreas-specific sustained increase in sub-harmonic contrast signal. (D) Screen shot showing representative images in B-mode and contrast mode in a NOD mouse prior to NB infusion using 12.5MHz frequency and 4% transmission power. (E) as in D in NOD mouse 1min after NB infusion indicating rapid increase in sub-harmonic contrast signal throughout abdomen.
Supplemental Figure S2: NB time course and quantification of 10w C57BL/6J mice. Wildtype C57BL/6J mice were used to examine NB accumulation in a non-diseased model. (A) Mean time-course of contrast signal in the pancreas and kidney of 10 week old female C57BL/6J mice following NB infusion. (B) Mean contrast signal averaged between 20-25 minutes following NB infusion, in A. (C) As in A displaying the signal minus background (S-B). Data represents n=3 B6 mice. *p<0.05, comparing groups indicated (paired Student’s t-test).
Supplemental Figure S3: Size distribution of NB sample. Following NB activation, the size distribution within the NB fraction was determined via resonant mass measurement (RMM) (Archimedes, Malvern Panalytical Inc., Westborough, MA, USA). (A) Median size distribution form n=3 sample measurements. (B) relative abundance of the nano-sized (<1μm) and micro-sized (>1μm).
Supplemental Figure S4: Lack of impact of continual imaging on NB contrast signal. (A) Mean time-course of contrast signal in the pancreas of 10 week NOD mice, following either continuous imaging following NB infusion (‘continuous’), as performed elsewhere in the study; or with only brief snap shot every 5min until 20min after NB delivery at which point continual imaging resumes (‘pause’). (B) Mean contrast signal averaged between 20-25 minutes following NB infusion. No significant difference in signal between 20 and 25 minutes was observed indicated (paired Student’s t-test).
Supplemental Figure S5: NB time courses. Time courses displaying Signal minus Background (S-B) for those time-courses where S-B/B is displayed which includes an additional background normalization. (A) Data in Fig.1E. (B) Data in Fig.1F. (C) Data in Fig.1G. (D) Data in Fig.3E. (E) Data as in Fig.3E. showing for the kidney. (F) Data in Fig.3F. (G) Data in Fig.4A. (H) Data in Fig.5B. (I) Data as in Fig.5B showing for 2w following splenocyte infusion.